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ICAR

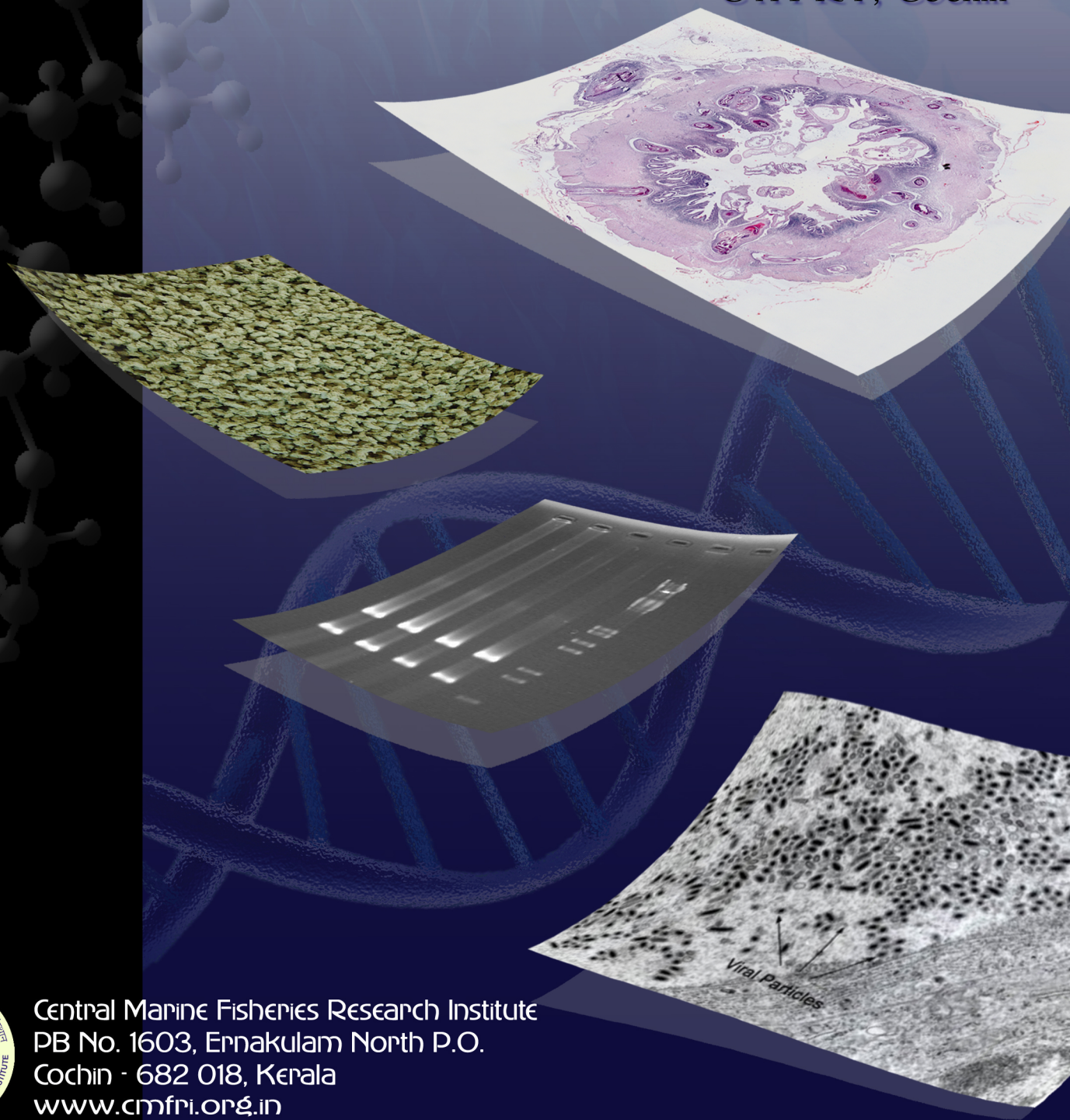
Winter School on

Vistas in Marine Biotechnology

5th to 26th October, 2010

at

Marine Biotechnology Division
CMFRI, Cochin



Central Marine Fisheries Research Institute
PB No. 1603, Ernakulam North P.O.
Cochin - 682 018, Kerala
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F O R E W O R D

Physisology, nutrition, pathology and genetics (PNP) Division of Central Marine Fisheries Research institute, (CMFRI), Cochin evolved into Marine Biotechnology Division (MBTD) two years ago. The change was long overdue. Before this change, the Division conducted several Winter/Summer Schools in the areas of Nutrition, Pathology and Genetics in isolation. Biotechnology integrates these areas and attracts more subjects and expertise. The need of the hour was this integration and a common platform from where these topics can be discussed right from the basics. Experts in the area will introduce the concepts and lead the participants to the labs. That is the genesis of this ICAR sponsored Winter School on Vistas in Marine Biotechnology for three weeks.

MBTD has prioritized R & D in the areas of genetics and genomics, nutrition, health and bioprospecting. This course manual is also organized on those lines.

We have taken efforts to include experts from sister institutions like Central Institute of Fisheries Technology (CIFT) and Central Institute of Brackishwater Aquacultute (CIBA), Chennai and Indian Institute of Spices Research (IISR), Kozhikode. Even experts from the Universities were also drawn in specific areas like toxicology (TANUVAS, Namakkal) and molecular approaches in parasitology (U C College Aluva, M G. University). The sister Institute CIFT was magnanimous in sparing us not only their expertise, but also their infrastructure (Trainees Hostel) for the conduct of this course.

Director CMFRI facilitated the programme along with the administrative and audit teams of the Institute. We appreciate on record all those who contributed to this team work.

The participants were chosen according to ICAR guidelines. They represent institutions from all over India. The response to call for applications far exceeded the number of positions. We regret we could not accommodate all, but now we know, there are opportunities in future also for the conduct of such programmes for which there is a demand.

Dr. K. K. Vijayan

Director, Winter School

C O N T E N T S

GENETICS AND GENOMICS

NUTRITION AND FEED TECHNOLOGY

BIOPROSPECTING

Genetics and Genomics

Perspectives in Marine Biotechnology Research and Development in India

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Introduction

In simple terms 'Marine Biotechnology' can be described as the sustainable commercial exploitation of marine life for the benefit of mankind. Marine biotechnology has continued to develop in recent years as a field of application of modern science and engineering of critical importance to the understanding, protection and exploitation of the potential resources of the sea, for the progress of fundamental science and benefit of humanity. Oceans comprise the biggest part of the biosphere and hold the most ancient and diverse forms of life. It is recognized that the sea's resources remain largely unexplored and marine organisms represent a vast untapped resource with potential benefits in many different areas of life, including mariculture, fisheries, industry, research tools and environmental applications. The combined expertise of many fields from molecular biology to chemical and physical oceanography contribute to the development of the knowledge platform upon which marine biotechnology applications render goods and services for public benefit.

With its vast human capital, long coast line and tropical seas, marine biotechnology in India has the great potential to emerge as the major cutting edge technology next to the information technology. Post green revolution era showed glaring inadequacies in the agricultural sector alone to produce the food for the ever-increasing Indian population. Hence, we have to look to alternatives such as mariculture (growing/farming animals and plants in marine environment) for meeting increasing demand for protein source. When the global mariculture production touched about 20 million tons with a value of $ 26.2 million and marine bioprospecting is estimated at somewhere between US $ 30-100 billion, the marine biological wealth of India remains largely untapped even today. The effort of farming of marine organisms for food and exploitation of sea for other commercial use is only in the nascent stage in India, initiated by institutions like Central Marine Fisheries Research Institute (CMFRI).

Marine biotechnological research and development in India

India is bestowed with vast stretches of marine and brackishwater habitats and their bountiful living resources. Indian coasts that extend to about 5,700 km on mainland and to about 8,118 km including two groups of islands (Lakshadweep and Andaman & Nicobar) have rich diversity of corals, fishes, crustaceans, molluscs and seas of microbes. The Western coastline (covering Gujarat, Karnataka, Maharashtra, Kerala and Goa) has a wide continental shelf having an area of about 0.31 million km, which is marked by backwaters and mud flats. The East coast consists of Tamil Nadu,

Pondicherry, Andhra Pradesh, Orissa and West Bengal, is flat, deltaic and rich in mangrove forests covering an area of about 1,430 km. Asia's largest brackish water lake Chilka, Andaman & Nicobar islands, Lakshadweep, Gulf of Kutch and Khambat and Gulf of Mannar are also important biodiversity reserves.

The Government of India, in order to promote biotechnology, relevant to the needs and priorities, constituted an agency, viz. the National Biotechnology Board (NBTB) in the year 1982 under the Ministry of Science and Technology, as an apex coordinating body to identify priorities, coordinate, oversee and plan for required manpower, integrated industrial development and large scale use of biotechnology products and processes later upgraded in the year 1986 into a full fledged separate Department under the Ministry of Science and Technology, viz. Department of Biotechnology (DBT). The DBT has set up a task force on aquaculture and marine biotechnology, under which the projects are funded for utilizing marine resources for exploitation and products and processes from marine organisms utilizing biotechnology tools and techniques. Marine biotechnology in India primarily targets research and development in the areas of mariculture, prospecting for beneficial biomolecules from marine sources and development of bio-processes for ensuring food security, sound health and clean environment to its people. The progress in some of these areas and the prospects of application of marine biotechnology are presented under different headings.

Genetic improvement of mariculture stock

Success in aquaculture/mariculture ventures purely depends on the sound management and use of superior germplasm with high production potential. Though one can bring about improvement in the production performance by environmental manipulations as well as through genetic manipulations, any improvement from the former cannot be transmitted to the next generation unless and until the improvement is inherited. Though a number of modern genetic manipulations techniques like genetic engineering as well as chromosomal engineering for production of polyploid, gynogenic and androgenic populations are available for genetic improvement, the quantitative genetics is still an attractive technique for production of genetically superior brood stock.

Stock improvement through quantitative genetic tools

Quantitative genetic approach for production of genetically superior brood stock is a time tested technique. In farm animals and plants, application of quantitative genetic principles and genetic tools has lead to increased production in accordance with the demands of the nation. The wide variations among the individuals of a population provide ample scope for genetic manipulations for developing genetically superior lines and strains.

Selective breeding

Selective breeding is a potential technique can be applied to develop genetically improved strains of finfish, shellfish and live feeds for aquaculture ventures. However, an in-depth knowledge of the genetics of the species in question is a pre-requisite for the formulation of suitable techniques for their improvement. Knowledge of the genotypic and phenotypic parameters is vital for any quantitative genetic improvement programmes. Estimates of parameters such as heritability, phenotypic and genetic correlations, heterosis, genotype environment interactions etc. are essential for planning a proper breeding strategy. Scientific breeding programme could be formulated only after careful consideration of these parameters. As for example, when there is relatively larger

additive genetic variance, simple selection methods like individual/mass selection should yield good progress. On the other hand if non-additive genetic variance is predominant, special selective breeding methods are to be formulated to exploit them. When over dominance is important for a trait, reciprocal or recurrent reciprocal selection needs to be employed. If heterosis is found to be high, cross breeding programmes could be given priority. Genotype environmental interactions of high magnitude call for developing different strains to suit each of the environments.

Genetic improvement of mariculture stock and live feeds are still remains a gray area in our country and only very limited reports are available on the application of quantitative genetics for genetic modification of marine species in India. The initiative in quantitative genetic work on species of mariculture importance has come from CMFRI, India, to study the quantitative genetic parameters as well as the genetic and phenotypic response to selection in *Artemia franciscana* from Great Salt Lake, Utah using mass selection for genetically altering the naupliar size (ìm) and was found very successful. The substantial genetic gains realized from selection indicated the usefulness of selective breeding for developing genetically altered lines. No comparable work in *A. franciscana* was available.

Inbreeding and crossbreeding (hybridization)

These are two traditional breeding approaches that have been successfully used for the improvement of crops and livestock. Inbreeding is often combined with hybridization to improve the results of the crossbreeding programme. Crossbreeding is a breeding programme that tries to find mating combinations between different populations of fish which produce superior offspring for grow-out, offspring that are said to exhibit hybrid vigor. Although crossbreeding is a tried and true method of increasing yields, the results of crossbreeding programmes are impossible to be predicted (unless the mating has been made previously). Many combinations often have to be evaluated before a combination that produces offspring with hybrid vigor is discovered. Crossbreeding programmes usually involve different strains within a species (intraspecific hybridization), but different species can also be hybridized (interspecific hybridization). To date, much of the breeding work in aquaculture has been devoted to hybridization among the different species of tilapia in an attempt to produce all hybrids for grow-out. In general, crossbreeding is used to produce superior fish for grow-out, while genetic selection is used to create superior brood fish. The hybrids that are created in a crossbreeding programme are usually grown and sold as food. On the other hand, brood fish that are created in a selective breeding programme are created to produce the next generation of genetically superior fish for grow-out and their offspring can, in turn, be retained and selected to continue the process.

Chromosomal engineering for production of superior stock

In recent years, chromosome engineering research had led to the development of animals with controlled sex and reproductive characteristics. These include the induction of polyploidy, androgenesis and gynogenesis.

Ploidy manipulation

Ploidy manipulation has received much attention, mainly because of its potential advantages in *Crassostrea virginica* successfully. Thereafter, triploidy was induced in *C. gigas, C.virginica, Saccostrea glomerata* and *Ostrea edulis*. In India, CMFRI has successfully developed triploid edible oyster and reared.

Gynogenesis

Gynogenesis is a special kind of reproductive process facilitating the inheritance of maternal genetic material alone into the progenies. Gynogenesis occurs naturally in some fish species but it can be induced artificially. The research in gynogenesis may help to (i) produce all-female populations, (ii) establish isogenic and homozygous lines of fish (iii) increase higher production at least in some species and (iv) understand the genetic regulation of development and construction of linkage maps.

Androgenesis

Androgenesis is a developmental process facilitating the inheritance of paternal genetic material. Androgenesis may prove useful technique for production of (i) viable YY supermale in male-heterogametic species, (ii) inbred isogenic lines and (iii) conservation of germplasm. Androgenesis has successfully induced viable YY supermales in a few cyprinids, cichlids and salmonids.

Biotechnological manipulation of sex and reproduction

Biotechnology can be applied to enhance reproduction and early development of cultivated aquatic organisms. This is particularly important because of the difficulty to depend on the vagaries of nature for procuring spawners for the year round hatchery operation. Many important cultured marine species fail to breed in captivity. This bottleneck has been overcome by biotechnological application, e.g., hypophysation using hormones and synthetically produced analogues. The most common breeding programmes in aquaculture are the production of sex-reversed brood stock to produce monosex populations for grow-out. This is done either because one sex is superior or more desirable or to prevent reproduction during grow-out. In aquaculture a particular sex preference is usually linked with desirable traits, such as faster growth rate. In India, attempts are being made to produce tetraploids by ploidy manipulation in *Oreochromis* spp. The rationale for the induction of such ploidy with differing genomic status in a number of fish is its potential to generate genetically sterile population and rapidly inbred lines, which could ultimately benefit the aquaculture sector.

Cryopreservation of gametes and embryos

Some important cultured species, such as Seabass (protoandrous) and groupers (protogynous) are sequential hermaphrodites in which getting the inverted sex in mature condition from the wild is almost always an uphill task. Here too, modern biotechnological innovations have significant role to play for ensuring the availability of reproductive elements from both sexes of broodstock ready at the same time for spawning. The problem of lack of synchronization of maturity in male and female fishes can be overcome through establishment of sperm or seed banks. Although sperm cryopreservation is well known, research is needed for cryopreservation of ova/embryos of cultivable species. Utilization of cryopreserved gametes in aquaculture would greatly reduce the cost of maintenance of broodstock and also permit free exchange of superior quality stock between different geographical origins. The studies on cloning through embryonic stem cells are the one of the emerging alternative technology, which can be used in the field of fish biodiversity and conservation of species.

Genetic engineering

Genetic engineering is being increasingly attempted for producing superior genetic stock. Production of transgenic with desirable traits such as faster growth, disease resistance and increased environmental tolerance is an active and important research area in aquaculture. The commercial

potential of transgenic fish lies in developing transgenic broodstock lines with desired traits. This requires combining transgenic technology with traditional selective breeding program to produce superior strains of fish. Transgenic salmon has been reported to adapt itself in cold climate when a gene encoding antifreeze protein was used for transgenesis. In India preliminary success has been reported in developing gene transfer technology in zebra fish, medaka and Indian catfish.

Although genetic engineering has generated much publicity, this type of breeding programme is very expensive, highly regulated and requires trained scientists. Commercialization of transgenic technology for food production has run into problems due to the concerns of safety and ethics. The gene transfer efficiency needs to be improved further along with the lookout for more useful novel genes. Due to the ethical issues related to production of transgenic food fishes, researches have diverted into the production of genetically modified ornamental fishes for aquarium keeping. The glaring examples is the use of tiny aquarium zebra fish, a popular laboratory animal, genetically modified to produce fluorescent pigments viz. red, green, yellow and is being promoted as a household aquarium pet, the 'glofish'. Ornamental transgenics in India has a promising future with large number of freshwater and marine ornamental fishes. The recent success of CMFRI in closing the life cycle of clown fish, a popular marine ornamental fish, is an achievement in this line of research.

Marker assisted selection (MAS)

A wide array of molecular markers, such as allozymes, mitochondrial DNA, RFLP, RAPD, AFLP, microsatellite, SNP and EST's are being used for characterization of aquatic animal populations. The use of molecular markers include identification of individual animals in broodstock populations, determination of genetic diversity between randomly selected animals, identification of the broodstock parents of post larvae or juvenile progeny displaying desired traits, identification of siblings and half-siblings in a mixed parentage spawn. **Identification of genetic relatedness,** genetic diversity, pedigree determination, **molecular tagging**, tracking family and population lines, strain identification are the potential application of markers. Determination of markers linked to **Quantitative Trait Loci (QTL) of economical important traits is envisaged to have beneficial role in the development of** genetically superior broodstocks.

As most of the production traits are controlled by multiple genes and inherited as quantitative traits, analysis of their associated quantitative trait loci (QTL) is emerging as a very important part of aquaculture genetics/genomics. Marker assisted selection refers to a selection process in which future breeders are chosen based on genotypes using molecular markers. To implement MAS, researchers need to produce high-resolution linkage maps, understand the number of QTL affecting a given performance or production trait and their mode of inheritance and relative contribution, determine the linkage and potential interactions of different QTL for the trait and for other traits and estimate the economic importance of each trait. This in turn would lead to even more precise selection by gene assisted selection (GAS), in which future breeders could be chosen according to favorable genotypes based on genes directly controlling performance traits, rather than on neutral markers associated with those traits via linkage.

Molecular Taxonomy

Molecular taxonomy is the identification of specimens based on molecular rather than morphological characters. Molecular techniques have become a major tool for systematic

ichthyologists at the species level and above. These approaches may also be useful to fishery biologists for taxonomic ambiguities ranging between the species and population levels.

DNA sequence analysis is a powerful tool for identifying the source of samples thought to be derived from threatened or endangered species. CMFRI has generated partial DNA sequences of mitochondrial cytochrome b and control region (D- loop) of 11 species of cetaceans (marine mammals), bottlenose dolphin, spinner dolphin, spotted dolphin, common dolphin, humpbacked dolphin, Risso's dolphin, finless porpoise, sperm whale, blue whale, Bryde's whale and dugong. Till date the Institute has released a total of 63 sequences of cytochrome b gene and control region of mtDNA from 40 individuals of 11 species in the GenBank (NCBI). Gender identification is of fundamental importance in the studies of population structure, social organization, distribution, behaviour or heavy metal accumulation in marine mammals. However, distinguishing the males and females among the marine mammals is difficult due to the poor sexual dimorphism, especially during their free ranging state. With the advent of DNA-based tools, such as PCR, it is possible to even identify the sex using tissue sample. Recently CMFRI has developed sex determination technique based on the amplification of genomic DNA extracted from the skin tissues of marine mammals. Molecular sexing was standardized by the Institute in several species of dolphins, finless porpoise, whales and dugong. Molecular taxonomy could also prove to be a promising tool in mussel farming for larval identification, regardless of developmental stage and can be used for the assessment and tracking of larval dispersion as well as assist in the identification of sites of seed settlement for their optimum exploitation.

Nutrition and feed biotechnology

Nutritional biotechnology applications in livestock feed sector itself is low in India. It is lowest in the aquaculture sector and in the area of marine biotechnology is only getting initiated. Major areas were biotechnology research outputs have translated into products available in the open market are, enzymes, probiotics, prebiotics, dietary aminoacids, toxin binders and nucleotides.

Enzymes

Improvement in nutrient availability and reduction in waste outputs are the major advantages of using enzymes in feed. These enzymes are mostly from microbes, which are genetically modified or biochemically refined to produce the enzymes in large quantities with desired properties to make them economically viable. Enzymes used for the formulated feeds need to be robust to stand variations in physiochemical parameters. They need to have high temperature stability to withstand pelletization and also have a long shelf life.

One of the most promising enzyme is phytase that breaks down the indigestible phytic acid (phytate) in plant based nutrient sources such as cereals and oilseeds and releases digestible phosphorus. This reduces the use of expensive supplemental inorganic phosphorus like dicalcium phosphate in feeds. Addition of inorganic phosphorus results in excessive excretion of phosphorus in manure, posing an environmental concern, especially in areas of intensive animal production. Phytase also releases minerals (Ca, Mg, Zn and K), amino acids and proteins, which are complexed with the phytate molecule.

Seaweeds utilization

The Indian coastal line offers wide scope and suitable environment for sea weed culture. Nearly 10 – 12% of the total marine algae available in different parts of coastal areas come under the category of important seaweeds belonging to green, red, blue and brown varieties. Most of the seaweeds are rich source of bioactive compounds, trace elements, minerals and proteins which finds their extensive application in the field of food & beverages and pharmaceutical industries. The cultivation of these seaweeds in turn will augment the social and economic status of coastal people

Probiotics

Microorganisms are natural inhabitants in the digestive system of the animals. Some microbes aid beneficial and others can cause pathogenesis. Understanding the microbial ecology of the gut merits great attention due to implication of gut ecology for nutrition, feed conversion and disease control. Use of antibiotics disturbs the microbiological balance of gut flora and which leads to the elimination of the major beneficial flora. On stopping the antibiotic treatment, pathogens begin to reestablish themselves in the intestine. Overgrowth of these organisms and subsequent invasion of the system by pathogenic organisms cause inflammatory, immunological, neurological and endocrinological problems. Probiotics are "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". Application of Probiotics can help build up the beneficial bacteria in the intestine and competitively exclude the pathogenic bacteria. These bacteria also release enzymes, which help in the digestion of feed. The concept of using probiotics or direct fed microbials in animal feed particularly poultry and aquaculture is slowly becoming popular. The common organisms in probiotic products are *Aspergillus oryzae, Lactobacillus acidophilus, L. bulgaricus, L. plantarium, Bifidobacterium bifidium, Streptococcus lactis* and *Saccharomyces cerevisiae*. These products can be administered through water or incorporated in the feed. Indian researchers have successfully isolated indigenous microbes from saline waters with probiotic properties with potential application in aquaculture/mariculture.

Prebiotics

The concept of prebiotics in feed is fairly recent. Prebiotics are basically feed for probiotics where they are resistant to attack by endogenous enzymes and hence reach the site for proliferation of gut microflora. Some of the prebiotics, which are currently used in animal feed, are Mannan-oligosaccharides (MOS), fructo-oligosaccharide and mixed oligo-dextran. The concept of using prebiotics has not yet been accepted but the advantages of prebiotics are that it can stand high pelletizing temperatures in the feed and also have a long shelf life.

Bioremediators

Beneficial microbes have also been used in a big way as pond cleaners or bioremediators in aquaculture. Bacteria directly uptake or decompose the organic matter or toxic material and improve the quality of water. The microbial cultures can produce a variety of enzymes such as amylase, protease, lipase, xylanase and cellulase in high concentrations than the native bacteria, which help in degrading waste. The pond probiotics also have a special blend of denitrifying bacteria that remove the algal primary source of food, nitrogen from the water. This drastic reduction in nitrogen concentration makes it difficult for the algae to bloom thereby maintaining pond health. Commercial

bioremdiators such as *Detrodigest* developed by research institution such as national centre for aquatic animal health (NCAAH), Cochin, using indigenous bacterial isolates has already in use by the aquafarmers in India.

Dietary amino acids

Essential aminoacids are added as supplement to the feed to get a balanced aminoacid profile. Since the aminoacid profiles of the ingredients do not match the profile of aminoacid requirement of the specific species, supplemental essential aminoacid are added. The new trend is to formulate diets on digestible amino acid levels thereby reducing the requirement of protein. So far lysine and methionine have been used as supplements. Genetically enhanced micro-organisms are being used to produce threonine and tryptophan on a commercial basis. Using all these amino acids it is possible to lower dietary crude protein level by 2 – 3 %, which is a substantial saving for the farmer.

Toxin Binders

Feed manufacturers have been incorporating various mold inhibitors in their diets to prevent mycotoxin formations. A variety of physical, chemical and biological approaches to counteract the mycotoxin problem have been reported, but largescale, practical and cost effective means for detoxification of mycotoxins containing feed stuffs are limited. Most of them are fungistats and not fungicides that is they only inhibit growth of moulds and do not inactivate any toxins already present. Present day methods are, use of organic acids and their salts like propionic acid or adsorbents like bentonites, zeolites, and hydroxyl aluminosilicates. In the future, biotechnology based products like microbes, herbal extracts or esterified glucomannan could be used. Aqua extracts of garlic, onion, turmeric, neem have been shown to exert antifungal activity or inhibit aflatoxin production.

Nucleotide nutrition

Nucleotide nutrition research in fishes is in its infancy and many fundamental questions remain unanswered, observations so far support the contention that nucleotides are conditionally or semi-essential nutrients for fishes and further exploration of dietary supplementation of nucleotides for application in fish culture is warranted. Hypothesized reason(s) associated with these beneficial effects include dietary provision of physiologically required levels of nucleotides due to limited synthetic capacity of certain tissues (e.g. lymphoid), inadequate energetic expenditure for de novo synthesis, immunoendocrine interactions and modulation of gene expression patterns. However, currently there are numerous gaps in existing knowledge about exogenous nucleotide application to fish including various aspects of digestion, absorption, metabolism and influence on various physiological responses especially expression of immunogenes and modulation of immunoglobulin production.Encapcell®., a patented nucleotide is a new, microencapsulated feed additive that enhances growth and aids in preventing disease. Incorporation in animal diets, it gives better weight gains, improves feed conversion, aids in reducing mortalities, improves general health and dramatically increases antibody production when used with vaccines.

Nutrigenomics

Functional genomics refers to how the genome of an organism regulates homeostasis and responds to stimuli. The application of functional genomics in nutrition research is known as

nutrigenomics. Influence of feed (nutrients) on the organism at a molecular level is the simplest explanation of nutrigenomics. With the development of cutting edge tools in molecular biology monitoring the affect of a nutrient at the molecular level is reality now. The regulatory control mechanisms of these processes can be based at all levels from genetics and gene expression to the feedback of specific metabolites. Modern technology is providing a new opportunity to monitor the regulation of these processes on a system wide basis. Nutrition researchers are just beginning to utilize these tools to ask key scientific questions about diet and its effects on the organism using functional genomics. The mechanism by which nutrients specifically regulate the expression of genes in vertebrates in general is poorly understood. These technologies have significant implications for nutrition research and include aspects of genomics (polymorphism), functional genomics (gene expression), proteomics (protein expression), and bioinformatics (data storage and integrated data analysis).

Fish Health management

Aquaculture health management using novel diagnostic tools for the early and rapid detection of disease causing pathogens and screening of the broodstock and seeds is very important for sustainable and economic viability of an aquaculture industry. The current knowledge on pathogens affecting marine organisms has mostly come from the disease conditions reported on the maricultured finfish and shellfish, from the overseas. Majority of these diseases are listed by international disease commission (OIE), as the epizootics that can cause serious damage to the rearing species. CMFRI has recorded initial success in developing the hatchery technology for marine ornamentals, bivalves such as green mussels, edible and pearl oysters and sand lobster and grow out for these species and hatchery technology for groupers, rabbit fishes are only under experimental stages. Effort to collect research data on the pathogen profile of these selected species in wild or in captivity is a priority as the entire broodstock of finfishes and shellfishes are sourced from the wild.

Conventional methods of pathogen detection have its own limitation with reference to specificity, sensitivity and speed. The classical methods such as, clinical examination, light microscopy and histopathology, culture methods for the isolation and identification of the microbes, electron microscopy and cell culture methods failed to deliver an early diagnosis in cases of subclinical and asymptomatic disease manifestations, especially in the cases of viral etiologies.

Nucleic acid based diagnosis

Nucleic-acid based diagnostics are the first line of biotechnology that found direct application in the fast growing aquaculture/mariculture arena. The very basic of the nucleic acid based diagnosis rests in the unique genetic make up or the genetic fingerprint of each organism or pathogen in the form of DNA or RNA. Information generated on the DNA sequence, can be directly used for the development of DNA/RNA based diagnosis.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) has been accepted as the most sensitive and useful diagnostic tool for the pathogen detection. This simple *invitro* DNA synthesizing technique has opened up new vistas in the disease diagnosis and health management. The DNA based diagnostic methods such as PCR and gene probes have emerged as the integral part of the fish health in aquaculture/

mariculture, in quarantine, biosecure systems, specific pathogen free (SPF) stocks, hazard analysis and critical control point (HACCP) and pathogen monitoring. In India institutions such as Central Marine Fisheries Research Institute (CMFRI), Kochi, Central institute of Brackishwater aquaculture (CIBA), Chennai and College of fisheries Mangalore are the fore runners in the development and commercialization of DNA based diagnostic kits.

DNA vaccines

DNA vaccines are made of a modified form of an infectious organism's DNA. This material, when introduced into an organism, leads to the expression of the genes within this modified, foreign DNA. This gene expression ultimately leads to synthesis of infectious organism proteins inside the host system. As a result, the defense system of the host responds in a protective manner almost the same way as would occur if the organism were actually infected by the true organism, itself. Most of DNA vaccines are still at the early investigative stages and have not yet reached the level of a product in the field.

Phage therapy

Phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections. The treatment uses a phage virus to infect and kill specific bacteria whilst not interacting with the surrounding tissue or with other harmless bacteria. The virus replicates quickly so a single, small dose is usually sufficient. Researchers from, Mangalore Fisheries College, National centre for aquatic animal health (NCAAH), Cochin and Central Institute of Brackishwater Aquaculture (CIBA), Chennai have already isolated phages of pathogenic vibrios, and efforts are underway for the development of treatment regimes against the pathogenic vibrios causing vibriosis in fish and shellfish rearing systems.

RNAi

RNA interference (also called "RNA-mediated interference", but abbreviated RNAi) is a mechanism for RNA-guided regulation of gene expression that is common in eukaryotic cells. RNAi involves short chains of double-stranded ribonucleic acid (dsRNA) interfering with the expression of genes with sequences complementary to this dsRNA. RNA interference is a form of posttranscriptional gene silencing in which dsRNA binds to specific mRNA and induce degradation of the homologous endogenous transcript, resulting in the reduction or loss of gene activity without there being detectable effects on the expression of genes unrelated in sequence. It is possible to silence specific genes in cell cultures, by transfecting the cells with predesigned dsRNA or siRNA with strict homology to the mRNA targets. An RNAi is also a promising tool for the control of viral disease among aquacultured organisms, through blocking the mechanism of viral multiplication.

GMOs for disease resistance

A genetically modified organism (GMO) is an organism whose genetic material has been altered using techniques in genetics generally known as recombinant DNA technology. The only transgenic fish that is commercially available today is not designed to be eaten, but a zebrafish that glows (GloFish™) for aquarium keeping. Research efforts are underway to induce genetic modifications, in various fish species, to develop fish and shellfishes with better resistance to infectious diseases.

Marine bioprospecting

Bioprospecting describes the systematic search for and development of new sources of chemical compounds, genes, micro and macro organisms, and other valuable products from nature and incorporates two fundamental goals (1) the sustainable use through biotechnology of biological resources and their conservation, and (2) the scientific and socioeconomic development of source countries and local communities. The potential applications offered by the screening of marine substances extend to pharmacology, agrochemistry and the environment. Marine organisms are the sources of: pharmaceuticals, enzymes, cryoprotectants, cosmaceuticals, agrichemicals, bioremediators, nutraceuticals etc. Marine drugs can be used as antioxidant, anti-fungal, anti-HIV, antibiotic, anti-cancer, anti-tuberculosis and anti-malarial.

Developing novel drugs for treating disease

Overuse of broad-spectrum antibiotics has resulted in the emergence of antibiotic-resistant pathogens, and also in dramatic (and dangerous) changes in the normal, protective microflora in humans and aquatic animals. There is a need to find new, effective antibiotic substitutes. This has prompted the constant search for and development of novel anti-infective agents.

Moreover, the use of combined approaches enhances these possibilities because marine molecules often belong to new classes without terrestrial counterparts; for example, halogenated compounds. Secondary metabolites produced by marine bacteria and invertebrates have yielded pharmaceutical products such as novel anti-inflammatory agents (e.g. pseudopterosins, topsentins, scytonemin, manoalide) anti-cancer agents (e.g. bryostatins, discodermolide, eleutherobin and sarcodictyin) and antibiotics (e.g. marinone). Melanins have a range of chromophoric properties that can be exploited for sunscreens, dyes and colouring. They also sequester different kinds of organic compounds, inducing fungicides and antibiotics, which may allow them to act as slow-release agents. The lysate from the horseshoe crab provides the major assay for bacterial endotoxin. Antioxidant peptides have been isolated from protease digests of prawn muscle, and the mycosporine amino acid (MAA) precursor, 4-deoxy-gadusol, has been isolated from seaweeds; these have applications as food additives and in cosmetics. A “living fossil”, the horseshoe crab, has furnished us with useful biochemicals and insights. Its circulating cells (amoebocytes) contain molecules that react with the lipopolysaccharide of Gram-negative bacteria, and thus have been of use in detecting early infection in humans as well as traces of LPS (pyrogens) in biotechnological products.

Enviromental biotechnology

Biosensors

Microorganisms provide the basis for development of sophisticated biosensors, and diagnostic devices for medicine, aquaculture and environmental biomonitoring. Intact cell preparations and isolated enzyme systems for bioluminescence are used as biosensors. The *lux* genes encoding these enzymes have been cloned from marine bacteria such as *Vibrio fischeri* and have since been transferred successfully to a variety of plants and other bacteria. The encoded enzymes are expressed only under defined environmental conditions, proving its use in pollution monitoring.

Environmental cleaners

Marine microorganisms, either as independent strains or as members of microbial consortia, express novel biodegradation pathways for breaking down a wide variety of organic pollutants. Marine microorganisms frequently produce environmentally friendly chemicals such as biopolymers and non-toxic biosurfactants that can also be applied in environmental waste management and treatment. Recent findings into the basis of cell-cell communication have shown that this process is involved in biofilm formation leading to environmental corrosion and plugging. This has generated a search for new bioactive molecules that prevent such communication and control subsequent fouling.

Products derived from marine ecosystems

- *Dogfish shark* have yielded squalamine, a potent antibiotic. Because squalamine works differently from today's antibiotics, it may also combat diseases that have become drug-resistant.
- *Sea squirt* produces Didemnin B, a compound that arrests cancers including leukemia, melanoma and those of the ovary, breast and kidney. It also may fight genital herpes and other viruses.
- *Moss animal* contains Bryostatin B, a cancer-fighting compound that has just entered human clinical trials and expected hit the market shortly.
- *Caribbean sponge* provides compounds from which a drug called AraC is synthesized to treat non-Hodgkin's lymphoma and acute myelocytic leukemia.
- *Gray encrusting sponge* emits manoalide, which appears to thwart the inflammation and pain caused by everything from bee stings to arthritis.
- *Bahama sponge* makes discodermolide, an effective immunosuppressive agent that could prevent tissue rejection after organ transplants.
- *Brown algae and kelp* manufacture a compound that seems to prevent duplication by the herpes and human immunodeficiency viruses (HIV). While researchers explore this, some obstetricians use sterilized strips of brown algae in place of metal instruments to dilate the cervix during invasive procedures such as IUD insertion.
- *Red algae* produce carrageenans or sulfate polysaccharides, used to treat peptic ulcers.
- *Horseshoe crab* has blue blood used to detect meningitis, septic shock and other forms of bacterially related conditions. Pharmaceutical companies routinely use the blood to screen for bacterial contamination.
- *Blue mussel* produces an adhesive that provided a model for Cell-Tak. Now used to repair corneas and retinas, it may soon work to secure dentures and dental fillings.
- *Blue crab* has an exoskeleton from which chitin, a crystalline polymer, can be extracted. Chitin is used in absorbable, nonallergenic sutures that remain stable in the alkaline environment of human intestinal and urinary tracts.

Future research needs of Indian Marine Biotechnology

- Profiling of major pathogens in candidates species identified for mariculture and DNA based diagnostics
- Use of probiotic in place of antibiotics to prevent infectious diseases
- Initiate genomics of farmed species with nutrigenomics and pathogenomics for the development of fast growing and disease resistant varieties

- Genetic and genomic studies of pearl oyster and other bivalves
- Quantitative genetic manipulations of cultured fish, shellfish and live feeds
- Development of molecular markers for MAS and taxonomical applications
- Development of ornamental transgenics
- High health formulated feed for better FCR and reduced disease problems
- Bioprospecting for novel products from biotic resources of sea
- Gene mining and metagenomics for developing novel biotech products
- Stem cell and tissue culture research
- Cell lines for marine finfsihes (vertebrates) and shellfishes (invertebrates)

Conclusion

India is yet to translate the benefits to be derived from marine organisms as sources of new products and develop viable strategies to conserve them as an investment opportunity through bioprospecting and marine biotechnology. Presently the potential bioprospecting of marine organisms is less than 1% and hence massive scale screening is essential by giving focus on few selected potential groups like microbes (bacteria and fungi), microalgae and macroalgae (seaweeds). There is urgent need to develop research and development projects with objective to study Indian Ocean flora, fauna, and microbes and to search for substances having physiological ac-tivity, which could serve as models for new drugs, antifouling, signal substances, pheromones, neurotransmitters, and antifertility compounds.

In a project jointly sponsored by the Oceanic Biology Program of the Office of Naval Research (ONR) of the United States Government, the Council of Scientific and Industrial Research (CSIR) and the Department of Science and Technology (DST) of the Government of India, four hundred and fifty extracts from marine organisms collected from the western and eastern coasts of India, Lakshadweep and the Andaman and Nicobar Islands have been evaluated for broad biological activities. Of these, 14 extracts exhibited anti implantation, 28 antiviral, 10 CNS depressant, 31 CNS stimulants, 33 diuretic, 10 hypoglycemic, 16 hypotensive, 11 oxytocic, 9 spasmogenic, and 3 spasmolytic activities**.**

Marine biotechnology is, thus, a cross-sectoral and evolving technology , a counterpart to terrestrial biotechnology and could provide fresh development momentum in several fields, including food production, pharmaceuticals, environment and even nanotechnology. The application of molecular biology can increase aquaculture/mariculture productivity, and is being sought to achieve a better balance with marine ecology. Further, with the exciting reach of genetics and genomics, global research has widened from just aquaculture applications to mainstream marine biotechnology, relates to a better understanding of human life and health. It may be humbling to accept that our nervous systems are functionally close to that of the jellyfish. But the structural simplicity of some marine animals helps explain how humans smell, see and think. Likewise, endowed with nature's largest photoreceptors, the nine-eyed horseshoe crab helps clarify the mystery of vision. Examples abound of marine organisms as biological and biomedical research models that provide insights into disease prevention and treatment.

While countries such as Japan, Norway, US, Australia are the global leaders in marine biotech, it is recognized that among the developing countries, India has considerable strengths, with its research in marine biology and ecology, marine bacteria, screening microorganisms for bioactive compounds, bioremediation and extra-cellular production by microorganisms, micro-algal production of beta carotene, proteins, etc. These lay strong foundation for acquiring global competitiveness in select biotechnological applications and products. This fascination is understandable, as Indian subcontinent bath with three oceans with vast EEZ of about 2.02 million sq km as well as its rich biological wealth remains largely untapped, even today. We cannot, however, ignore that biotechnology confers the ability to manipulate and change the ultimate blueprint of life. So the dangers of unbridled biotechnological interference should never be lost sight of. It is therefore, of paramount importance to debate safety, ethical and intellectual property issues and resolve them amicably.

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An overview of mariculture techniques

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Introduction

It is widely accepted that the catch and catch rates of many marine fishery resources are declining mainly due to overcapitalization and overexploitation.. In this context, it is very much relevant to resort to resource augmentation methods through mariculture and allied techniques to enhance the seafood production. Mariculture is the farming and husbandry of marine plants and animals in marine environments. On a global basis, aquaculture is the fastest growing animal food production sector with per capita supply increasing from 0.7 kg in 1970 to 7.8 kg in 2006 with an average annual growth rate of 6.9%. Most of the global aquaculture production of fish, crustaceans and molluscs continues to come from inland waters (61% by quantity and 53% by value). Mariculture contributes 34% of the total aquaculture production and 36% of the value. While much of the marine production is contributed by high value finfish, relatively low priced mussels and oysters are also widely farmed. While the overall share of farmed fish in marine finfish production has stayed much low, for the species that are farmed, cultured fish dominates the market. This is the case of Asian seabass, gilthead sea bream, red drum, bastard halibut and cobia. It is also a fact that for such species, the quantities now produced by aquaculture are often substantially higher than the past highest catch recorded by capture fisheries. In the last decade, salmonids have overtaken shrimp as the top aquaculture group in Latin America and the Caribbean as a result of outbreaks of disease in the major shrimp producing areas (FAO,2009).

Aquaculture in the Asia-Pacific region has been growing steadily over the last few decades and to satisfy the demand of the local and export markets, many countries are expanding their aquaculture activities in the sea, including offshore areas where competition is less. Mariculture in this region is exceptionally biodiverse and relies on many species and hence the nature of mariculture is rapidly changing in this area (Rimmer, 2008). Some of the countries like China, Vietnam, Australia, Indonesia, and Japan. Korea DPR, Korea Rep, Malaysia, Phillippines, Thailand are much ahead in mariculture in this region and agencies like NACA should take intergovernmental regional programmes so as to develop mariculture in the region as a whole.

Mariculture -Indian scenario

The dwindling catch rates in capture fisheries and rampant unemployment in the coastal region focus towards the development of mariculture and coastal aquaculture as a remunerative alternate

occupation. Recent estimates quantify the per capita fish consumption in India around 8-10kg per year and is likely to grow to 16.7kg by 2015. Although about 1.2 million hectares are suitable for land based saline aquaculture in India, currently only 13 % is utilized. Farmed shrimp contributes about 60% by volume and 82% by value of India's total shrimp export. Share of cultured shrimp export is 82,600 tonnes. The farming of shrimp is largely dependant on small holdings of less than 2 hectares, as these farms account for over 90% of the total area utilized for shrimp culture. Coastal aquaculture is mainly concentrated in the states of Andhra Pradesh, Tamil Nadu, Orissa and West Bengal. In recent years, the demand for mussels, clams, edible oysters, crabs, lobsters, sea weeds and a few marine finfishes is continuously increasing and brings premium price in the international market. The long coastline of 8129 km along with the adjacent landward coastal agro climatic zone and the sea-ward inshore waters with large number of calm bays and lagoons offer good scope to develop mariculture in the country.

In this context, the Central marine Fisheries Research Institute (CMFRI) is the pioneering institution in the country which has initiated mariculture research and has been developing appropriate mariculture technologies in India (Devaraj *et al*, 1999, ICAR, 2000, Pillai and Menon, 2000, Pillai *et al*, 2003, Mohan Joseph 2004 Modayil *et al.* 2008, Gopakumar *et al* 2007). In India till date mariculture activities are confined only to coastal brackish water aquaculture, chiefly shrimp farming. The other coastal aquaculture activities are green mussel farming which is confined to Malabar Coast in Kerala producing about 10,000 tonnes and seaweed farming along Ramanathapuram and Tuticorin coasts of Tamilnadu producing 5000 tonnes annually.

The potentially cultivable candidate species in India include about 20 species of finfishes, 29 crustaceans, 17 molluscs, 7 seaweeds and many other species of ornamental and therapeutic value. Many mariculture technologies are very simple, eco-friendly and use only locally available infrastructure facilities for construction of farm, feed and seed and hence the entire farming can be practiced by traditional fishermen. Another advantage is that most of our brackish and coastal areas are free from pollution and suited for aquaculture. But hardly 10% of the potential cultivable area is presently used for aquaculture in spite of growing demand for cultured shrimp, bivalves, crabs, and lobsters etc., all of which are in high demand in the export market. In addition a fast growing trade of marine ornamental fishes and other tropical marines has also emerged in the recent years which open up the possibility of culture and trade of these organisms. . The policies pertaining to advent of alternative avocations to fishers by providing the awareness, training and initial resource capabilities can do better in the way of providing flexibility to other sectors. Employment in aquaculture (inland and marine) has been increasing and is now estimated to account for about 25 percent of the total. (Govt. of India, 2001).

Coastal aquaculture is a significant contributor to marine fish production, constituting mainly the shrimps like *Penaeus monodon* and *P. indicus.* However, vast water bodies highly suitable for aquaculture and the varied biodiversity that has the potential to capture new markets with a wide range of seafood products, have prompted consideration of other candidate species like oysters, mussels, crabs, lobsters, scampi, sea bass, groupers, sea cucumber, ornamental fishes and sea weeds in the new aquaculture scenario in the country. Hatchery and rearing techniques have also been standardised for many of these organisms. (ICAR, 2000)

Existing major mariculture practices

Shrimp seed production and culture

Shrimps being a highly valued export commodity, shrimp farming is considered a lucrative industry. Production-wise *Penaeus monodon* contributed 75% and *F. indicus* 20%. Depending on the area of the pond; inputs like seed, feed and management measures like predator control, water exchange through tidal effects or pumping, etc., farming systems have been classified into four groups: extensive, modified extensive, semi-intensive and intensive. Currently, 80 per cent of the shrimp production comes from small and marginal holdings, with farms of less than 2 ha constituting 49.2 per cent of the total area under culture, between 2-5 ha (15.8 per cent), 5-10 ha (13 per cent) and the rest >10 ha. The farming community has now become more responsive to the concepts of environment-friendliness and sustainable aquaculture. Disease problems are being overcome through adoption of closed system of farming (recirculation system, zero water exchange) in grow outs, application of probiotics, secondary aquaculture of selected fishes like mullets, milkfish, molluscs and seaweeds in reservoirs and drain canals, adoption of indigenous, good quality seed and feed and reduction in stocking density.

Lobster farming and Fattening

Increasing demand for live lobsters in the export market led the farmers and entrepreneurs to collect juvenile lobsters from the wild and grow to marketable size in ponds and tanks by feeding trash fishes and other discards. In many maritime states juvenile lobsters of *Panulirus homarus, P. ornatus* and *P. poyphagus* are grown in captivity and the eyestalk ablated lobsters attained 180 – 200 g in 5 – 6 months period. This type of lobster fattening at a stocking density of 10 – 15 young ones per square meter yielded appreciable growth rates with a profit margin of Rs.50, 000/- from a pond of 70 m^2. Fattening and grow out trials with artificial pellet feeds has been successfully completed. Cage farming of spiny lobsters was successfully demonstrated by CMFRI at Vizhinjam, Mandapam and Veravel. Recently major breakthrough in breeding and hatchery production of two species of scyllarid lobsters, *Thenus orientalis* and *Petrarctus rugosus* was achieved. Successful hatchery production of seeds of *T. orientalis* and its compatibility with *F. indicus* at high density race way culture with very high production rates of 3-5kg/sq.m is highly promising..

Crab farming / fattening

Live mud crabs (*Scylla serrata*, *S. tranquebarica)* being a much sought after export commodity, mud crab fattening is considered the best alternative. Seed stock consists of freshly moulted crabs (water crabs) of 550 g which are stocked in small brackishwater ponds at a stocking density of 1/sq. m or in individual cages for a period of 3-4 weeks while being fed thrice daily with low value fish @ 5-10 per cent of their biomass. Selective harvesting is done according to size, growth and demand and the venture is profitable because of low operating costs and fast turnover. Monoculture (with single size and multiple sizes stocking) and polyculture with milkfish and mullets are being carried out on a small scale, as the seed supply is still mainly from the wild. Hatchery technology for breeding and seed production of the blue swimming crab, *Portunus pelagicus*, has also been developed and four generations of crabs have been produced by domestication. Fattening and grow out trials with artificial pellet feeds has been successfully completed. The hatchery seed is being mainly utilized for stock enhancement programmes along the east coast.

Edible Oyster Farming

CMFRI has developed methods for edible oyster (*Crassostrea madrasensis*) culture and has produced a complete package of technology, which is presently being widely adopted by small scale farmers in shallow estuaries, bays and backwaters all along the coast. In the adopted rack and ren method, a series of vertical poles are driven into the bottom in rows, on top of which horizontal bars are placed. Spat collection is done either from the wild or produced in hatcheries, on suitable cultch materials. Spat collectors consist of clean oyster shells (5-6 Nos.) suspended on a 3 mm nylon rope at spaced intervals of 15-20 cm and suspended from racks, close to natural oyster beds. Spat collection and further rearing is carried out at the same farm site and harvestable size of 80 mm is reached in 8-10 months. Harvesting is done manually with a production rate of 8-10 tonnes/ha. Oyster shells are also in demand by local cement and lime industry and culture production has increased to 800 tonnes in the year 2000.

Mussel Farming

The Institute has developed technologies for culture of bivalves like raft method (in bays, inshore waters), rack method (in brackishwater, estuaries) or long line method (open sea) are commonly adopted for mussel farming (*Perna indica* and *P. viridis*). Mussel seeds of 15-25 mm size collected from intertidal and sub tidal beds are attached to coir/nylon ropes of 1-6 m length and enveloped by mosquito or cotton netting. Seeds get attached to rope within a few days while the netting disintegrates. The seeded ropes are hung from rafts, racks or longlines. A harvestable size of 70-80 mm is reached in 5-7 months and production of 12-14 kg mussel (shell on) per metre of rope can be obtained. Attempts to demonstrate the economic feasibility of mussel culture has led to the development of group farming activities in the coastal communities (especially rural women groups) with active support from local administration and developmental agencies like Brackishwater Fish Farmers Development Agency (BFFDA) and State Fisheries Department. Cultured mussel production has increased from 20 tonnes (1996) to 18,000tonnes (2009) mainly through the rack culture system in estuarine area.

Pearl Oyster Farming and Pearl Production

In India, the marine pearls are obtained from the pearl oyster, *Pinctada fucata*. Success in the production of cultured pearls was achieved for the first time in 1973 by CMFRI Raft culture and rack culture in nearshore areas are the two methods commonly adopted for rearing pearl oysters and recently attempts have been made to develop onshore culture methods. Shell bead nucleus (3-8 mm) implantation is done in the gonads of the oyster through surgical incision while graft tissues are prepared from donor oysters of the same size and age group. Implanted oysters are kept under observation for 3-4 days in the labs, under flow through system and then shifted to the farm in suitable cages for rearing. Periodic monitoring is done and harvest is carried out after 3-12 months. Pearls are categorized into A, B and C types depending on colour, luster and iridescence. 25 per cent pearl production has been successfully demonstrated in a series of farm trials at various locations along the Indian coast. Research is also directed towards development of a technology for *in vitro* pearl production using mantle tissue culture of pearl oyster. The technology for mass production of pearl oyster seed and pearl production has paved the way for its emergence as a profitable coastal aquaculture activity at certain selected centres along the coast. Village level pearl oyster farming

and pearl production, through direct involvement of small scale fishermen have been carried out successfully as part of technology transfer programme along the Valinokkam Bay on the east coast. Recently success has been obtained in the production of Mabe pearls and tissue culture of pearls. Success was achieved in the organ culture of mantle of pearl oyster and abalone. A breakthrough has been achieved by developing a tissue culture technology for marine pearl production using the pearl oyster *Pinctada fucata* and abalone *Haliotis varia* for the first time in the world. This technology can be easily extended to other pearl producing molluscs and gives ample scope for manipulation of pearl quality and also increased pearl production. Mabe pearl production was standardised for production of base images with ten different types of moulds. Technology for production of jewellery from Mabe pearl was also standardised.

Clam Culture

Package of clam culture practices has been developed for the blood clam *Anadara granosa* and *Paphia malabarica*, where production of 40 tonnes/ ha/6 months and 15-25 tonnes/ha/4-5 months have been achieved in field trials. Induced spawning and larval rearing to setting of spat has been perfected for clams like *P. malabarica*, *Meretrix meretrix* and *Marcia opima*.

Abalone Culture

Abalones are marine gastropods of the genus Haliotis. They are known for the production of gem quality pearls and also for their succulent meat. *Haliotis varia* is the commercially important species along the Indian coast. CMFRI has developed methods for the seed production and culture of this species.

Marine Finfish Culture

In the area of marine fish seed production and culture, the country is still in the experimental phase only..Seed production technology is available only for the Asian seabass *Lates calcarifer*. The Central Institute of Brackishwater Aquaculture (CIBA) has developed an indigenous hatchery technology for Asian seabass. The Rajiv Gandhi Centre for Aquaculture (RGCA) has also been propagating the seed production and farming techniques in the country. Recently CMFRI has successfully demonstrated the cage farming of sea bass at different parts of the coast. The broodstock development and spawning of the grouper *Epinephelus tauvina* was achieved at CMFRI. Attempts are being made to develop suitable hatchery and farming technology for cobia, mullets, pearl spot, rabbitfish, groupers, snappers, breams and pompano. The broodstock development of cobia in cages and induced spawning and fingerling production was achieved for the first time in India at Mandapam Regional Centre of CMFRI. The standardisation of fingerling production of cobia can lead to the development of cobia aquaculture in the country.

Ornamental Fish Culture

On a global basis a lucrative marine ornamental fish trade has emerged in recent years which have become a low volume high value industry. There are a wide variety of ornamental fishes in the vast water bodies and coral reef ecosystems along the Indian coast, which if judiciously used, can earn a sizeable foreign exchange. A long term sustainable trade of marine ornamental fishes could be developed only through hatchery produced fish.

The Central Marine Fisheries Research Institute has intensified its research on breeding, seed production and culture of marine ornamental fishes. One of the milestones in this programme is the recent success in the hatchery production technology of clown fish (Gopakumar *et al.,* 2001a, Ignatius *et al.,* 2001, Madhu 2002, Madhu 2006). Success was also obtained on the broodstock development, larval rearing and seed production of 7 species of damsel fishes (Gopakumar *et al,* 2001b, Gopakumar, 2005). The marine ornamental fishes for which breeding and seed production technologies were developed by CMFRI are the following.

1) *Amphiprion sebae*
2) *Amphiprion percula*
3) *Amphiprion ocellaris*
4) *Premnas biaculeatus*
5) *Pomacentrus pavo*
6) *Neopomacentrus filamentosus*
7) *Neopomacentrus nemurus*
8) *Dascyllus aruanus*
9) *Dascyllus trimaculatus*
10) *Chromis viridis*
11) *Pomacentrus caeruleus*
12) *Chrysiptera cyanea*

The technologies developed have to scale up and demonstrated for commercial level production. Hatchery production and culture of marine tropical ornamental fish can prove to be more economically feasible than that of marine food fish culture, due to the high price per unit of ornamental fish. The clown fishes and damselfishes of the family Pomacentridae offer immediate scope for hatchery production due to the availability of seed production methodologies. .

Seaweed Culture

Around 60 species of commercially important seaweeds with a standing crop of one lakh tonne occur along the Indian coast from which, nearly 880 tonnes dry agarophytes and 3,600 tonnes dry alginophytes are exploited annually. Seaweed products like agar, algin, carrageenan and liquid fertilizer are in demand in global markets and some economically viable seaweed cultivation technologies have been developed in India by CMFRI and Central Salt and Marine Chemical Research Institute (CSMCRI). CMFRI has developed technology to culture seaweeds by either vegetative propagation using fragments of seaweeds collected from natural beds or spores (tetraspores/ carpospores). It has the potential to develop in large productive coastal belts and also in onshore culture tanks, ponds and raceways. The rate of production of *Gelidiella acerosa* from culture amounts to 5 tonnes dry weight per hectare, while *Gracilaria edulis* and *Hypnea* production is about 15 tonnes dry weight per hectare. Recently the culture of the carageenan yielding sea weed *Kappaphycus alvarezii* has become very popular due to its fast growth and less susceptibility to grazing by fishes and is being cultivated extensively along the Ramanathapuram and Tuticorin coasts of Tamil Nadu Commercial level cultivation of *K. alvarezi* has been practised along different parts of Tamil Nadu coast contributing nearly 5000 t dry weight annually.

Open sea cage culture

For the first time in India a marine cage was successfully launched and operated at Visakhapatnam, in the east coast of India by the Central Marine Fisheries Research Institute. Asian seabass (*Lates calcarifer*) was stocked during the first stocking as a trial. Successful harvesting was done after four months. A few demonstration cages are deployed in different parts of our coast with fishermen participation and successful harvests could be made at some places. The standardisation sea cage farming methods along with the commercial level production of fish seed can augment the mariculture production in the country.

Frontier areas of Biotechnological interventions in Mariculture

Broodstock Development

It is well understood that the first step towards seed production technology is the development of best quality broodstock. The ability to manipulate growth rates through the introduction of additional growth hormone (GH) can be applied to develop better broodstock instead of the conventional selective breeding. Dramatic growth enhancement has been shown using the technique in salmonids (Du *et al*., 1992; Delvin *et al*., 1994). An 'all fish' gene construct consisting of ocean pout antifreeze protein (AFP) promoter fused to Chinook salmon GH cDNA was injected into salmonid embryos and due to the availability of transcription factors required for its activation, enhancement of growth in adult salmon to an average size of 3-5 times the size of non-transgenic controls was achieved. Some individuals, especially during the first few months of growth, reached as much as 10-30 times the size of controls (Du *et al*., 1992, Delvin *et al*., 1994). These fish generally appeared healthy, and some produced second and third generation offspring (Saunders *et al.,* 1998). The enhanced growth phenotype was inherited along with the genotypes. The economic advantage of this type of manipulation is obvious and in comparison with selective breeding methods takes very little time for attaining similar success (Melamed *et al*., 2002).

Sex Change

Sex change is common among certain groups of fishes of aquaculture importance like groupers and sea bass and hence knowledge of the mechanisms involved is essential for endocrinological manipulations to induce sex reversal for broodstock development. Simultaneous hermaphrodites function concurrently as both male and female and are capable of releasing viable eggs and sperms during the same spawning event (Helfman *et al*. 1997). In contrast, sequential hermaphrodites function as a male in one life phase and as female in another (Warner, 1988). If the male phase develops first with later sex change into a female, the fish is protandrous; if the female phase develops first, with later sex change into a male, the fish is protogynous. Changing the sex serves to increase the fish's lifetime reproductive success. The ability to change sex is present in at least 23 teleostean families (Helfman *et al.,* 1997) including over 350 species (Munday, 2001) of which most inhabit coral reefs (Reinboth, 1988).

The families of fish renowned for sequential hermaphroditism include the Sparidae, Serranidae, Pomacentridae, Scaridae and Labridae. In many of the reef dwelling species, such as the protogynous saddleback wrasse *Thalassoma duperrey*, and the protandrous anemonefish *Amphiprion melanopus*, individuals form discrete units of social organisation (Nakamura *et al.,* 1989; Godwin and Thomoas,

1993). Within these units, intraspecific social interactions mediate sex change. Fishes with recognizable social groups are therefore particularly useful models for investigating sexual regulation in fishes so that manipulation techniques for sex reversal can be developed. Most investigations on sex change endocrinology have been done in species which are important to aquaculture such as sparids and serranids. The development of sex change technology is instrumental for improving the efficiency of broodstock development by overcoming shortages of either male or female broodstock (eg. male grouper) which are rare or difficult to catch.

For over 30 years biologists have hypothesized about the involvement of steroid hormones in sex change (Frisch, 2004). However recent biotechnological applications like radio immunoassay (RIA) techniques and enzyme-linked immunosorbent assay (ELISA) have enabled the rapid and accurate determination of steroid concentrations. A variety of experimental techniques have been developed to induce sex change, thus enabling measurements of hormone metabolism during the sexual transition period. The discovery that the sex change in certain fishes is controlled by social interaction (Robertson, 1972) has enabled researchers to stimulate sex change by manipulation of fish's social environment (Godwin and Thomas, 1993; Ohta *et al*., 2003). Either a member of the terminal sex (i.e. a male in protogynous species or a female in protandrous species) is removed from the social unit (i.e. sex change by release of suppressive dominance) or by introducing multiple numbers of the initial sex together in captivity can bring about sex change (i.e. sex change by induction). In both the situations, at least one individual of the initial sex is expected to undergo sexual transition (Shapiro, 1984; Munoz and Warner, 2003). These methods have been applied in the broodstock development of clownfishes and damselfishes which are highly valued coral reef fishes in the ornamental fish trade. The second method of manipulation of sex change is the administration of sex steroids (e.g. testosterone), derivatives thereof (e.g. methyl testosterone) or inhibitors of steroidogenic enzymes (eg. fadrozole). These technologies have been instrumental in the successful development of broodstock of many commercially important marine finfishes such as seabass and groupers.

Endocrine Manipulations of Spawning

Acquisition of seed stock from the wild (larvae or fry or gametes from gravid broodstock) during the seasonal spawning period of fish is unreliable and unpredictable and hence not suitable for commercialization of aquaculture. If reproduction can be controlled, a steady supply of seed can be produced by off-season spawning (Bromage and Roberts, 1995) and genetic manipulations can be employed to enhance their growth, survival and meat quality (Thorgaard, 1995). But many fishes exhibit reproductive dysfunctions when reared in captivity. These dysfunctions are due to the fact that the fish in captivity do not experience the conditions of spawning grounds and as a result there is a failure of the pituitary to release the maturational gonadotropin, luteinising hormone (LH). Most commonly, females fail to undergo final oocyte maturation (FOM) and thus ovulation and spawning (Zohar, 1988, 1989a, b; Peter *et al*. 1993), while males produce small volumes of milt or milt of low quality (Billard, 1986, 1989). In many species hormonal treatments are the only means of controlling reproduction reliably. Over the years, a variety of hormonal techniques have been used successfully.

Most research and development efforts on the use of hormones to control finfish reproductive cycles in aquaculture have focused on the induction of FOM, ovulation, spermiation and spawning in fish that do not complete these processes in captivity. But, hormonal manipulations have important

applications in commercial aquaculture, even for fishes that undergo FOM and spermiation spontaneously in captivity. For example in many salmonid hatcheries, ovulation is induced with hormones in order to synchronize and optimize egg collection and fry production, thereby minimizing the handling and stress to the fish and reducing labour requirements. Development of genetic selection programmes often requires artificial fertilization and hormonal manipulations can be used to enable proper maturation and timely collection of gametes. Hence hormonal manipulations for the induction of ovulation, spermiation and spawning will continue to play an important role in commercial broodstock management of marine finfishes(Zohar and Mylonas, 2001).

The earliest techniques employed freshly ground pituitaries collected from reproductively mature fish, which contained gonadotropins (mainly LH). Eventually purified gonadotropins became available, both of piscine and mammalian origin (eg. carp or salmon gonadotropin and human chorionic gonadotropin). In the 1970s, spawning induction methods began employing the newly discovered gonadotropin releasing hormone (GnRH) which induces the secretion of fish's own gonadotropin from the pituitary. Development of highly potent, synthetic agonists of GnRH (GnRHa) constituted the next generation of hormonal manipulation therapies and created a surge in the use of hormones to control reproductive processes in aquaculture. The most recent development is the incorporation of GnRHa into polymeric sustained –release delivery systems, which release the hormone over a period of two weeks. These delivery systems alleviate the need for multiple treatments and induce long term elevation in sperm production and multiple spawning in fish with asynchronous or multiple –batch group-synchronous ovarian physiology. Based on the recent discovery of GnRH multiplicity in fish and the increasing understanding of its functional significance, new GnRH agonists can be designed for more potent, affordable and physiologically compatible spawning induction therapies. These methods have contributed significantly to the development of more reliable and less species-specific methods for the control of reproduction of captive broodstock. Future strategies for improved spawning manipulations will be based on understanding the captivity-induced alterations in the GnRH system, and on new approaches for their repair at the level of GnRH gene expression and release.

Live feed research

Most marine finfishes have altricial larvae and when yolk sac is exhausted, they remain in an undeveloped state. The digestive system is rudimentary, lacking a stomach and much of the protein digestion takes place in the hindgut epithelial cells. Altricial larvae cannot digest formulated feeds and hence live feed is vital for their survival. Live feeds are able to swim in water column and are thus constantly available to the larvae. The movement of live feed in water stimulates larval feeding responses. Live feed organisms with a thin exoskeleton and high water content may be more palatable to the larvae when compared to the hard formulated diets (Stottrup and Mc Evoy, 2003).

The hatchery production of juveniles of marine finfish is achieved globally by the use of 'greenwater technique' and the live feeds like rotifers and copepods.

(i) Greenwater technique

Microalgae are used in the 'greenwater technique' employed for marine finfish larviculture and play a critical role in the larviculture of marine finfishes. Microalgae are generally free living, pelagic and in the nannoplankton range (2-20µm). Batch cultures are generally run according to production cycles of 3-7 days. The cultures obtained in hatcheries seldom exceed a density of 6 x 10^6 cells ml^1

at the end of 5 days. In Industrial facilities specialized in the production of microalgae in controlled conditions such as photobioreactors, the cost of production can be reduced considerably. The productivity of microalgal systems used in aquaculture hatcheries is 10 – fold lower than that of photobioreactors, which is in turn 10 fold lower than that of fermentation techniques. But aquaculture operators are reluctant to take up these technologies mainly because of the significant investment involved. It is likely that microalgae for fish aquaculture will be produced in the near future by specialized companies implementing high technology.

Microalgae have been shown to play a significant role in larviculture of marine finfish. When phytoplankton was included in larval rearing tanks, the survival, growth and food conversion index of many marine finfish species were better than in clear water condition. The green water technique (larviculture in an endogenous bloom of phytoplankton and rotifers) and the 'pseudo green water technique' (larviculture in a tank supplemented daily with exogenous phytoplankton and rotifers) have much commercial application in marine finfish larviculture (Divanach and Kentouri, 2000). Micro algae can also influence live feed and larval microbiology. It has been found that exudates of some algal species can either enhance or inhibit the feeding activity of copepods in cultures (Van Alstyne, 1986). These substances are also involved in the settlement of micro flora required in the gut of fish larvae to prevent intestinal opportunistic bacteria from causing disease. Bacteria associated with live feed can be transmitted to larval fish during feeding (Benavente and Gatesoupe, 1988). As live prey actively ingest bacteria, it is possible to introduce favourable bacteria as probiotic. In the 'green water technique' of larviculture micro algae contribute to maintaining the nutritional quality of live food and also positively influence on the settlement of a healthy intestinal micro flora in fish larvae (Skjermo and Vadstein 1993). Micro algae can also possibly influence the endotrophic stages (egg and pre–larvae) and early exotrophic stages. Micro algal background has an important effect on the timing and intensity of first zooplanktonic feeding. Micro algae also play a role in intestinal transit and gut repletion. Improvement in the survival at first feeding is the main result of larviculture with micro algae. Improvement in growth efficiency during rotifer period is another result of micro algal background in larval tanks. Early enhancement of digestive and assimilative functions improves the survival and growth of fish larvae and favours the transition to exotrophy. The use of micro algae in tanks increases the production of pancreatic and intestinal digestive enzymes and improves the quality of gut flora. Even after the endo-exotrophic phase, micro algae have a positive effect on larviculture and may increase the resistance of larvae to further stressing or adaptive conditions. The indirect effects of micro algae on larvae are mainly related to water quality, luminosity, the bacteriology of water and the quality and accessibility of rotifers. It is thus evident that strategic use of micro algae in hatcheries during the very early life of marine fish improves the success of first feeding, a prerequisite for efficient survival, growth and quality in fish larviculture. A lot of research focus is needed in future on microalgal biotechnology for larviculture.

(ii) Rotifers

Rotifers have been used as live feed for cultured marine fish, since four decades. It is well known that a continuous, stable and reliable supply of nutritionally adequate rotifers is the key to the larviculture of marine finfish. Rotifers of the species *Brachiounus rotundiformis* and *B. plicatilis* are almost indispensable for larval rearing of most marine finfish (Gopakumar and Jayaprakas, 2001; 2003; 2004).

The success of rotifer cultivation is dependent on selecting the most suitable rotifer species or strain for local culture conditions, maintaining water quality in culture tanks and choosing the most appropriate culture technique. Size, the type of reproduction and reproductive rates are species or strain specific. Culture temperatures, salinities, type of food and its quality - all influence the type of reproduction and its rates. Mass production of rotifers is achieved by encouraging rotifers to reproduce asexually, since sexual reproduction results in males and resting eggs. New high density culture technologies for rotifers, such as closed recirculation systems are offering new possibilities for continuous supplies of high quality rotifers at ten times higher than in batch cultures. Evaluating the physiological state of rotifer culture is very important in hatcheries since larval production depends on a predictable and reliable supply of rotifers. Six parameters *viz* egg ratio, swimming velocity, ingestion rate, viscosity, enzyme activity and diseases are employed for assessing the state of health of rotifer cultures.

The nutritional quality of rotifers is improved by enrichment, in which rotifers are collected or harvested from culture tanks into containers where they are kept at very high densities and incubated for 8 – 20 hours with enrichment dietary components like HUFA. In addition to nutritional enrichment, rotifers can be enriched with antibiotics (Verpraet *et al*, 1992) or with probiotic bacteria (Markridis *et al*, 1999, 2000). The nutritional value of rotifers depends on their dry weight, caloric value and chemical composition (Lubzens *et al*, 1989).

Various methods of storing rotifers have been studied. Frozen rotifers are not usually adequate as feed because of leaching of nutrients. Live *B .plicatilis* can be stored at 4^0C at relatively high densities for at least one month (Lubzens *et al*, 1990). Rotifers can be kept at -1^0 C without feeding or water exchange for about 2 weeks (Lubzens *et al*, 1995). *B. rotundiformis* strains are less tolerant to 4^0 C than *B .plicatilis* rotifer strains and the strains known as SS type are most susceptible and showed lowest survival. Amictic eggs of rotifers can be preserved by cryopreservation in liquid nitrogen after they have been impregnated with cryoprotective agents like dimethyl sulfoxide (DMSO) (Hadani *et al*, 1992). This method ensures full preservation of genetic traits of importance to aquaculture. Cryopreservation is not a suitable method for preservation of large numbers of rotifers for direct use as feed.

Artificially produced rotifer eggs have been tried as an alternative to daily production of rotifers. The production of these eggs can be manipulated by environmental factors, such as salinity, food quality and quantity, rotifer culture density, exchange of culture media and temperature and varies between *B. plicatilis* and *B. rotundiformis*. The cost of producing resting eggs is very high and therefore not yet been extensively adapted in hatcheries.

It is evident that rotifer cultures will continue to be indispensable in marine finfish hatcheries. Current methodologies of producing and enriching rotifers are meeting the requirements of the industry. The need to have very small sized rotifers is difficult to achieve, although several super small strains have been found and cultured (Hagiwara *et al*, 2001). Improved methods for predicting the health of cultured rotifers may be useful in preventing culture crashes. Using preserved rotifers may eliminate the dependence on daily production of rotifers. Cheaper methods of resting egg production and high density culture techniques are the major areas which requires research attention in future.

(iii) Copepods

The rapid expansion of hatchery production of seeds for farming of many marine food fishes and the hatchery rearing of marine ornamental fishes to replace wild caught fishes in the trade, could not be met by conventional live feeds such as rotifers and *Artemia*. Thus interest in copepods has been generated and the use of copepods as live feeds in finfish hatcheries is gaining impetus. Copepods are employed mainly because they are the only acceptably sized prey for small larvae of many species of marine finfish and ornamental species. Copepods have a larger size range from first nauplii to adult copepodites and offer good size ranges for the entire hatchery phases for certain species of finfish. When compared to rotifers and *Artemia* nauplii, copepods can improve the larval growth, survival and the ratio of normally pigmented juveniles when fed either alone or in combination with conventional live feeds.

It is well understood that the mass culture of copepods has several limitations especially due to its low multiplication rate when compared to rotifers. The species that are mass cultured fall under three orders – Calanoida, Harpacticoida and Cyclopoida. In calanoids, species belonging to the genera *Acartia*, *Centropages* and *Eutemora* are in most widespread use in mono and mixed cultures. Among harpacticoids, species belonging to the genera *Euterpina*, *Tigriopus* and *Tisbe* have been widely used. Under cyclopoids, *Oithona spp*. and *Apocyclops spp*. are recognized as suitable for marine finfish larvae.

Improved growth, survival and /or rates of normal pigmentation have been documented for several marine fish species fed copepods alone or as supplement to the traditional diets of rotifers or *Artemia* nauplii compared with traditional diets alone(Kraul 1983; Heath& Moore 1997;Mc Evoy *et al*. 1998; Naess & Lie 1998; Nanton and Castell 1999). In many hatcheries, malpigmentation of the reared juveniles constitutes a major problem .Flatfish larvae fed natural or laboratory reared zooplankton exhibit higher rates of normal pigmentation than larvae fed *Artemia* nauplii (Seikai *et al*. 1987, Naess *et al.* 1995, Mc Evoy *et. al*. 1998). Larval nutrition is suggested to be the major factor determining pigmentation patterns. The documented improvements in larval growth, survival and rates of normal pigmentation are generally attributed to levels of DHA, EPA and/or arachidonic acid (ARA) in the diet (Castell *et. al.,* 1994; Reitan *et.al*.,1994; Zheng *et. al.,*1996; Sargent *et al.* 1997) and in particular to the DHA : EPA ratio in the diet (Bell *et al.* 1995b; Sargent *et al*. 1997: Nanton and Castell 1998) and EPA : ARA ratio (Bell *et al.,* 1995a; Sargent *et al.,* 1997; Estevez *et al* 1999). DHA can be synthesized from shorter chain precursors in some marine fish larvae, but at rates insufficient to meet requirements for their normal growth and survival. A minimum of 0.5 to 1.0% of dry weight as n-3 HUFA is required for juvenile marine fish and higher amounts are required for rapidly growing fish larvae. Marine copepods, the principal diet for most marine fish larvae in nature, contain high levels of DHA and other PUFA, either obtained through their phytoplankton diet or accumulated despite low PUFA levels in the diet. DHA levels in wild copepods can be more than 10 times higher than in enriched *Artemia* (Mc Evoy *et*. *al.,*1998).DHA is important in maintaining structural and functional integrity in fish cell membranes, in neural development and function, and especially in retinal development and vision (Bell & Tocher 1989; Bell & sergent 1996). It is suggested to play an important role in the development of normal pigmentation when provided in sufficient quantities at particular times during the larval stage(Reitan *et al*., 1994). EPA cannot be synthesized by most marine fish and it is therefore essential in the diet of the fish. EPA gives rise to less biologically active

eicosanoids than those produced from ARA. Since it competes metabolically for the same enzyme systems required for ARA derived eicosanoid production, EPA is very important in modulating the production of these highly biologically active eicosanoids. This metabolic interaction necessitates an optimal EPA : ARA ratio in the diet. Eicosanoids of n-6 origin are important for the normal function of vital organs such as kidney, gill, intestine and ovaries of marine fish. Levels of ARA in copepods are high in both calanoids and harpacticoids. Apart from the superior fatty acid composition in copepods, they contain high amounts of polar lipids (Fraser *et al*. 1989). Polar lipids are more easily digested by larvae and may also facilitate digestion of other lipids in the undeveloped gut of marine fish larvae. Varying concentrations of the carotenoid astaxanthin were found in the various copepods and its possible value for fish is as a precursor to Vitamin A. Copepods are also an important source of exogenous digestive enzymes and are thought to play an important role in fish larval digestion (Munilla –Moran *et al.,* 1990).

The apparent inability to be cultured in high densities is the major constraint for the commercial use of copepods as live feed in hatcheries. Copepod cultures rarely exceed 2 per ml for adults and ten per ml for nauplii (Stottrup *et al*. 1986; Mc Kinnon *et al*. 2003). Although much experience has been gained in culturing different calanoid species a lot of research is further needed on achieving stable cultures and finding the optimal conditions for maximum production. This includes the optimal feeding regime for cultures, the optimal quality (size and nutrition) and how to ensure the food availability of copepods to maximize production. A method that includes all these features in the most efficient manner and ensuring stability would be a big leap in calanoid culture. Work towards improving the quality of cold stored non-diapause eggs is also needed to increase the benefits of cold storage. Harpacticoids can be cultured in higher densities than calanoids. The densities may reach more than 100 per ml (Fleeger 2005). It should be possible to develop semi automated systems that would minimize labour and make culturing of harpacticoids more efficient. Reliable rearing systems for mass production of small sized copepods that can meet the needs of marine finfish larviculture is the key area of research to be focused in the immediate future for commercializing the seed production of many high value finfishes with altricial type of larvae. Eventhough many potential species of copepods for culture have been studied, the possibility of an ideal species which can produce higher densities of nauplii per ml can be another important aspect of future research. Production of resting eggs for sale on a commercial scale can revolutionize the seed production of many high value finfishes for mariculture.

Recirculating Aquaculture System (RAS)

A recirculating Aquaculture System (RAS) can be defined as an aquaculture system that incorporates the treatment and reuse of water with less than 10% total water volume replaced per day. The concept of RAS is to reuse a volume of water through continual treatment and delivery to the organisms being cultured. Water treatment components used in RAS need to accommodate the input of high amounts of feed required to sustain high rates of growth and stocking densities. Generally RAS consists of mechanical and biological filtration components, pumps and holding tanks and may include a number of additional water treatment elements that improve water quality and provide disease control in the system.

Conclusion

Research and development on commercial level seed production technologies of high value finfish and shellfish, popularisation of sea cage farming and evolving suitable policies for sea farming are the key areas to be focused urgently to make mariculture as a significant seafood production sector in India. In this context, biotechnological interventions in controlled reproduction, induction of spawning, live feed technology and recirculating aquaculture systems can go a long way in the improvement of mariculture technologies. Mariculture and allied post harvest technologies if scaled up with spatial and seasonal variations with community participation the coastal productivity and economy can prosper.

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3

Molluscs and Biotechnology

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Animals of the phylum Mollusca occupy every trophic level in the marine environment. Three classes of Mollusca are of fisheries interest, namely, Gastropoda, Bivalvia and Cephalopoda. The exploitation of these shelled animals in India, range from the very old and historic records of exploitation of pearl oysters and chanks to the very modern trawling for cephalopods. Available production statistics reveals that annually more than 1,00,000 tonnes of cephalopods are harvested from the Indian seas, besides another 1,00,000 tonnes of gastropods and bivalves. The economic importance of cephalopods to the country is considerable as they earn more than Rs. 1400 crores (more than 16% in terms of quantity and more than 15% in terms of value of total marine products exports) every year through exports. In fact, every second cephalopod consumed by Europeans originates from India.

Research on molluscs in the country is more than a century old. However, it is only recently that biotechnology as a tool has found many applications to deepen our understanding of mollusc populations and their biology. In CMFRI, the Molluscan Fisheries Division has been collaborating with the Marine Biotechnology Division of the institute in the following areas:

- Resolving taxonomic ambiguities through molecular taxonomy – Case of *Sepia pharaonis*
- Disease diagnosis – Case of protozoan parasite *Perkinsus olseni* in pearl oyster and other bivalves
- Accurate predictions of spatfall of oyster and mussels in bivalve farming through PCR based identification of specific bivalve larvae from a plankton cocktail

Phylogeography of the pharaoh cuttle *Sepia pharaonis* based on partial mitochondrial 16S sequence data

The pharaoh cuttle *Sepia pharaonis* Ehrenberg, 1831 (Mollusca: Cephalopoda: Sepiida) is a broadly distributed species of substantial fisheries importance found from east Africa to southern Japan. Little is known about *S. pharaonis* phylogeography, but evidence from morphology and reproductive biology suggests that *Sepia pharaonis* is actually a complex of at least three species. To evaluate this possibility, we collected tissue samples from *Sepia pharaonis* from throughout its range. Phylogenetic analyses of partial mitochondrial 16S sequences from these samples reveal five distinct clades: a Gulf of Aden/Red Sea clade, a northern Australia clade, a Persian Gulf/Arabian Sea clade, a western Pacific clade (Gulf of Thailand and Taiwan) and an India/Andaman Sea clade.

Phylogenetic analyses including several *Sepia* species show that *S. pharaonis sensu lato* may not be monophyletic. It was suggested that "*S. pharaonis*" may consist of up to five species, but additional data will be required to fully clarify relationships within the *S. pharaonis* complex.

Map showing the type localities for Sepia pharaonis and sampling localities for the study: 1 = Red Sea (RS), 2 = Gulf of Aden (GofA), 3 = Persian Gulf (PG), 4 = Arabian Sea (AS), 5 = Gulf of Oman (GofO), 6 = Veraval (VRL), 7 = Kochi (CFH), 8 = Vishakapatanam (VSK), 9 = Phuket (PH), 10 = Prachuap (PR), 11 = Chumphon (CHU), 12 = Taiwan (TAI), 13 = Gulf of Carpenteria (GofC), 14 = Northeast Queensland (NEQ)

Diagnosis and specific identification of pathogens in Pearl oyster

The pearl oyster, *Pinctada fucata* (Gould), is a commercially important bivalve distributed in the Gulf of Mannar along the southeast coast of India and had supported a healthy, traditional pearl fishery until the 1950s. But, during the past few decades, the natural pearl oyster beds in the Gulf of Mannar have showed a sharp decline leading to the closure of the traditional pearl fishery and was presumed to be due to overexploitation and pollution. No major disease/pathogens were reported from molluscs from the Indian subcontinent and the pathogen profile of *P. fucata* from the region was not known. A screening of pearl oyster samples revealed heavy infections with the protozoan parasite *Perkinsus olseni*,in the population and this turned out to be the first report of P. olseni, an OIE listed pathogen in the wild and cultured *P. fucata* populations from the Indian subcontinent. PCR Screening of the tissues using the *Perkinsus* genus specific internal transcribed spacer (ITS) 85 and ITS 750 primers, amplified the product specific to the genus *Perkinsus* (ca. 700 base pairs) and further, the specific identity of the parasite was determined by sequencing the amplified PCR products and was confirmed as *Perkinsus olseni*. This preliminary investigation suggests a possibility

that perkinsosis could be one of the major reasons for the decline of the *P. fucata* beds in the Gulf of Mannar over a period of time.

Accurate predictions of spatfall of oyster and mussels for farming

In oyster and mussel farming knowledge of the time of spatfall is very important for farmers to decide on the time for setting spat collectors. This is particularly important when the current farming practice is wholly dependent on natural spat as seed. Through a project funded by the DBT, the CMFRI has achieved preliminary success in developing a PCR based protocol for identification of mussel and oyster larvae from a cocktail mix of various holo and mero plankters (as found in a plankton collection).

New areas in mollusc biotechnology

Because of the unique life history patterns of many molluscs, they face a wide range of environmental, behavioural and metabolic challenges and these have been met with unusual chemical strategies. Examples include metal accumulation, adhesion, chemical defences, novel amino acids, structural materials, luminescence, pigments, novel proteins and peptides, immune responses, plus a wide range of unusual secondary metabolites of unknown properties.

1. Metal Accumulation

Many bivalves are able to sequester heavy metal ions from their environment, either using metal binding proteins such as metallothioneins, specific metal binding proteins, or by sequestering the metal to structural components such as shell or connective tissue. Accumulation of lead, cadmium, and copper, by binding to metallothioneins appears to be quite common whilst zinc accumulation, at least in sea urchins, seems to be due to a distinct protein. Accumulation of heavy metals can occur in the shells of molluscs and may be a form of decontamination of the immediate environment of the organism.

2. Adhesion

Many invertebrates rely on adhesion for stability, for example marine molluscs attached to rocks. Bioadhesives can act both as lubricants and adhesives, as in the case of mollucsan slime, which may for example facilitate the movement of a slug over dry ground, whilst also maintaining its position on smooth vertical surfaces. Adhesion is usually accomplished by means of proteins with varying amounts of carbohydrate depending on the function. Limpets for example produce two kinds of mucous; adhesive and non-adhesive, the former containing higher amounts of carbohydrate and each mucous having different proteins. The adhesive protein of the mussels facilitates the very strong adhesion of the animal to rocky intertidal zones where adhesion both in wet and dry conditions is required. The protein with 20% lysine residues is highly basic in nature and consists of repetitive sequences, reminiscent of structural proteins, and is rich in hydroxyproline and dihydroxyphenylalanine.

3. Biochromes, Fluorescence & Bioluminescence

Many marine organisms are a rich source of biochromes including carotenoids, chromans, flavonoids, porphyrins, melanins, phenoxaxines, purines, pteridines, isoalloxazines and quinones, as well as biochromic proteins. Aside from visual and primary metabolic functions these biochromes

function as chemical protectants against enemies and the environment, and as bioluminescent agents. Luminescence and fluorescence are distributed throughout the invertebrata. The calcium-dependent fluorescent protein aequorin from the jellyfish *Aequoria victoria* been used in research for many years as an indicator of intracellular calcium movements and more recently since the isolation and characterization of the aequorin gene, has become known as GFP (green fluorescent protein) and has been expressed in fungi, bacteria and plants as a useful marker gene. GFP is a fluorescent protein which emits green light upon excitation with ultraviolet to blue light. Same form of luminescence or fluorescence can be found in molluscs such as limpets, nudibranchs, clams, squid and octopods. There are no luminous flowering plants, birds, reptiles, amphibians or mammals, so invertebrates have this property pretty much to themselves.

4. Antimicrobials

Antimicrobial compounds have been found in many organisms and clearly form part of the defensive strategy of many invertebrates. Antibacterial substances have been found in the mucous of slugs and snails. They tend to be large glycoproteins which are permanently produced by the animal rather than induced by infection. Achacin, the antibacterial glycoprotein from the giant African snail, is active against both Gram-positive and Gram-negative bacteria, apparently by disrupting the integrity of the cytoplasmic membrane. Marine invertebrates produce a range of antiviral compounds including the dideminins; cyclic depsipeptides found in tunicates. Tunicates are also the source of small antiviral compounds including patellazole, active against Herpes simplex and eudistomin whilst avarone and avarol, which have potent antiviral activity against immunodeficiency virus have been isolated from a sponge.

5. Toxins, Venoms and other Chemical Defences

Invertebrates have evolved a wide range of chemical defences, far too many and diverse to be covered here. The chemistry of these defences ranges from small reactive molecules, such as the benzoquinones ejected by some insects, millipedes and centipedes, to peptide or polypeptide toxins such as those produced by marine coelenterates. In molluscs the best known venoms are those of the cone snails, the conotoxins.

The venom of these snails can contain up to 100 pharmacologically active agents which bears favourable comparison to the numbers of bioactive compounds found in some plants. The most active compounds are short peptides of 10-30 amino acids. The peptides target ion channels in membranes and have a range of specificities for channel types. The salivary glands of some marine snails have been shown to produce low molecular weight toxins including serotonin from *Nucella lapillus* and tetramethyl ammonium chloride from *Neptunea antiqua*.

6. Biomaterials – natural plywood

As by definition invertebrates contain no backbone, they have evolved a range of novel structural materials, to protect themselves and their offspring, and for a range of other functions such as shelter, and food gathering. Some of these structural solutions comprise entirely unique and often beautiful materials. Two obvious examples are the shells of molluscs and the cuticle of insects.

The calcareous shells of molluscs are reinforced with carbohydrate/protein matrices and often overlaid with a highly structurally ordered proteinaceous periostracum. A number of molluscs produce

a proteinaceous egg case or capsule to protect the developing eggs. These materials are mechanically remarkably strong, resistant to chemical, physical and bacterial degradation, and are composed of long protein chains mutually rotated to form a natural "plywood".

Marine Bioprospecting – the hunt for *blue gold*

Exploitation??

The answer probably lies in the emerging science of lower invertebrate mariculture

The above represents just a glimpse of the chemical diversity found in the mollusca; some areas such as chemical communication are just too vast and complex to cover other than to say that a wide range of diverse chemical types are used in inter and intraspecies signalling. Is there however any prospect of exploiting such chemical resources without damaging the resources and the environment? The answer probably lies in the emerging science of lower invertebrate mariculture. Marine bioprospecting is typically associated with public-private partnerships, due to the astronomical expenses, state of the art technology and specialized expertise associated with marine exploration. It is estimated that marine exploration costs at least $30,000 per day and $1 million for 30 days, expenditure well beyond the means of public institutions.

The regulation of marine bioprospecting is complex, involving a range of issues. These include grappling with the challenges of ownership of common property, intellectual property rights and benefit t sharing; conservation and protection of marine biodiversity; technology transfer and capacity building; jurisdictional issues in monitoring and enforcement; as well as the difficulties inherent in regulating marine over-exploitation and activities that occur beyond national jurisdictions. These issues have been discussed in various international meetings including those held under the auspices of the Convention on Biological Diversity, the United Nations Convention on the Law of the Sea, the International Seabed Authority and the United Nations General Assembly on Oceans and Law of the Sea.

Further Reading

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Introduction to Nucleic Acids

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Introduction

The primary aim of this lecture is to give a brief introduction to the basic structure and physical and chemical properties of nucleic acids so as to easily understand the in vitro amplification of DNA through Polymerase Chain Reaction (PCR). The knowledge on these basic principles will help to manipulate the reaction to suit ones need and effectively use the PCR technology in shrimp disease diagnosis. This will also help in understanding and solving the various problems one may face during the application of this technology. Therefore, I have avoided the cumbersome chemistry of the nucleic acids, but the most relevant principles that are required for the understanding the PCR are discussed and explained.

DNA was isolated way back in 1869

DNA was first isolated from pus cells and from salmon sperm by Friedrich Meiescher in 1869. Since it was isolated from nuclei it was called nuclein. DNA from different cells and viruses vary in their nucleotide sequence, nucleotide ratio and molecular weight. In fact nucleic acids are the major component of the cell. The genomic content of the cell varies from 0.01 pg in prokaryotes to 0.3 to 10 pg in higher plants and animals. However, the vast majority of the nucleic acid in cells is present as complexes with proteins. Prokaryotic DNA form complexes with polyamines and proteins while eukaryotic DNA is associated with histones and various non histone proteins. The amount of DNA in any given species of cell or organism is constant and can not be altered either by environmental or nutritional or metabolic conditions. The germ cells (sperm/egg) of higher animals possess only one half of the amount of DNA found in somatic cells of the same species.

Nucleotides are the building blocks of Nucleic acids

Just as the amino acids are building blocks of peptides (proteins), the nucleotides are the building blocks for nucleic acids. The monomeric units of DNA are called deoxyribonucleotides. Each of nucleotide contains three characteristic components. (a) a heterocyclic nitrogenous base, derivative of either a pyrimidine or purine. (b) a pentose sugar molecule and (c) a molecule of phosphate. There are four different deoxyribonucleotides which serve as the major building blocks of DNA macromolecule. They are all similar except for the nitrogenous base. Each nucleotide is named after the base. The purine derivatives are adenine (A) and guanine (G) while the pyrimidine derivatives are cytosine (C) and thymine (T). Similarly, four different ribonucleotides are the building blocks for the RNA. They are the purine bases adenine and guanine. The pyrimidine bases are

cytosine and uracil (U). The pentose sugar is different in DNA and RNA, DNA contains 2-deoxy ribose sugar, while RNA contains ribose sugar.

Nucleic Acids exist in different forms

Nucleic acids exist in two major types, namely DNA and RNA. Though DNA exists in one type they exist in different forms, as linear, circular, single stranded and double stranded forms. On the contrary mostly RNA exists as single stranded form. There are three types of RNA present in a living cell- messenger RNA (mRNA), Ribosomal RNA (rRNA) and transfer RNA (tRNA). Even though RNAs are single stranded, they form extensive secondary structures as in the case of tRNAs and rRNAs. Messenger RNAs of eukaryotic cells are unique that they contain long stretches of poly (A) sequences at the 3' ends (the carbon atoms are numbered by adding prime to the number, for eg. 2^{nd} carbon atom in the sugar is written as 2', 3^{rd} as 3' etc). In general DNA contains the genetic information; however, in certain viruses RNA contains the genetic information.

DNA/RNA are formed by covalent links of Deoxy/Oxy ribonucleotides

A nucleic acid is polynucleotide - that is a polymer consisting of nucleotides. The pentose sugar is a cyclic five carbon ribose sugar in case of RNA and 2'deoxyribose sugar in the case of DNA. A purine or pyrimidine base is attached to the 1' carbon atom of the pentose sugar by an N-glycosidic bond. A phosphate is attached to the 5' carbon of the sugar by phosphoester bond. It is this phosphate which gives the strong negative charge for the nucleotides and the nucleic acids. This property is used in agarose gel electrophoresis of nucleic acids. The nucleotides in nucleic acids are covalently linked by a second phosphoester bond that joins the 5' phosphate of one nucleotide and the 3' OH group of adjacent nucleotide. This phosphate plus its bonds to the 3' and 5' carbon atoms is called a phosphodiester bonds.

DNA exhibits base equivalence

Edwin Chargaff and his colleagues using quantitative chromatographic separation methods analyzed the base composition of nucleic acids and proposed the Chargaff's rule for DNA. (1) The base composition of DNA varies from species to species. (2) DNA specimens isolated from different tissues of the same species have the same base composition. (3) The base composition of DNA in a given species does not change with age, nutritional state or changes in environment. (4) The number of adenine residues is always equal to the number of thymine residues, A=T. Similarly Guanine residues is always equal to cytosine residues, G=C. (5) DNAs extracted from closely related species have similar base composition whereas those of widely different species have widely different base composition.

Watson and Crick model of DNA

In the year 1953 Watson and Crick proposed a structure for the DNA based on the crystallographic structure. It was an epoch making proposal, which revolutionized the world, for which they got the Nobel Prize. The structure proposed by Watson and Crick explained the long known Chargaff's rule. The DNA exists in a double helix state. The two strands run in opposite direction (anti parallel). The polarity of the DNA strand is 5' to 3'. These two strands are held together by H-bonding between complementary N- bases. A bonds with T and G bonds with C. Phosphate and sugar form backbones on the outside and hydrophobic N-bases are inside, stacking on top of one another. The double

helix is 20Aº in diameter. The helix makes a complete turn within 10 nucleotides at a distance of 34Aº. The raise per nucleotide is 3.4Aº. It is important to note that nucleic acids have polarity. This means that their ends (termini) are not the same. One end of the polynucleotide chain bears a 5' phosphate group whereas the other end bears a 3' hydroxyl group. By convention the sequence of the nucleic acid is written in the 5'-3' direction, the 5' terminus always being to the left.

DNA can exist in different forms

The geometry of the DNA double helix was deduced from X-ray diffraction studies using DNA fibers. Analysis of such patterns revealed the existence of three different conformations of the DNA. They are called as A-DNA, B-DNA and C-DNA. The Watson and Crick model is nothing but the B-DNA. A-DNA differs from the B structure in the following respects. The base pairs, although parallel to one another and spaced by the same amount (0.30nm) as in the B form, are inclined by about 20 degrees to the planes perpendicular to the helix axis. The number of bases per turn is 12 and hence the structure is fatter, but longitudinally more compact. The two grooves that run around the outside helix are approximately the same size in A-DNA. But the grooves vary in their size in B-DNA, called major and minor grooves. The base pairs overlap in B-DNA such that the top view of the helix appears to be full of base pairs but A-DNA has a hole down the middle. C-DNA is a distorted B structure with a non-integral number of bases per full turn and the base pairs are somewhat more inclined to planes parallel to the axis. There is also another form of (synthetic) DNA the Z-DNA. It is left handed. The base pairs are inclined to 7 degrees and there are 12 base pairs per full turn. The biological significance of Z-DNA is still uncertain.

Secondary structure of DNA is drastically different from the primary structure

Linear nature of a DNA sequence is regarded as the primary structure. In the case of DNA, secondary structure consists of two independent, covalently linked chains coiled around a common axis and forms double helix. In the case of RNA molecule which is primarily single stranded, a number of intra-strand H-bonding can be found. This intra strand bending of the helix axis in different directions is called the secondary structure. They are termed differently as hairpin loops, hairpin bends, stem loops cruciform etc. Secondary structures of RNA are believed to play important biological roles such as recognition regions for certain enzymes.

DNA is stabilized by various forces

What holds the DNA double helix together? One of the factors is the hydrogen bonding. The Watson-Crick Model is thermodynamically correct. A:T base pairing has two hydrogen bonding, while G:C pairing has three hydrogen bonding which is more stable than the former. Hydrogen bonding is not the only stabilizing factor. Hydrophobic interactions in base stacking interactions between aromatic rings inside the helix are the major stabilizing forces against repulsion by negatively charged phosphates. The presence of counter ions such as Mg+ + and K+ also play a role in stabilization of DNA double helix.

DNA is very stable compared to RNA

Compared to DNA, RNA is less stable. This is primarily due to the single stranded nature. The nature of the pentose sugar also plays a role in the stability. RNA can be easily hydrolyzed by dilute alkali. The sugar moiety contains 2' hydroxyl group. Dilute sodium hydroxide produces a mixture of

nucleosides, 2' and 3' phosphates. Cyclic 2', 3' monophosphates are the first products of the action of alkali on RNA. They are further hydrolyzed by alkali, which attacks either one of the two P-O-C linkages to yield a mixture of 2' and 3' nucleoside phosphates. Since DNA has no 2' OH group it can not be hydrolyzed by alkali.

DNA can be denatured by heat and acids but not by alkali

Gentle acid hydrolysis of DNA at pH 3.0 causes selective hydrolytic removal of all its purine bases without affecting the pyrimidine deoxyribose bonds or the phosphodiester bonds of the backbone. The resulting DNA derivative, which is devoid of purine bases is called apurinic acid. Selective removal of the pyrimidine baes, accomplished by some what different chemical conditions produces apyrimidinic acid. As mentioned above alkali cannot hydrolyse the DNA. Nucleases also hydrolyse the nucleic acids. Nucleic acids can be denatured by heating. This process is called melting. When nucleic acid is denatured the stacking is lost and hence the UV-absorbance increases. This increase is called hyperchromic shift. For total denaturation of the double stranded DNA, the hyper chromic effect is of the order of 30%. The temperature at which the solution contains 50% denatured and 50% double stranded DNA is called the melting temperature (Tm) .The value of Tm is the function of the nature of the DNA, ions in the solutions and the ionic strength. Renaturation is not simply the reversal of denaturation. If the denatured DNA solutions are maintained 5-10 ºC below the melting temperature, the complementary strands will slowly re-associate and the double helical structures will reform. The whole process is concentration dependent. Thermal denaturation is one of the properties exploited in polymerase chain reaction (PCR).

DNA replicates by semi-conservative mechanism

The most striking feature of the Watson-Crick model of DNA, from the genetic point of view is that the two strands of double helical DNA are complementary. The replication of each to form new complementary strands results in formation of two daughter duplex DNA molecules, each of which contains one strand from the parental DNA. This process is called semi conservative replication. This model was conclusively proved by Meselson and Stahl in 1957 by ingenious experiment using bacteria. The same is true in other dividing cells.

DNA polymerase is the key enzyme in DNA replication

The enzymatic mechanism by which the DNA is replicated was elucidated by A. Kornberg and his colleagues in 1956. The enzyme involved in this process is DNA polymerase I. Later it was found out that, other enzymes, (Pol. II. Pol. III) were also involved in replication. Now it is shown that Pol III is the major enzyme concerned in the replication process, although Pol I participates. This also functions in repair of DNA. It also has the 3'-5' and 5'-3' exonuclease activity. The most striking and the characteristic property of DNA polymerase is that it requires the presence of some pre-existing DNA called primer, in the absence of which the purified enzyme will not be able to make any DNA at all.

Pre existing DNA primer and template is essential for DNA replication

Okazaki and his colleagues discovered that nascent DNA occurs in short pieces, called Okazaki fragments. These fragments are found in viral, bacterial and eukaryotic cells during DNA replication. Replication of DNA in short steps is a device that permits replication of both strands of DNA by DNA

polymerase that replicates only in 5'-3' direction. These short pieces are quickly joined by covalent bonds. It was also shown that the purified DNA polymerase can not utilize the native DNA strand as a primer. Therefore, DNA replication is preceded by the formation of a short strand of RNA complementary to a section of double strand DNA. This priming RNA is generated by a DNA directed RNA polymerase. Once the priming RNA strand has been made, DNA polymerase begins to add nucleotides to form DNA from 5'-3' direction. This is the principle that is involved in the necessity of primers for the PCR.

RNA too can act as a template for DNA synthesis.

There are many viruses whose genetic material is made of RNA. These viruses replicate their genome via synthesis of DNA. This is mediated by an enzyme called reverse transcriptase (RT). This enzyme was first isolated by Temin and Baltimore. It is primarily an RNA dependent DNA polymerase. Such enzymes are purified from RNA tumor viruses. This enzyme is used in molecular biology to synthesize complimentary DNA (cDNA) from mRNAs. They are also used to amplify RNA viral genes through RT-PCR. This technology is used for detecting the presence of RNA viruses.

Polymerase Chain Reaction (PCR) is the result of successful exploitation of the properties of DNA and its replication.

DNA polymerase uses single stranded DNA as a template for the synthesis of a complementary new strand. These single stranded DNA templates can be produced by simply heating double stranded DNA to temperatures near boiling. DNA polymerase also requires a small section of double stranded DNA to initiate (prime) synthesis. Therefore the starting point for DNA synthesis can be specified by supplying an oligonucleotide primer (a small piece of DNA with 15-40 nucleotides) that anneals to the template-DNA at that point. This is the first important feature of the PCR- that DNA polymerase can be directed to synthesize a specific region of DNA. Both DNA strands can serve as templates for synthesis, provided an oligonucleotide primer is supplied for each strand. For a PCR, the primers are chosen to flank the region of DNA that is to be amplified so that the newly synthesized strands of DNA, starting at each primer, extend beyond the position of the primer on the opposite strand. Therefore, new primer binding sites are generated on each newly synthesized DNA strand. The reaction mixture is again heated to separate the original and newly synthesized strands, which are then available for further cycles of primer hybridization, DNA synthesis and strand separation. The net result of a PCR is the amplification of the DNA-molecules in geometric proportion. This is all possible due the DNA polymerase from a heat stable bacterium *Thermus aquaticus* (Taq). Using this technology it is now possible to synthesise DNA in the purest form, which is the basic requirement for any genetic manipulations. PCR technology now forms the most preferred, rapid, sensitive and specific diagnostic tool both in human and animals.

RNA can also base pair with RNA

Similar to DNA, RNA can also form duplexes by base pairing with another strand of RNA with complementary sequence. mRNA can form duplexes with complementary RNA called antisense RNA. Antisense RNA is so called because its sequence of nucleotides is a complement of the sense or messenger strand. When such a duplex is formed, translation of mRNA is hindered thereby blocking protein synthesis. This translational repression occurs because (a) ribosomes cannot bind to the mRNA strand or (b) such RNA duplex formation can also trigger another phenomenon called

RNA interference also known as posttranscriptional gene silencing. In a cell, long dsRNAs can arise from self-annealing cellular transcripts and the transcription of convergent genes. dsRNA molecules are also encountered during replication of RNA viruses, transposons or experimental transfection. When a double stranded RNA (dsRNA) is encountered in a cell, a nuclease called Dicer cleaves the dsRNA into short 21-25 nucleotide fragments called small interfering RNAs (siRNAs). The siRNA-Dicer complex is incorporated into a protein complex called RNA induced silencing complex (RISC). RISC then identifies the homologous mRNA in the cytoplasm and cleaves the target mRNA resulting in silencing of gene expression. Both antisense technology and RNAi are used in the laboratory to study gene function by gene knock-down studies. These technologies are currently being explored as potential therapeutics for a wide range of diseases. For example, in shrimp aquaculture RNAi is being currently explored as a possible antiviral therapeutic for the White spot disease (WSD). Researchers prepare dsRNA corresponding to genes of White Spot Syndrome Virus (WSSV) and inject them in shrimp. When dsRNA-treated shrimps are infected with WSSV, they show higher survival rates.

Conclusion

The basic knowledge on the structure and function of nucleic acid is the basis of many revolutionary developments in biology. PCR is one such technology that has revolutionized molecular biology. RNAi is another novel nucleic-acid based therapeutic which has wide applications in animals and plants against viral diseases. The deeper understanding of the chemistry of nucleic acids will help not only to understand the novel technologies but also to develop new technologies in future.

5

Polymerase chain reaction and its various modifications

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Introduction

Polymerase Chain Reaction or PCR is a molecular technique which allows *in vitro* synthesis of billions of copies of a target DNA fragment within hours using a simple enzymatic reaction. This is achieved by using a pair oligonucleotide primers that hybridize (anneal) to the opposite strands of the target DNA at positions flanking the region to be amplified. New strands are made through the simultaneous extension of both the primers by addition of nucleotides to the primers. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by the enzyme DNA polymerase results in the exponential accumulation of the DNA whose termini are defined by the 5' ends of the primers. Since the primer extension products synthesized in one cycle can serve a template for the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR can yield about a million-fold amplification. The method is simple, as the PCR can be performed in a single tube. It can be performed on relatively crude DNA containing samples. These factors have made the PCR an attractive method for amplification of specific sequences. This method is extremely rapid; it takes only 3 hours to amplify a known sequence of interest. PCR generates sufficient copy numbers of target DNA sequences for their routine visualization through standard procedures such as electrophoresis followed by staining with ethidium bromide. The PCR products may be sequenced to determine the exact sequence of the nucleotides within the amplified product. As a result, PCR permits routine analysis of DNA from single egg and larvae, and from non-invasively secured tissues such as fin clips and scales. Even partially degraded DNA from poorly preserved sources can be analyzed if sufficiently small PCR products are identified.

Discovery of PCR

The concept of PCR was first conceived by Dr. Kerry Mullis in 1983, first reported in1985, while working at the Cetus Corporation in Emeryville, CA, along with other researchers at Cetus Corporation (Molecular Station, 2006). Kerry Mullis discovered that by harnessing one component of molecular reproduction technology, ie a basic principle of replicating a piece of DNA using two primers, a target DNA of interest could be amplified exponentially. This DNA amplification procedure was an *in vitro* process (meaning in a test-tube). The first ever PCR product was the 110 base pair DNA fragment of a cloned segment of the human beta-globulin gene at the company labs, being the beginning of PCR as a basic technique in molecular biology (Mullis et al.,1986, Mullis and Faloona 1987). Dr. Mullis was awarded the Nobel Prize in Chemistry in 1993 for his development of the Polymerase

Chain Reaction (PCR), a central technique in biochemistry and molecular biology. Dr. Mullis subsequently was awarded the Japan Prize that same year.

Materials and reagents for PCR

The components required for the PCR are the template (the DNA to be amplified), a pair of primers, thermostable polymerase, the four types of de-oxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and appropriate reaction buffer containing magnesium ions (KCl, Tris-HCl (pH 8.4), $MgCl_2$ and gelatin). They are assembled in a tube and the amplification reaction is carried out by manipulating the temperature within the reaction tube, in cyclic manner, using a thermal cycler. For any given pair of primers, the optimal concentrations of all the above ingredients and parameters have to be standardized. Even though there is no single set of conditions and concentrations that will be optimal for all reactions, the parameters outlined below defines a common starting point from where modifications can be attempted.

Target DNA (Template): An advantage of PCR is that it can amplify relatively impure DNA or DNA from blood spots, archival material and ancient DNA. Concentration of template DNA also affects the degree of amplification. The sample DNA generally contains 10^2 to 10^5 copies of template. Too high or too low concentration will result in poor amplification. Therefore, it is useful to optimize the template concentration in a PCR reaction to obtain maximum product. While typically DNA quantity is measured in ng, the relevant unit is actually moles, i.e., how many copies of the sequence that will anneal with the primers are present. Thus, the amount of DNA in ng that is needed to add is a function of its complexity. In theory, a single molecule of DNA can be used in PCR but normally between 1000 and 100,000 molecules for eukaryotic nuclear DNA are used. The nucleotide composition of target DNA also affects the PCR amplification. Extremely GC rich DNA strands are difficult to separate. Addition of denaturing agents like formamide or DMSO can help to overcome the problem.

Primers: Primers are the most important components of PCR, and the success of a PCR largely depends on the primers. Primers are short, single stranded DNA molecules which will bind (anneal) to either ends of region to be amplified (one on each strand) and serve as the starting point for building a new complementary nucleic acid strand. Primers are generally made in pairs, called “forward” and “reverse”. These primers are complimentary to the regions flanking the DNA segment to be amplified such that they can be extended toward one another with DNA polymerase, forming new DNA molecules. The most important property of a primer is its sequence specificity, which determines what nucleic acid sequence it can bind to, how well it will bind, and how well it will serve as a site for extension of new nucleic acid molecules. Generally, a “specific” primer is designed to target a DNA sequence in a closely related group of organisms, while not matching organisms outside that group. “Universal” primers are designed to target DNA sequences shared by any species that contains the sequence of interest. Thus, care should be taken while designing the primers for a particular experiment. Oligonucleotide primers in the range of 18 to 30 bases are generally used for the PCR. Though there are no set rules that will ensure the synthesis of an effective primer pair, the following guidelines are useful.

(a) Wherever possible, select primers with a random base distribution and with a GC content similar to that of the fragment being amplified. Avoid primers with stretches of polypurines, polypyrimidines or other unusual sequences.

(b) Check the primers against each other for sequence complementarity. Use primers with low complementarity to each other. Avoid primers with 3' end overlaps in particular. This will reduce incidence of "primer dimers". Most primers are generally 18 to 30 bases in length and the optimal length to be used in an amplification will vary. Longer primer may be synthesized but are seldom necessary. If shorter primers or degenerate primers are used, the thermal profile should be modified considering the lower stability of the primed target. However, the 3' end of the primer should match the template exactly. Generally, concentrations ranging from 10 to 50 p moles of each primer should be used.

The optimum length of a primer depends upon its (A+T) content, and the Tm of its partner. A prime consideration is that the primers should be complex enough so that the likelihood of annealing to sequences other than the chosen target is very low. For example, there is a ¼ chance (4^{-1}) of finding an A, G, C or T in any given DNA sequence; there is a 1/16 chance (4^{-2}) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a given sixteen base sequence will statistically be present only once in every 4^{16} bases (= 4 294 967 296, or 4 billion): this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*. Thus, the association of a greater than 17-base oligonucleotide with its target sequence is an extremely sequence-specific process. Generally, 17-mer or longer primers are routinely used for amplification from genomic DNA of animals and plants. Long primers will result in mismatch pairing and non-specific priming even at high annealing temperatures

Melting temperature (Tm) of primers: The annealing temperature is dependent on the **Tm** of primer. Annealing temperature can be the Tm value calculated using the following formulae:

(1) **Tm** = [(number of A+T residues) x 2 °C] + [(number of G+C residues) x 4 °C]

This formula was determined originally from oligonucleotide hybridization assays, which were performed in 1 M NaCl, and appears to be accurate in lower salt conditions only for primers less than or about 20 nucleotides in length.

(2) **Tm** p = 22 + 1.46 ([2 x (G+C)] + (A+T))

This formula is reportedly useful for primers of 20-35 bases in length. The calculated annealing temperature is only a reference temperature from which to initiate experiments. The actual annealing temperature may be 3-12 °C higher than the calculated Tm. The actual annealing temperature condition should be determined empirically. The optimum annealing temperature which gives the best PCR product should be used.

Deoxynucleotide triphosphate: The dNTPs are the building blocks of DNA. Once the primer binds to its target site, synthesis of the complementary strand of DNA takes place through primer extension by linking of nucleotide to its 3'end with the help of Taq DNA polymerase. Precursor dNTPs can be obtained as a neutralized solution, which are stable at –20ºC for months. The doxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) is generally used at concentrations of 200 mM (0.2mM) each. Higher concentrations may lead to mis-incorporations. Low dNTP concentration reduces mispriming at non-target sites. The lowest dNTP concentration appropriate for the length and composition of the target must be standardized. As a thumb rule, 20mM of each dNTP in a 100 ml reaction is sufficient to synthesize 10 p Mol of a 400 bp sequence. In the standard

reaction, all four triphosphates are added to a final concentration of 0.8mM; this leaves 0.7 mM of the original 1.5mM $MgCl_2$ not complexed with dNTP. Therefore, if dNTP concentration is changed significantly, a compensatory change in $MgCl_2$ may be necessary.

Taq* DNA polymerase:** The discovery of thermostable DNA polymerase has revolutionized the PCR technology. They are obtained from organisms that thrive in extreme temperatures and have an optimum activity at 72° C. It is able to withstand the denaturing conditions (over 90 °C) required during PCR cycling. Thus, unlike thermo-labile polymerase, with *Taq* polymerase there is no need for extra addition of enzymes during cycling process where strands separation required heating to over 90°C .There are now a plethora of commercially available enzymes to choose from that differ in their thermal stability, processivity, and fidelity. The choice of the DNA polymerase employed by PCR is determined by the goals of the experiment. The most commonly used thermostable polymerase is *Taq* DNA polymerase isolated from the bacterium *Thermus aquaticus* which inhabit the hot springs with extremely high temperatures. Isolation of the DNA polymerase from this bacterium yielded a PCR polymerase that was not rapidly inactivated at high temperatures. In 1986, Dr. David Gelfand and Ms. Susanne Stoffel of Cetus Corporation purified such a thermostable DNA polymerase, referred to as native *Taq* (T****hermophilus* ***aq****uaticus* in short), from the organism *Thermus aquaticus*. Taq polymerase was shown to work successfully in PCR, enabling the process to be performed much more easily. Today, almost all PCR is done using recombinant *Taq*, a cloned version of the enzyme, as it is less expensive to manufacture than the native form of the enzyme (Roche Diagnostics, 2007). *Taq* was the first polymerase that was able to withstand the denaturing conditions (over 90 °C) required during PCR cycling. *Taq* has an enzymatic half life at 95°C of about 40 min. *Taq* DNA polymerase is unique in that it produces PCR products with A (Adenine) overhangs. This was found to be quite useful, and was exploited to produce TA Cloning and TOPO cloning. One of *Taq* polymerases' major disadvantages is its low replication fidelity. As Taq does not have 3' to 5' exonuclease proofreading mechanism to replace an accidental mismatch in the newly synthesized DNA strand, Taq produces more errors than proofreading polymerases, such as Pfu (Roche Diagnostics, 2007) *Pyrococcus furiosus*, where it functions in vivo to replicate the organism's DNA. Pfu's have superior thermostability and 'proofreading' properties compared to other thermostable polymerases. Unlike *Taq* DNA polymerase, Pfu DNA polymerase possesses 3' to 5' exonuclease proofreading activity and corrects nucleotide-misincorporation errors. Thus Pfu DNA polymerase-generated PCR fragments will have fewer errors than Taq-generated PCR inserts. It also results in blunt-ended PCR products.

The required concentration of *Taq* DNA polymerase is between 1 and 2.5 units per 100 ml reaction when other parameters are optimum. When optimizing a PCR, enzyme concentration ranging from 0.5 to 5 units/ 100ml are tried and resultant products are visualized by agarose gel electrophoresis. If the enzyme concentration is too high, non-specific background products may accumulate and if too low, an insufficient amount of desired product is made.

Examples of thermostable DNA polymerases:

DNA Polymerase	**Source**
Taq	*Thermus aquaticus*
Amplitaq®	*T. aquaticus*

Amplitaq (Stoffel fragment)®	*T. aquaticus*
Hot *Tub*™	*Thermus flavis*
Pyrostase™	*T. flavis*
Vent™	*Thermococcus litoralis*
Deep Vent™	*Pyrococcus GB-D*
Tth	*Thermus thermophilus*
Pfu	*Pyrococcus furiosus*
ULTma™	*Thermotoga maritima*

The Reaction Buffer: The PCR buffer contains KCl, Tris HCl (pH 8.4), $MgCl_2$ and gelatin. The components of PCR buffer, particularly the concentration of $MgCl_2$ have a profound effect on the specificity and yield of an amplification product. Success of PCR is dependent on $MgCl_2$ concentration in the reaction to a great extent. Mg_2+ ions form a soluble complex with dNTPs which is essential for dNTP incorporation, stimulate polymerase activity and increase the Tm (melting temperature) of primer / template interaction (i.e. it serves to stabilize the duplex interaction). Concentration of about 1.0 to1.5 mM is usually optimal (when 200uM each of dNTPs are used). Excess of Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield. Optimization by titration of $MgCl_2$ concentration is recommended to establish an optimum concentration for a particular reaction.

Several buffer formulations have been published & a consensus is emerging. The recommended PCR buffer should contain 10mM Tris-HCl (pH 8.4) also. KCl up to 50mM can be included in the reaction mixture to facilitate primer annealing. Excess KCl inhibits Taq polymerase activity. Gelatin or bovine serum albumin (100 g/ml) and nonionic detergents such as Tween- 20 and NP40 (0.05 - 0.1%) are included to help stabilize the enzyme. The nonionic detergents can be replaced by 0.1% Triton X-100, but some detergent is essential.

Thermal Cycles for PCR:

Amplification of a target DNA is achieved by repeated cycles of denaturation, primer annealing and extension. These events are controlled by manipulation of temperature. The above three major steps in a PCR are repeated for 35 to 40 cycles. This is done using an automated thermal cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation: Double stranded DNA used for the PCR is separated into single strands in the initial denaturation step. Typical denaturation temperature is $94^{O}C$ for 15 to 60 seconds. Higher temperatures e.g. $97^{0}C$ may be necessary for G + C rich targets. Denaturation steps that are too long or too high lead to unnecessary loss of enzyme activity. Denaturation of nucleic acid (NA) is carried out to make it single-stranded for the purpose of annealing with primers. It is done by heating it to a point above the “melting temperature” of the double or partially double stranded form, and then flash-cooling it: this ensures the “denatured” or separated strands do not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than 150 mM NaCl, the melting temperature is generally less than $100^{o}C$ - which is why PCR works with denaturing temperatures of 91-$97^{o}C$. The main reason of importance of denaturing temperature and time in relation to number of cycles is because Taq polymerase has a half-life of 30 min at $95^{o}C$. This half life supports not more than about 30 amplification

cycles. However, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased. "Time at temperature" is the main reason for denaturation / loss of activity of *Taq*. Thus, with reduction of time, increases the number of cycles are possible, whether the temperature is reduced or not. It is possible, for short template sequences, to reduce this to 30 sec or less.

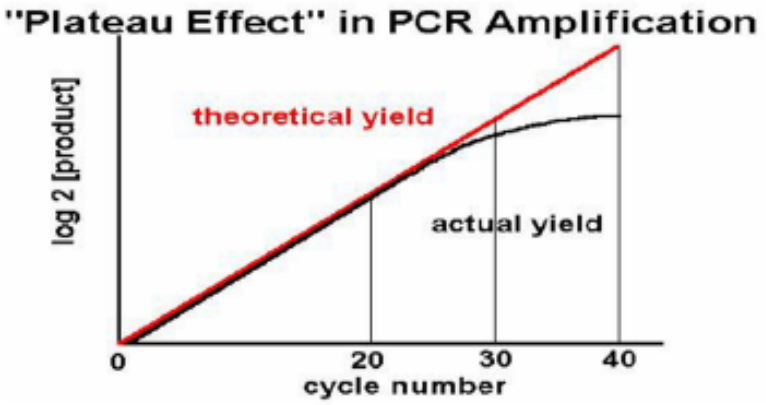
Primer annealing: At temperatures ranging from 47ºC to 62ºC, the primers anneal to its complimentary region on the template. The complimentary sequences will form hydrogen bonds between their complimentary bases (G to C, and A to T or U) and form a stable double stranded, anti-parallel molecule. During PCR, the primers are moving around, caused by the Brownian motion in the reaction mix. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bond lasts a little bit longer (primer that fit exactly) and on that little piece of doubling stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break any more. This is usually performed at temperatures between 47°C and 65°C for 30 to 60 seconds.

The temperature and length of time required for primer annealing depends upon the base composition, length and concentration of the primers. As a rule of the thumb, annealing temperature (Ta) of 5^0C below the lowest melting temperature (Tm) of the amplification pair of primers can be attempted. The annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). Annealing temperature in the range of 55 to 65^0C generally yield the best results. At the optimal primer concentration annealing will require only a few seconds. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces mis-extension of incorrect nucleotides at the 3' end of the primers. Therefore, stringent annealing temperature, especially during initial few cycles will help to increase specificity.

Primer Extension: The DNA polymerase works ideally at temperature 72ºC. The annealed primers, to which a few bases have been added, have a stronger attraction to the template, created by hydrogen bonds, than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and do not give an extension of the fragment. The nucleotides (complementary to the template) are linked to the primer on the 3'side by the polymerase, from 5' to 3', reading the template from 3' to 5' side and bases are added complimentary to the template. Extension time depends on the length and concentration of the target sequence and upon the temperature. Primer extensions are usually performed at 72^0C. The rate of nucleotide incorporation at 72^0C varies from 35 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and the nature of the DNA template. The length of the elongation step (30 seconds to three minutes) is determined by the speed of the enzyme, its ability to continue moving down the template DNA and the length of the DNA segment to be amplified. At around 70ºC, the polymerase activity is optimal, and primer extension occurs at upto 100 bases/sec. A general guideline is 1 minute / kb of product length. An extension time of one minute at 72^0C is considered sufficient for products up to 2 Kb size. Longer products require longer times: approximately 3 min for 3kb and longer products.

Cycling could include an initial denaturation at 94^OC and a final extension at 72^OC for 5 min. At the end reactions are stopped by chilling at 4^OC or by addition of EDTA at 10mM.

Cycle number: The optimum number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA when other parameters are optimal. Because both strands are copied during PCR, there is exponential increase of the number of copies of the gene. For example if the PCR is initiated with one copy of the gene, after one cycle there will be 2 copies, after two cycles there will be 4 copies, three cycles will result in 8 copies and so on. Too many cycles may increase the amount and complexity of non-specific background products. Too few cycles give low product yield. Innis and Gelfand (1990) recommend from 40 - 45 cycles to amplify 50 target molecules, and 25 - 30 to amplify $3x10^5$ molecules to the same concentration. This non-proportionality is due to a so-called *plateau effect* (Rybicki, 2001), which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3 - 1.0 nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs - former a problem with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product (Innis and Gelfand, 1990).



(Source: Rybicki, 2001)

Detection and analysis of PCR product: The PCR product will be DNA fragments (amplicons) of defined length. The simplest way to check the PCR product is to load a portion of it into an agarose gel containing ethidium bromide along with molecular weight markers and carry out an electrophoresis. The DNA fragments generated by the PCR should be readily visible over an ultraviolet transilluminator. Hybridizing the PCR product with suitable DNA probe is also in practice for conformation.

Common Types of PCR

PCR has been adapted to fit many different applications and hence there are many different types and each one is unique to the application for which it was designed. There are six common types of PCR: conventional PCR, multiplex PCR, reverse transcription (RT)-PCR, nested PCR, real-time PCR and random primed PCR. And there are many other types for specific purposes.

Conventional PCR: Conventional PCR uses a thermostable DNA polymerase to amplify a region of the DNA defined at each end by a specific primer. The exponential replication of the same target sequence produces enough DNA product or amplicons for use in subsequent analyses. PCR typically consists of three basic steps, as mentioned earlier.

Multiplex PCR: Multiplex PCR is a modification of conventional PCR in which two or more different PCR products are amplified simultaneously within the same reaction. This type of PCR consists of the same steps as conventional PCR, except that multiple sets of primers are used, each one priming a PCR product. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. The advantage is that it requires less time and effort in amplifying multiple target templates or regions than individual reactions and may be a useful screening assay. However, significant optimization is required to obtain all of the products with equal efficiency and sensitivity. By simultaneously amplifying more than one locus in the same reaction, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. Since its first description in 1988 (Chamberlain *et al*., 1988), this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse transcription PCR.

Nested PCR: Nested PCR is a very specific PCR amplification and is a variation of the conventional PCR, in that two pairs (instead of one pair) of PCR primers are used to amplify a fragment. The first PCR utilizes a pair of primes flanking the gene in question while the second PCR uses another pair of primers having complementarity to an internal segment of the gene, which was amplified in the first PCR. The fragment produced by the first reaction is used as the template for the second PCR. The second set of primers called nested primers (as they lie / are nested within the first fragment) is specific to the DNA sequence found within the initial PCR product. The use of a second amplification step with the "nested" primer set results in a reduced background due to the nested primers' additional specificity to the region. The amount of product produced, which is shorter than the first one, is increased as a result of the second round of amplification. Carrying out nested PCR can further enhance the reliability of the PCR. Therefore, when information on the sequence of specific genes is available, amplification and visualization of that gene using a nested PCR could be carried out for confirmation.

Reverse Transcription Polymerase Chain Reaction (RT-PCR): This is based on the processes of reverse transcription and polymerase chain reaction. RT-PCR is a two step process. The first step consists of the formation of complementary or copy DNA (cDNA) from RNA (generally mRNA). This is followed by the second step which is a conventional PCR using the cDNA as the template. The first step referred to as the "first strand reaction" uses enzyme reverse transcriptase for the production of the cDNA from the RNA. In the second step, the cDNA sequence is amplified by using primers specific to it. The RT-PCR forms a high sensitivity detection technique, where low copy number or less abundant RNA molecules can be detected. It is also used to clone mRNA sequences in the form of complementary DNA, allowing cDNA libraries to be created which contain all sequences of all the genes expressed in a cell. It allows the creation of cDNA constructs for the gene expression studies.

Real Time PCR: Real-time PCR is different from other PCR as it quantifies the initial amount of the template instead of detecting the amount of final amplified product (Freeman *et al*., 1999; Raeymaekers, 2000). Real Time PCR is characterized by the point in time during cycling when amplification of the PCR product of interest is first detected *rather* than the amount of the PCR product which has accumulated at the end point. Real Time PCR does this by using fluorescent

dyes such as Sybr Green, or fluorophore-containing DNA probes such as Taq Man which get incorporated into each of the new strand, and monitoring the amount of fluorescence emitted during the PCR. This acts as an indicator of the amount of PCR amplification that occurs during each PCR cycle. Thus, in Real Time PCR machines, one can visually see the progress of the reaction in "real time". Quantification using real-time PCR can be 'relative or absolute.

Random /Arbitrary primed PCR: Random primed PCR conceived by Williams *et al* (1990) is unique in that only single short primer (usually 10 bases long) is used instead of the primer pair in the conventional PCR. Prior knowledge of the sequence of the target DNA is not required and primer with any sequence can be employed. The underlying theory in AP-PCR is that the primer may find complimentary sequence at different locations on the two DNA strands used as template, and amplify the intervening regions at low PCR stringency conditions (36 – 40°C).

This is used to generate Random amplified polymorphic DNA (RAPD) profile which is increasingly being used as a method for the DNA finger printing and genetic characterization where prior knowledge of the sequence of the target DNA is not required. RAPD is used as a marker system, where sequence of the target DNA is not known. This is a rapid technique and can be useful for species/strain identification. Genomic variations between and within species could be identified as the difference in the molecular size and number of DNA fragments amplified. The PCR products variations shall be resolved by agarose gel electrophoresis.

Modifications of PCR for specific purposes:

Degenerate PCR: Degenerate PCR is in most respects identical to ordinary PCR, but with one major difference. It is in a situation where the sequence of the gene to be amplified is not known, insert "wobbles" in the PCR primers are inserted. So, instead of using specific PCR primers with a given sequence, mixed PCR primers are used. For example, when a protein motif is back-translated to the corresponding nucleotide motif (Protein —> Sequence), there will be more than one codon coding for particular amino acid, due to degeneracy of genetic code. Thus there will be more than one nucleotide sequence deciphered for a particular protein sequence.

Asymmetric PCR: Asymmetric PCR is used to preferentially amplify one strand of the original DNA more than the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary stands is required. PCR is carried out as usual, but with a great excess of the primers for the chosen strand. Due to the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR): Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is a fast and efficient method initially developed to amplify unknown sequences adjacent to known insertion sites in Arabidopsis. Nested, insertion-specific primers are used together with arbitrary degenerate primers (AD primers), which are designed to differ in their annealing temperatures. Alternating cycles of high and low annealing temperature yield specific products bordered by an insertion-specific primer on one side and an AD primer on the other. Further specificity is obtained through subsequent rounds of TAIL-PCR, using nested insertion-

specific primers. The increasing availability of whole genome sequences renders TAIL-PCR an attractive tool to easily identify insertion sites in large genome tagging populations through the direct sequencing of TAIL-PCR products. For large-scale functional genomics approaches, it is desirable to obtain flanking sequences for each individual in the population in a fast and cost-effective manner.

Hot Start PCR: The technique may be performed manually by heating the reaction components to the melting temperature (e.g., 95ÚC) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature. In Hot Start PCR, polymerase activity during PCR reaction preparation is inhibited by using included chemical modifications, wax-barrier methods, and inhibition by a *Taq*-directed antibody. By limiting polymerase activity prior to PCR cycling, non-specific amplification during the initial set up stages are reduced and the yield of desired PCR product is increased.

Touchdown PCR: Non-specific primer binding obscures polymerase chain reaction results, as the non-specific sequences to which primers anneal in early steps of amplification will "swamp out" any specific sequences because of the exponential nature of polymerase amplification. Touchdown PCR or touchdown style polymerase chain reaction is a variant of PCR that aims to reduce non-specific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5ÚC) above the Tm of the primers used, while at the later cycles, it is decreased in increments for every subsequent set of cycles (the number of individual cycles and increments of temperature decrease is chosen by the experimenter) a few degrees (3-5ÚC) below the primer Tm. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.

Inverse PCR: Inverse PCR (IPCR), variant of PCR, was first described by Ochman *et al.* (1988). It is used when only one internal sequence of the target DNA is known. It is therefore very useful in identifying flanking DNA sequences of genomic inserts. Inverse PCR uses standard polymerase chain reaction, however it has the primers oriented in the reverse direction of the usual orientation. The template for the reverse primers is a restriction fragment that has been ligated upon itself to form a circle. Applications of Inverse PCR in molecular biology include the amplification and identification of sequences flanking transposable elements, and the identification of genomic inserts.

Long PCR: Long PCR is used when large segments of DNA (frequently over 10 kb) is to be amplified. For the accuracy of PCR, special mixtures of proficient polymerases such as *Pfu* are often used, which possesses 3' to 5' exonuclease activity or proofreading activity. The efficiency drastically declines when incorrect bases are incorporated. The 3' to 5' exonuclease activity removes these mis-incorporated bases and makes the further reaction proceed smoothly. Therefore, the amplification of long DNA fragments can be achieved. Long PCR is often used to clone larger genes or large segments of DNA which standard PCR cannot.

Gradient PCR: When a set of PCRs are run with different annealing temperatures all in the same block of the thermal cycler, simultaneously it is referred to as Gradient PCR. This is generally carried out for

standardizing PCR conditions for heterologous primers. In many models of thermal cyclers, blocks are available with gradient annealing temperatures. This saves time and multiple blocks are not needed.

AFLP PCR: Amplified Fragment Length Polymorphism PCR, also called AFLP PCR was originally described by Zabeau and Vos, 1993. AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci using a minimal number of PCR reactions.

Alu PCR: PCR using a primer that anneals to *Alu* repeats to amplify DNA located between two oppositely oriented *Alu* sequences. Used as a method of obtaining a fingerprint of bands from an uncharacterized human DNA.

Colony PCR: Colony PCR is mostly used after a transformation, to screen colonies for the desired plasmid. Primers which generate a PCR product of known size are used. Thus, colonies which give rise to an amplification product of the expected size are likely to contain the correct DNA sequence. Colony PCR is used for the screening of bacterial (*E. coli*) or yeast clones for correct ligation or plasmid products. Selected colonies of bacteria or yeast are picked inserted into the PCR master mix or pre-inserted into autoclaved water. PCR is then conducted to determine if the colony contains the DNA fragment or plasmid of interest.

***In Situ* PCR:** *In Situ* PCR (ISH) is a polymerase chain reaction that actually takes place inside the cell fixed on slide. *In situ* PCR amplification can be performed on fixed tissue or cells. ISH applies the methodology of the nucleic acid hybridization technique to the cellular level. Combining cytochemistry and immunocytochemistry, it allows the identification of cellular markers to be identified and further permits the localization of to cell specific sequences within cell populations, such as tissues and blood samples.

Single Cell PCR: The advent of the polymerase chain reaction (PCR) has revolutionized the way in which molecular biologists view their task at hand, for it is now possible to amplify and examine minute quantities of rare genetic material: the limit of this exploration being the single cell. It is especially in the field of prenatal diagnostics that this ability has been readily seized upon, as it has opened up the prospect of preimplantation genetic analysis and the use of fetal cells enriched from the blood of pregnant women for the assessment of single-gene Mendelian disorders. However, apart from diagnostic applications, single-cell PCR has proven to be of enormous use to basic scientists, addressing diverse immunological, neurological and developmental questions, where both the genome but also messenger RNA expression patterns were examined. Furthermore, recent advances, such as optimized whole genome amplification (WGA) procedures, single-cell complementary DNA arrays and perhaps even single-cell comparative genomic hybridization will ensure that the genetic analysis of single cells will become common practice, thereby opening up new possibilities for diagnosis and research.

Single nucleotide polymorphism PCR (SNP PCR): SNP PCR involves real-time PCR using single nucleotide polymorphisms (SNPs) as markers. It is a very sensitive and accurate method to quantify the percentage of recipient and donor cells to monitor the effect of stem cell transplantation (SCT) and sequential adoptive immunotherapy by donor lymphocyte infusions (DLI).

Digital PCR: Digital PCR represents an example of the power of PCR and provides unprecedented opportunities for molecular genetic analysis in cancer. The technique is to amplify a single DNA template from minimally diluted samples, therefore generating amplicons that are exclusively derived from one template and can be detected with different fluorophores or sequencing to discriminate different alleles (e.g., wild type vs. mutant or paternal vs. maternal alleles). Thus, digital PCR transforms the exponential, analog signals obtained from conventional PCR to linear, digital signals, allowing statistical analysis of the PCR product. Digital PCR has been applied in quantification of mutant alleles and detection of allelic imbalance in clinical specimens, providing a promising molecular diagnostic tool for cancer detection.

Assembly PCR: Assembly PCR is the artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments thereby selectively producing the final long DNA product.

Helicase-dependent amplification: This technique is similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA Helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

Intersequence-specific (ISSR) PCR: A PCR method for DNA fingerprinting that amplifies regions between some simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.

Ligation-mediated PCR: This method uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA fingerprinting.

Methylation-specific PCR (MSP): The MSP method was developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine, and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCR reactions are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

PCR applications

PCR has transformed the way that most studies requiring the manipulation of DNA fragments and DNA cloning may be performed as a result of the simplicity and usefulness of PCR. Cell-free DNA amplification by PCR is able to simplify many of the standard procedures for DNA cloning, DNA analysis, and the modification of DNA. Previous molecular biology techniques for isolating a specific piece of DNA had relied on gene cloning, which is a tedious and slower procedure. An alternative to cloning, PCR, can be used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known. This method of amplifying rare sequences from a mixture has numerous applications in basic research, human genetics testing and forensics. Some of the PCR applications include site-specific mutagenesis studies, amplification and detection of

DNA *in situ* from cells for rapid diagnosis, genomic subtraction, analysis of protein functions and intermolecular assembly, DNA fingerprinting (RAPD/AFLP/VNTR/) for evaluation of genetic heterogeneity & relationship, paternity verification, forensic application, generation of single chain antibody fragments for immunology, sensitive disease diagnosis, cDNA synthesis from RNA for cDNA library construction, production of clones for sequencing, molecular epidemiology, molecular taxonomy and many more.

Electrophoresis: Principles and Types

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Each fish species is chemically composed of different proteins at varying levels, so techniques that separate proteins may help to identify different species. Of these techniques, electrophoresis is the most important one. Many biological molecules such as proteins are made up of amino acids with electrically charged side chains. Basic amino acids such as arginine, histidine and lysine are positively charged while the acidic amino acids such as aspartic acid and glutamic acid carry negative charges. Thus, virtually all proteins have a net charge depending on the relative proportions of amino acids, unless they are at their "iso-electric point" (pI), the definite pH at which the net charge of the protein molecule is zero. The basis of electrophoretic separation is that proteins of different net charge and different molecular size will migrate at different rates within an electric field and it is a very useful technique for the separation of cellular proteins and DNA.

The term electrophoresis comes from the Greek, and means, "transport by electricity" and has been known since the end of 19th century. In 1807, a Russian Physicist, Alexander Reuss observed a novel phenomenon - when electricity was passed through a glass tube containing water and clay, colloidal particles moved towards the positive electrode. The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Charged molecules are having either positive or negative charge. At a given pH, the biological molecules exist in solution as electrically charged particles. Under the influence of an electric field, these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The theory of movement of a particle in electrophoresis is as follows: When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient, **E**, which is the applied voltage (**V**) divided by the distance, **d**, between the electrodes. The force that drives a charged molecule towards an electrode is the product of potential gradient, and the charge of **q** coulombs on the particle. However, the frictional force that retards the movement of a charged molecule, is function of hydro-dynamic size of the molecule, shape of the molecule, the pore size of the medium in which electrophoresis is taking place and the viscosity of the buffer.

The velocity (**v**) of charged molecule in an electric field-

$$V = \frac{Eq}{f}$$

Where f = frictional coefficient, which depends upon the mass and shape of the molecule.
E = electric field (V/ cm)
q = the net charge on molecule
v = velocity of the molecule.

Most of the large molecules possess both cationic (basic positively charged) and cationic (acidic-negatively charged) groupings as part of their structure and hence are termed as "amphoteric molecules" or "Zwitterions". The actual charge of protein molecule is the result of the sum of all single charges. Because dissociation of the different acidic and basic groups takes place at different hydrogen ion concentrations of the medium, pH greatly influences the total charge of the molecule. At lower pH, they migrate to the negative pole (cathode) and at higher pH to the positive pole (anode). Ionic strength also affects the migration, low ionic strength permits high rate of migration. The choice of buffer strength may be seen to be crucial, since it determines the amount of electrical power that can be applied to the system. The rate of migration will also depend upon the charge density (the ratio of charge to mass) of the proteins concerned; the higher the ratio of charge to mass, the faster the molecule will migrate.

Gel electrophoresis

In this type of electrophoresis, gel is used as the support media instead of thin paper sheets etc. Most electrophoresis is carried out on the inert media.

Types of gel electrophoresis

I. Based on Buffer System

A. Continuous buffer System:

Continuous buffer electrophoresis is the simplest and most commonly used method. The same buffer is used to provide electrical contact between the platinum electrodes and the support medium and to soak the gel. In the gradient pore method an acrylamide gel is prepared other. Protein solutions are applied at the end of the gel where pore size is largest and allowed to migrate in an electrical field until they reach the point where pore size prevents further movement. Separation is purely on the basis of molecular size and electrical charge is used only to induce movement.

B. Discontinuous buffer System:

In discontinuous buffer or multiphasic electrophoresis, the electrode chambers contain a different buffer to that in the gel. The front at which the two buffers meet concentrates different proteins so that they enter the main electrophoresis system as a very narrow zone. This will enhance the resolution.

II. Based on Support media

Supporting media:

A variety of anti-convection media are being used in zone electrophoresis. They exhibit several properties. An ideal medium should have the following features:

Chemical nature	inert
Availability	easy

Electrical conductivity	high
Adsorptivity	low
Sieving effect	desirable
Porosity	controlled
Transparency	high
Electro-endosmosis (EEO)	low
Rigidity	moderate to high
Preservation	feasible
Toxicity	low
Preparation	easy

The original material, filter paper, has now been replaced by a variety of gels. Cellulose acetate and agar have large pore size and are used for separation of large protein molecules and for immunodiffusion. Starch and polyacrylamide have a pore size to the molecular size of many proteins, so there is also a molecular sieving effect. The pore size of acrylamide gels is adjustable. Protein resolution depends on the pH and ionic strength of the buffer, the pore size of the gel and the current applied. Buffer pH is usually 8 to 9 ensuring that all proteins are negatively charged and migrate to anode. Heat is produced in proportion to the product of Volt x Ampere so temperature must be controlled by using cooling devices. The details of various media are discussed below.

A. Starch gel electrophoresis:

In this type of electrophoresis, starch is used as the support media. The molecular sieving properties of starch make it good choice for the separation of complex mixtures of structural molecules and physiologically active proteins. Starch gel is prepared by mixing hydrolyzed potato starch with an electrolyte buffer, cooking until a gel of uniform consistency is achieved, followed by pouring the gel into a mold. The ends of the longer section of the gel are placed gently on the absorbent towels or filter paper in the lower (anodal) buffer tank. Each tank is approximately one-third filled with chilled electrode buffer. Voltage, current and duration of the electrophoresis and other requirements vary widely among buffer systems for particular electrophoretic separations. Gels should be run in the cold chambers to produce sharp and straight lines of migration and to minimize heat production in overnight conditions usually requiring a constant 200V and variable current of not more than 30mA. An important application of Starch gel electrophoresis is the analysis of isozyme pattern.

B. Polyacrylamide gel electrophoresis (PAGE):

1. Native polyacrylamide gel electrophoresis

Acrylamide monomer (CH_2= CH CO NH_2) is co-polymerized with a cross linking agent, usually N.N'- methylene bisacrylamide {CH_2 (NH CO CH = CH_2)$_2$}, in the presence of a catalyst accelerator chain initiator mixture. This mixture consists of freshly prepared ammonium per sulfate as (0.1 to 0.3% w/v) initiator together with about the same concentration of a suitable base, for example, dimethyl aminopropionitrile (DMAP) or N, N, N', N' - tetra methylenediamine (TEMED) as catalyst. Out of the two, the most used is TEMED and proportional increase in its concentration speeds up the rate of gel polymerization. Gelation occurs due to vinyl polymerization. Prior degassing of solution is required since molecular oxygen inhibits chemical polymerization. The relative proportion of

acrylamide monomer to cross-linking agent determines the porosity of a gel. Gels may be defined in terms of the total percentage of acrylamide present. Gels may be prepared containing from 3% to 30% acrylamide, corresponding to pore sizes of 0.5nm and 0.2nm, diameter respectively. Polyacrylamide gels may be prepared with a high degree of reproducibility and the precise porosity. This feature makes the method particularly suitable for resolving mixtures of proteins. This feature makes the method particularly suitable for resolving mixtures of proteins. Other features of polyacrylamide gels include their minimal absorption capacity, their lack of electro-endosmosis and their general suitability for *in situ* quantitative analysis (as they do not absorb UV) and for various types of histochemical analysis. For running of the polyacrylamide gels, the gel slab (earlier gel rods made in glass tubes of uniform diameter were used) is loaded with the sample and attached to the lower submarine unit, filled with the buffer and connected to the power pack for the DC supply.

2. Denaturing Polyacrylamide gel electrophoresis:

In the electrophoretic technique previously discussed, the mobility of biological molecules is influenced by both charge and size. But, if protein samples are treated with certain chemicals so that they have a uniform charge, the electrophoretic mobility then depends primarily on size. The molecular weights of proteins may be estimated if they are subjected to electrophoresis in presence of detergent, SDS and disulfide reducing agent mercaptoethanol. When protein molecules are treated with SDS, the detergent disrupts the secondary, tertiary and quaternary structure, leaving the molecule to produce polypeptide chain in a random coil, imparting an overall negative charge and masking the individual variation in charge. The presence of mercaptoethanol assists in protein denature by reducing all disulfide bonds. In essence, polypeptide chains of constant charge/mass ratio and uniform shape are produced. The electrophoretic mobility of the SDS-protein complexes will be influenced primarily by molecular size; the larger molecules will be retarded by the molecular sieving effect of the gel, while the smaller molecules will have greater mobility. In practice, a protein of unknown molecule weight and structure is treated with 1% SDS and 0.1 mercaptoethanol in electrophoresis buffer. A standard mixture of proteins with known molecular weights must also be subjected to electrophoresis under the same conditions. After electrophoresis followed by staining, the molecular weight may be determined.

C. Agarose electrophoresis:

The electrophoretic technique used to characterize DNA and RNA is through agarose gels. The mobility of nucleic acid in agarose gels is influenced by agarose concentration and the size and shape of the nucleic acid. Agarose concentration of 0.5 to 3% is most effective for nucleic acid separations. Gels with agarose concentration less than 0.5% are rather fragile and must be used in a horizontal slab arrangement or in a refrigerated chamber. Like proteins, nucleic acids migrate at a rate that is inversely proportional to the logarithm of their molecular weights; hence molecular weight can be estimated from electrophoretic results using standard nucleic acids of known molecular weight. Passage of a molecule through a gel is influenced by the shape and size of the molecule. A small, compact molecule would be expected to have a greater mobility than rod like, linear molecules.

Most agarose gel electrophoresis experiments are carried out with horizontal slab gels. This method is chosen over vertical mode because low agarose concentration can be used for maximum mobility.

D. Cellulose acetate gel electrophoresis:

In this type of electrophoresis, protein migration essentially takes place in the buffer film on the gel surface. The medium has therefore no influence on the electrophoretic mobility.

E. Isoelectric Focusing (IEF):

Isoelectric focusing uses a polyacrylamide gel with large pore size containing a mixture of polyamino, poly carboxylic acids with different isoelectric point (pI) s. These form a stable pH gradient along the gel in an electric field. Strong acid applied at the anode and strong base in the cathode contain and stabilize the gradient. Proteins migrate under the influence of their charge until they reach the point in the gel where the pH is equivalent to their isoelectric point and so their charge is neutralized. At the isoelectric point, proteins in the electrical field do not migrate to either of the poles. High resolutions are achieved permitting separation of proteins differing only by 0.01 pI.

F. Two - dimensional (2D) electrophoresis:

The techniques of isoelectric focusing and polyacrylamide gel electrophoresis have been combined to produce two-dimensional separation of proteins. This technique is increasingly used now a days and its great resolving power is due to the use of two independent properties of proteins. The proteins are first separated by isoelectric focusing (this is the first dimension), which separates proteins according to their charge (isoelectric point). The proteins are subsequently separated by SDS-PAG electrophoresis (this is the second dimension) at right angles, which separates proteins according to their size (molecular weight). This technique results in a series of spots distributed throughout the polyacrylamide gel.

Source of current:

The source of DC is a simple battery. However, for prolonged and constant supply, alternating current (AC) after rectification to DC is employed. Unlike in DC, the electron flow in AC is not unidirectional. But this is rectified by equipment called electrophoresis power supply. These equipments are made to supply constant voltage, current or power.

Constant Voltage: Almost all power supplies provide constant voltage. Voltage gradient of 15/cm is generally set for electrophoresis at room temperature (25^0C). But, when higher voltage is employed, heat generation is unavoidable. Hence for all types of electrophoresis using agarose gel, which is heat labile, a constant low voltage is given. Increased resistance during the run is reflected in the decrease of mA.

Constant Current: This provision is available in imported power supplies, and is generally required for Disc-PAGE to generate localized voltage gradient. Upto 5 mA/gel rod and 25 mA/gel slab is provided. Voltage and temperature rise during electrophoresis can be lowered by buffer circulation through a coolant.

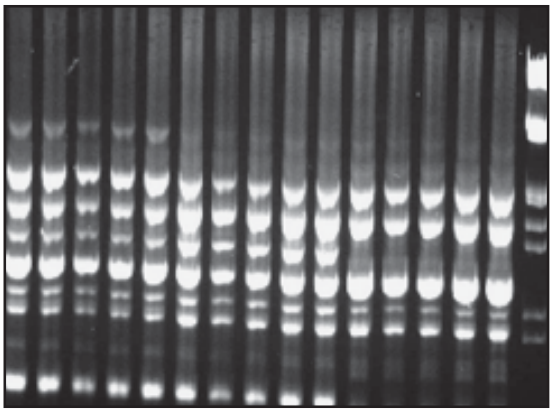
Buffers:

It is a solution of a weak acid and one of its salts. It resists changes in H^+ and OH^- ion concentrations and maintains constant pH. Each buffer has its own 'buffering capacity' (the rate of change of pH to the number of equivalents of acid or base added). The following are the commonly employed buffers and their pH values with regard to electrophoresis.

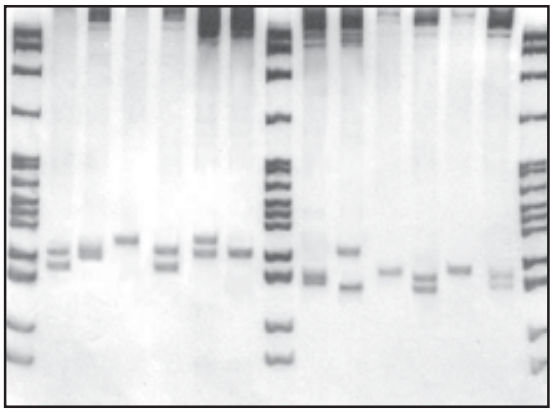
Buffer	**pH value**
Phosphate buffer	around 7.0
Tris-Borate-EDTA buffer (TBE)	around 8.0
Tris-Acetate EDTA buffer (TAE)	above 8.0
Tris Glycine buffer (TG)	more than 8.5
Tris -Citrate-EDTA buffer (TCE)	around 7.0
Tris -EDTA buffer (TE)	around 8.0
Tris -Maleic acid -EDTA buffer (TME)	around 7.5
Lithium Borate - buffer (LB)	around 8.6

Thus, electrophoresis, a ubiquitous biochemical method that allows separation as well as visualization of macromolecules. This may be considered as the core technique of all molecular based studies. In the filed of genetics, it may be utilized as the base technique in examining the genetic diversity of individuals/population, which in turn may help in establishing genetic relatedness between taxa to provide major role in conservation and management strategies.

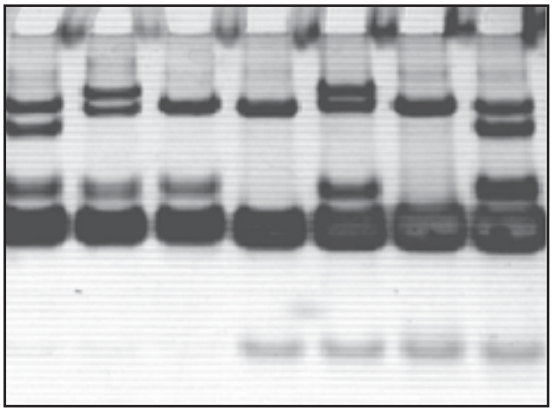
Images of different types of gel electrophoresis



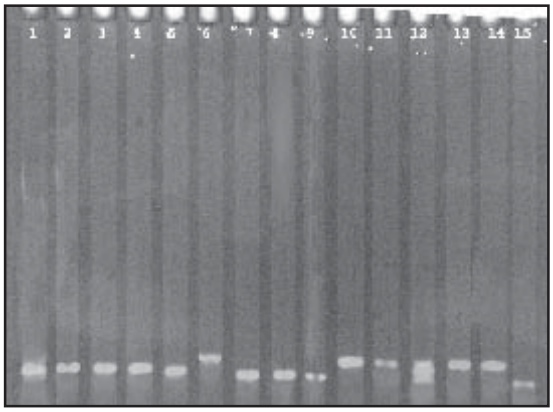
RAPD pattern of fish DNA with Operon primer Agarose (1.5%) electrophoresis



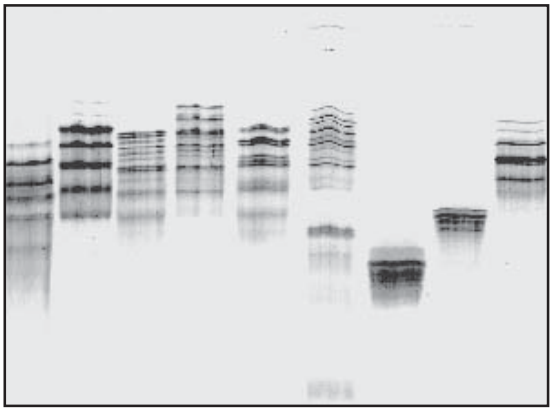
Microsatellite pattern in PAGE



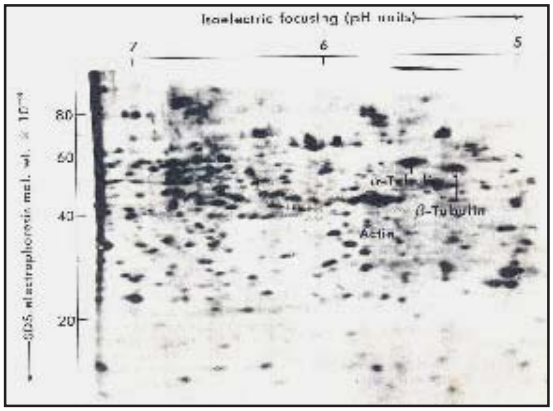
Allozyme (Esterase) pattern in PAGE



Allozyme (SOD) pattern in PAGE



Ultra-thin IEF of fish haemoglobin



2D gel electrophoresis of frog oocytes (IEF and SDS PAGE at right angles)

Molecular Genetic Markers

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Establishment of genetic markers is the prerequisite for stock structure analysis. The markers can detect genetic variations and they can be explained and analysed within the limits of genetic principles. Based on their mode of transmission and evolutionary dynamics genetic markers can be categorized into (**1**) protein markers such as general proteins and allozymes and (**2**) DNA markers such as (2.1) mitochondrial DNA, (2.2) chloroplast DNA and (2.3) nuclear DNA markers like randomly amplified polymorphic DNA (RAPDs) and variable number of tandem repeats (VNTRs) loci such as minisatellites and microsatellites.

Types of molecular genetic markers and their principles

All organisms are subject to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation (polymorphism). In conjunction with selection and genetic drift, there arises genetic variation within and among individuals, species, and higher order taxonomic groups. For this variation to be useful to geneticists, it must be (1) heritable and (2) discernable to the researcher, whether as a recognizable phenotypic variation or as a genetic mutation distinguishable through molecular techniques. At the DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphisms (SNPs), insertions or deletions of nucleotide sequences (indels) within a locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest. Through long evolutionary accumulation, many different instances of each type of mutation should exist in any given species, and the number and degree of the various types of mutations define the genetic variation within a species. DNA marker technology can be applied to reveal these mutations. Large deletions and insertions (indels) cause shifts in the sizes of DNA fragments produced upon digestion by restriction enzymes, and are among the easiest type of mutations to detect, mainly by electrophoresis of the fragments on an agarose gel; smaller indels require DNA sequencing or more elaborate electrophoretic techniques to determine smaller changes in size. Inversions and rearrangements that involve restriction sites can be easy to detect because they disrupt the ability of a restriction enzyme to cut DNA at a given site and thus can produce relatively large changes in DNA fragment sizes. Point mutations are more difficult to detect because they do not cause changes in fragment sizes. Several marker types are highly popular in genetics. In the past, allozyme and mtDNA (restriction fragment length polymorphism (RFLP)) markers have been popular genetics research. More recent marker types that are finding service in this field include, mtDNA sequence information, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers.

Type I versus type II markers and polymorphic information content (PIC)

Molecular markers are classified into two categories: type I are markers associated with genes of known function, while type II markers are associated with anonymous genomic segments. Under this classification, most RFLP markers are type I markers because they were identified during analysis of known genes. Likewise, allozyme markers are type I markers because the protein they encode has known function. RAPD markers are type II markers because RAPD bands are amplified from anonymous genomic regions via the polymerase chain reaction (PCR). AFLP markers are type II because they are also amplified from anonymous genomic regions. Microsatellite markers are type II markers unless they are associated with genes of known function. EST markers are type I markers because they represent transcripts of genes. SNP markers are mostly type II markers unless they are developed from expressed sequences (eSNP or cSNP). Indels are becoming more widely used as markers since they often are discovered during genomic or transcriptomic sequencing projects; they can be either type I or type II markers depending on whether they are located in genes. The significance of type I markers was not fully appreciated in the early stages of aquaculture genetics, though it is becoming clear that these markers are extremely important. In addition to their functions as markers in population studies, type I markers are becoming very important in studies of genetic linkage and QTL mapping. Type I markers have utility in studies of comparative genomics, genome evolution, candidate gene identification, and enhanced communication among laboratories. Due to evolutionary constraints on the genome, many genes and their organization are conserved among species. Comparative genomics deals with the similarity and differences found among genomes. Much time, money, and effort can be saved in developing markers for use in aquaculture genetic studies if genetic information is already available for closely related species. To date, full understanding of aquaculture genomics depends heavily on information from well-studied species such as human, mouse, and zebra fish. Type I markers serve as a bridge for comparison and transfer of genomic information from a map-rich species into a relatively map-poor species. Such interspecific comparisons can also be made based on type II markers, but the extent to which the comparison can be made is limited to closely related taxa. The requirement for such comparisons lies in sequence conservations. For the most frequently used microsatellite markers, such comparative studies depend on conservation of the flanking sequences used for the design of PCR primers. In contrast, sequence conservation within genes are high, allowing type I markers to serve as anchor points for genomic segments to be compared among species. For instance, if 15 genes are located between type I markers A and B in zebra fish, it is likely that the majority of the 15 genes also reside between markers A and B in catfish, even though the exact number of genes, gene order, and orientation are not necessarily identical. Currently, large insert bacterial artificial chromosome (BAC) libraries are already available for several leading fish species in aquaculture genomics including channel catfish (http:// bacpac.chori.org/catfish212.htm; Quiniou *et al*., 2003), tilapia (Katagiri *et al.,* 2001), Atlantic salmon (http://bacpac.chori.org/salmon214.htm), and rainbow trout (Thorgaard *et al.,* 2002). In general, type II markers such as RAPDs, microsatellites, and AFLPs are considered to be non-coding and therefore selectively neutral. Such markers have found widespread use in population genetic studies whose characterizations of genetic diversity and divergence within and among populations are based on assumptions of Hardy–Weinberg equilibrium and selective neutrality of the markers employed. Type II markers also have proven useful in genetics for species, strain and hybrid identification, in breeding studies, and more recently as markers linked to QTL. The usefulness

of molecular markers can be measured based on their polymorphic information content (PIC). PIC refers to the value of a marker for detecting polymorphism in a population. PIC depends on the number of detectable alleles and the distribution of their frequencies, and equals 1 minus the sum of the square of all allele frequencies. For instance, the PIC of a microsatellite marker with two alleles of frequency 0.5 each should be1- $[(0.5)^2 + (0.5)^2] = 0.5$, while PIC for a microsatellite marker of two alleles with allele frequencies of 0.9 and 0.1 is 0.18. Thus, the greater the number of alleles, the greater the PIC; and for a given number of alleles, the more equal the allele frequencies, the greater the PIC. Comparison of PIC values can give researchers a rough idea of the power of the various marker types discussed below to address specific questions in genetics.

Protein Markers:

Allozymes: Isozymes are functionally similar and separable forms of enzymes encoded by one or more loci. Isozyme products of different alleles at the same locus are termed as allozymes. The most important quality of allozyme data is the co-dominant nature of inheritance of gene products and thus genetic interpretation (genotype) of the phenotype is facilitated because all products are normally visible and not masked by dominance of one over another. Other advantages include function of most of the proteins are known and extensive database is available for many fish species. Allozyme electrophoresis has been used in defining genetic markers for stock identification on the basis of differences in allelic frequencies between stocks in many species. Using allozyme markers, it is possible to determine whether a population is a random mating one with equilibrium genotypes frequencies or sample comprises of an assembly of genetically distinct units. Their allele frequencies primarily respond to mutation, gene flow and drift. One of the limitations of enzyme variants as genetic markers is the low level polymorphism observed in some species and populations. The extensive allozymes studies undertaken on fish stocks have not only proven valuable for estimating population divergence, but also have focussed attention on the underlying evolutionary forces that promote differentiation.

Sarcoplasmic proteins (water-soluble proteins): The soluble proteins of the sarcoplasm, located within the sarcolemma are referred to as sarcoplasmic proteins. Among them, some albumins and so called myogens; to which belong most of the glycolytic enzymes are the real water-soluble proteins. (The other fractions of sarcoplasmic proteins are soluble in low salt concentrations). The genetic differences between species are more pronounced in this than in other group of proteins, as they are responsible for widely divergent enzymatic transformations in the muscle cell. Hence, the separation patterns of profiles obtained on electrophoresis or isoelectric focusing (IEF) can be used for the unequivocal identification of the species.

Myofibrillar proteins (salt-soluble proteins): They are salt soluble proteins present in the myofibrils of the muscle fibre. Of the different myofibril proteins, myosin and tropomyosin find application in fish species identification by electrophoresis. Fish myosin, similar to myosin of other vertebrates, is a hexameric protein consisting of two identical heavy chains and four light chains, of which two of them are identical. Electrophoretic pattern of heavy chains from different species are similar whereas that of the light chains is different for different species. Hence, an electropherogram of myosin light chain isolated from fish muscle is used for species identification. Electrophoresis of most of the fish

muscle tropomyosin gives a single band whose electrophoretic mobility is different for different species. Tropomyosin is a heat stable protein that can be extracted from heat-treated fish products, thus useful in identifying the species of fish of the product by studying the SDS-electrophoretic pattern of tropomyosin.

Eye-lens Proteins: The soluble proteins of the eye-lens have great value in taxonomic studies, because they are synthesized only one cell type present in the eye as a single layer. Three saline soluble eye lens proteins are distinguishable by electrophoretic and immunological techniques. There are alpha, beta and gamma crystallines in order of decreasing electrophoretic mobilities, each of which constitutes a family of similar, but no identical proteins. Protein with alpha-crystallin characteristics have been found in all vertebrate species and regarded as a classical organ-specific protein. The beta- and gamma- crystallin patterns are species-specific and can be used to resolve taxonomic disputes using ultra-thin IEF technique.

Isoelectric Focusing (IEF) of proteins: Isoelectric focusing uses a polyacrylamide gel with large pore size containing a mixture of polyamino, poly carboxylic acids with different isoelectric point (pI) s. These form a stable pH gradient along the gel in an electric field. Strong acid applied at the anode and strong base in the cathode contain and stabilize the gradient. Proteins migrate under the influence of their charge until they reach the point in the gel where the pH is equivalent to their iso-electric point and so their charge is neutralized. At the iso-electric point, proteins in the electrical field do not migrate to either of the poles. High resolutions are achieved permitting separation of proteins differing only by 0.01 pI and the technique using muscular and eye lens proteins is highly useful in generating species-specific profiles of both finfish and shellfish.

DNA markers:

The development of DNA amplification using the polymerase chain reaction (PCR) technique has opened up possibility of examining genetic changes in populations over the past 100-years or more even using archive material. In PCR reaction, a DNA sequence can be amplified many thousand folds to provide sufficient product for restriction analysis or direct sequencing. Once appropriate primers are available, large number of individuals can be assayed quickly thus facilitating large population screening for variability. Based on the source of DNA, markers can be classified into (1) mitochondrial (2) nuclear and (3) chloroplast DNA markers.

Mitochondrial DNA (mt DNA):

The mitochondrial genome is a small and double stranded circular DNA molecule. It is haploid *i.e.* each mitochondrion contains only one type of mt DNA which is cytoplasmically inherited, these making it predominantly maternally transmitted. It is non-recombinant because there is little or no paternal contribution of mtDNA in organisms and no recombination have been reported. Since the cytoplasm of an ovum/fertilized egg is derived from the female, mtDNA is maternally inherited in most of the cases with some rare exceptions like in a marine bivalve, *Mytilus* spp. that has a form of bi-parental inheritance or doubly uniparental inheritance (DUI) of mtDNA. It has high mutation rate as compared to single copy nuclear DNA (scnDNA). These factors in combination reduce the effective population size for mt DNA to one fourth in comparison to nuclear DNA. Small effective population size results in greater genetic differentiation between isolated gene pools, making it an attractive marker for studying population specificity. There may be up to several thousand copies per cell,

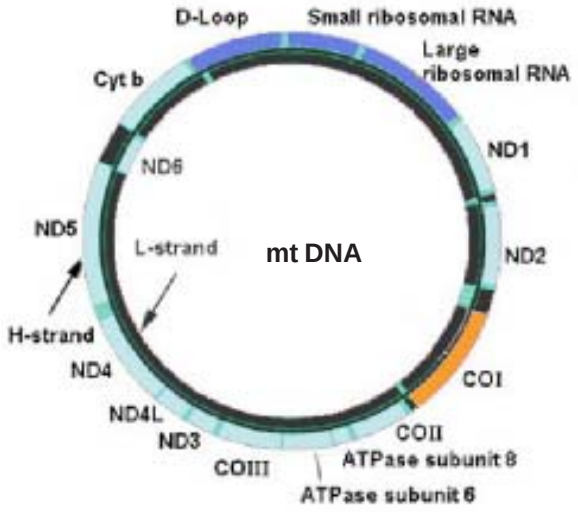
depending on the cell type. In higher animals, mtDNA typically is around 16,000 base pairs (bp) long, although there is some length variation. The largest mtDNA molecule found in higher animals is that of scallops (*Placopecten magellanicus*), which is more than 39,000bp long. Variability in the size can be found not only between species, but also intra-specifically. Structurally, most animal mitochondrial genomes contain the same 37 genes [13 genes coding for proteins, two genes coding for ribosomal RNAs (12S and 16S rRNA), 22 genes coding for transfer RNAs (tRNAs), and one noncoding control region (also called D-loop in vertebrates)], and among vertebrates the gene order is highly conserved. The coding genes code for enzyme subunits involved in electron transport and oxidative phosphorylation. The ribosomal RNAs and the transfer RNAs participate in protein translation on mitochondrial ribosomes. The control region is about 1,000bp long and contains the origin of mtDNA replication. Mitochondrial gene order differs slightly among vertebrates, and the piscine gene order does not differ from the vertebrate consensus order. Usually the differences found are due to tRNA gene translocations.

Generally, somatic and germ cells of an individual animal contain a single type of mtDNA, a state known as *homoplasmy*. However, there are a number of reported cases of *heteroplasmy,* that is, of presence of more than one type of mtDNA in an individual. In most cases of heteroplasmy; the two mtDNA variants differ in their size (length), typically due to tandem duplications in some portion of the molecule, largely in the control region. But there also have been reports of site-heteroplasmy in which the two variants differ in their sequence due to point mutations. Generally; the existence of heteroplasmy does not constitute a serious complication in the genetic analysis. Despite the conservation of the mitochondrial gene content and order over long evolutionary time, the nucleotide sequence of mtDNA evolves rapidly. The divergence of mtDNAs of taxa that shared a common ancestor is believed to be 2 % per million years and to remain linear for 8 to 10 million years. Some portions of the control region and ATPase evolve exceptionally rapidly and are very useful for high-reso1ution analysis of population structure. The slowly evolving genes like 16SrRNA, 12SrRNA, COI, II, III, and Cytochrome *b* are more suitable for comparisons at the interspecific or higher level.

Mitochondria provide the primary source of cellular ATP in eukaryotes via the process of oxidative phosphorylation. In animals, extranuclear mitochondrial genomes are typically circular, and with few exceptions, code for 13 subunits of the oxidative phosphorylation machinery as well as genes for two rRNA subunits and 22 tRNAs. Mutations in mitochondrial DNA (mtDNA) have a number of known deleterious effects. At least 50 base substitutions and hundreds of insertion/deletion mutations have been identified in human mtDNA, with effects ranging from degenerative diseases to aging to cancer. In addition to their role as the powerhouse of the cell, mitochondria are also involved in regulating programmed cell death (apoptosis) and mutagenic reactive oxygen species are generated in the process of energy production. Pathologies can result directly from the loss of ATP production in affected tissues, the build-up of oxygen radicals due to downstream blockage of the oxidative phosphorylation pathway, or unregulated apoptosis. Hundreds of mitochondria and thousands of mtDNAs are inherited maternally through the cytoplasm of the oocyte. If a zygote receives more than one form of mtDNA (heteroplasmy), different forms can be randomly distributed to daughter cells during cell division and, over many cell generations, can drift to high or low frequencies in various cell lineages. Thus, if one of the mutant forms is deleterious, disease may affect lineages where it reaches sufficiently high frequency. Somatic mutations in mtDNA appear to behave similarly

and may be a significant source of mitochondrial disease. Given the central role of mitochondria in cell physiology, mutations (either inherited or somatic) are probably responsible for many developmental abnormalities. Although a high frequency of mutant mtDNA molecules is likely to be lethal during embryogenesis, oocytes with moderate to low levels of heteroplasmy occur at detectable levels. Mitochondrial mutations probably affect a number of both general and tissue-specific developmental processes; however, the role of mitochondria in early development has not been well characterized.

Vertebrate mitochondrial DNA genomes are very compact and have been characterized as an "extreme example of genetic economy" because there are no introns within the coding genes, no repetitive DNA, and essentially no intergenic spacer sequences between genes. The only significant non-coding sequence is the control region, which is involved in regulating transcription and replication and is usually <5% of the total genome size. Since its endosymbiotic origin around 1.5 billion yr ago, a substantial fraction of original mitochondrial genes have moved to the nucleus. The products of many of these genes remain essential for oxidative phosphorylation or housekeeping functions and are selectively transported into the mitochondria after translation in the cytoplasm. A result of this evolutionary trend is that some mitochondrial abnormalities are due to mutations in genes that now reside in the nucleus and are inherited in a Mendelian fashion, rather than through the maternal, haploid inheritance of mtDNA. Knowledge of gene location (and thus mode of inheritance) is therefore essential for accurate characterization of the developmental– genetic basis of mitochondrial abnormalities. Mitochondrial genomes from several species have now been sequenced. Their small size and relative autonomy from the nucleus makes mitochondrial genomes valuable windows on the process of genome evolution and with respect to cytonuclear interactions. Mitochondrial sequences have also proven to be of great utility in molecular phylogenetic studies, and complete genome sequences have provided valuable insights into deeper-level phylogenetic problems. In plants, however, the vastly different mitochondrial genome, characterized by a large size, slow nucleotide substitution rates and extensive levels of intra-molecular recombination, has been of limited use in similar studies.



In contrast to the largely independent sources of information from different allozyme loci, the mitochondrial DNA molecule is effectively a single locus with composite genotypes equivalent to alleles. Earlier studies of mt DNA variation required large tissue samples and time consuming protocols but use of mt DNA probes and PCR amplification of selected regions have made examination of mt DNA much faster and easier. However, the contribution of mitochondrial studies to stock concept in fisheries although similar to allozymes in nature has been less informative overall.

Nuclear DNA Markers:

Random Amplified Polymorphic DNA (RAPD): The principle behind Randomly amplified polymorphic DNA (RAPD) analysis is that at low annealing temperatures or high magnesium concentrations, a primer is likely to find many sequences within the template DNA to which it can anneal. Depending on the length and complexity of genome of an organism, there can be numerous pairs of these sequences and they will be arranged inversely to and within about two kilobases of each other. Considering this, PCR will amplify many random fragments that can vary in sizes when different species, subspecies, populations or individuals are analysed and this will constitute the basis of identification.

RAPD analysis has several advantages. These include relatively shorter time (1-2 days) required to complete analysis after standardization; no need of prior information on the genome of an organism; availability of series of primers for analysis; minimal operational cost requirement; relatively smaller amount (20 ng) of high molecular weight DNA; simpler protocol and involvement of non-invasive sampling for tissue analysis. However, the application and interpretation of RAPD – PCR in population genetics is not without technical problems and practical limitations. The main negative aspect of this technique in is the necessity of extensive standardization to obtain reproducible results. In addition, most of the RAPD polymorphisms segregate as dominant markers and individuals carrying two copies of an allele cannot be distinguished from individuals carrying one copy of an allele. The limited sample size in each population and the specific RAPD primers utilized can also have an influence over the results. Even then RAPD technique is used in microbes, plants and animals for resolving taxonomic ambiguities and stock identification.

RAPD-PCR technique can also generate species-specific, sex-specific and population specific fragments. These fragments are useful in developing specific "**Sequence Characterized Amplified Region** (**SCAR**) **Markers**". For this, SCAR primers need to be synthesized from specific RAPD fragments. Usually, fragments above 1000 bp and less than 300 bp are not considered to develop SCAR markers owing to difficulties arising from co-migration and the lesser possibility of designing suitable primers from smaller fragments. The identified fragments are excised from the gel, purified and sequenced; and based on the sequence information, suitable SCAR primers are synthesized. These primers will amplify only specific fragments that are useful in settling taxonomic disputes and identifying sex or distinct populations. However, to identify specific RAPD fragments, screening of large number of samples and RAPD primers are required.

Multilocus fingerprinting: Protein or mtDNA markers are based on changes in DNA sequence generally as a result of point mutations involving base substitutions. Recently attention has turned to another type of variation that of differences in number of repeated copies of a segment of DNA called Variable Number of Tandem Repeat (VNTR) loci. On multilocus DNA fingerprinting, the length

variation is surveyed at many VNTR loci simultaneously. Due to the large number of loci examined and the extremely variable nature of this particular class of repeated DNA, each profile of bands (the so called “fingerprinting”) is usually highly informative and individual specific.

Numerous probes are available that hybridize to different VNTR loci processing similar repeat unit sequences. Even probes that cross-hybridize in distant taxa are also available. However, multilocus fingerprinting is not usually the method of choice for population level applications as the DNA profiles are often very complex and it is usually not possible to estimate allelic frequencies, necessary in many population analyses. In addition, they often do not generate reproducible results. Even under carefully controlled conditions, the intensity and presence of bands can also vary between gels.

Minisatellites and single locus VNTR profiling: Minisatellites are tandemly repeated DNA, consisting of shorter repeat units (10 – 64 base pairs) which are repeated from two to several hundred times at a locus. In single locus VNTR profiling, allelic variation is surveyed at individual minisatellite loci using two methods. The first method involves restriction endonuclease digestion of genomic DNA, separation of fragments by electrophoresis through agarose gels and southern blotting onto DNA binding membranes. Membranes are then probed with denatured labelled DNA from a single VNTR locus, preferably the unique flanking region. The second method is to PCR amplify the locus using primers flanking the array. The PCR products are separated by standard gel electrophoresis and visualized. Single locus minisatellite profiling is commonly used for maternity and paternity assessment of offspring but in population applications it is much more difficult to estimate the possible range of allele sizes likely to be encountered. Also intra-allelic duplication or deletion, inter-allelic recombination and gene conversion occur at some minisatellite loci causing the variations highly complex. Due to this, devising realistic mutation modes for such loci will be exceedingly difficult.

Minisatellites are less common than microsatellites in genomes, probably present at hundreds or thousands of different loci per genome. Unlike microsatellites which are distributed throughout the entire genome at regular 10kbp intervals, minisatellites are frequently clustered on chromosomes, DNA fingerprinting examines allelic diversity at minisatellite loci which contain much longer repeat units (>10bp) than those at microsatellite loci. Many hypervariable minisatellite loci show some variation in the precise sequence of repeat units. An allele at a typical hypervariable minisatellite locus is not perfectly repeated, but instead is a mixture of two or more repeat units. Minisatellite mutations probably result from a combination of processes including unequal recombination at meiosis, gene conversion, and slippage at replication forks.

Microsatellites: Microsatellites are repeated DNA sequences having a unit length of 1-6 base pairs tandemly repeated minimum 6 times usually; maximum several times at each locus. That means that a simple DNA motif, for example, AC, is arranged head-to-tail and in perfect repeats usually without interruption by any other motif or base. They are also known as “Short Tandem Repeat (STR)” DNA or “simple sequence (SSR)”. They are found in all prokaryote and eukaryote genomes investigated to date. Individual alleles at a locus differ in the number of tandem repeats of the unit sequence owing to gain or loss of one or more repeats and they as such can be differentiated by electrophoresis according to their size.

There are four types of microsatellites

1.Perfect	:	Perfect tandem repeat sequences.
2.Imperfect	:	Tandem repeat sequences with intervening sequences.
3.Compound	:	More than one kind of repeats, adjacent ones.
4.Complex	:	More than one kind of repeats, with intermediary sequences.

Based on the number of base pairs in a repeat unit, microsatellites can be again classified into mono (*e.g.* C or A), di (*e.g.* CA), tri (*e.g.* CCA), tetra (*e.g.* GATA) repeat unit microsatellites. Microsatellites that are used in stock identification studies typically contain di- $(AC)_n$, tri-$(ACC)_n$, or tetranucleotide $(GATA)_n$ repeats. There may be from 6 to 100 tandem copies of a repeat motif at a single microsatellite locus. The most common ones are dinucleotide repeats. Though they are widely employed in stock identification studies, appearance of stutter bands often cause problems in scoring alleles. Tetra-nucleotide microsatellites are gradually replacing dinucleotide loci as the preferred genetic marker for stock analysis.

Microsatellite loci are abundant in all eukaryote genomes and it has been estimated that there are from 10^3 to 10^5 microsatellite loci dispersed at 7- to 10-^{100} kilobase pair (kb) intervals in the eukaryotic genome. Fish genomes may contain more microsatellite loci than most other invertebrate and vertebrate taxa. Mapping studies suggest more or less even distributions of microsatellites throughout genomes, although they are somewhat rarer within coding sequences. Estimates of microsatellite mutation rates vary considerably among taxa and varieties of microsatellites. From pedigree analyses, microsatellite mutation rates were 10^{-3} to 10^{-4} in mice, 1×10^{-3} to 5.6×10^{-4} in humans, and 1.5×10^{-4} in zebrafish events per locus per generation. Also, because allele variants at microsatellite loci are codominant and are inherited in Mendelian fashion, traditional approaches such as Hardy-Weinberg equilibrium can be used in initial data analysis.

Properties of Microsatellites: Several features of VNTR render them invaluable for examining fish population structure. Microsatellites are codominant in nature and inherited in Mendelian fashion, revealing polymorphic amplification products from all individuals in a population. They contain information, which are directly related to the effective number of alleles at each locus. PCR for microsatellites can be automated for identifying simple sequences repeat polymorphism. Small amount of samples of blood or alcohol preserved tissue is adequate for analysing them. Because they are highly variable in nature, abundant variants are ensured for characterisation of populations. However, sample size in excess of 50 may be required to represent the genotype frequencies. The microsatellites are non-coding and therefore variations are independent of natural selection. These properties make microsatellites ideal genetic markers for defining heterozygosity, genetic diversity and distance measures.

How and why do microsatellites vary among individuals? Panels of microsatellite loci have been variable and sometime hypervariable, in all taxa investigated to date. The number of copies of repeats at a microsatellite locus often differs among individuals within a population and serves as the basis for microsatellite allelic variation. The origin of such high levels of variability is probably due to frequent slippage events during DNA replication. The functional significance of variation at microsatellite loci is largely unknown; consequently, microsatellites have usually been considered to be neutral genetic markers. However, evidence has accumulated that microsatellites serve functional

roles as coding or regulatory elements. The conserved presence among species of particular microsatellite motifs in the regulatory regions of specific genes, binding of proteins to these microsatellite sequences, and alterations in gene expression when the microsatellites are artificially removed provides strong support that they are sometimes important in regulation of gene transcription. Additionally, instability in number of tandem repeats at individual microsatellite loci in humans, particularly trinucleotide repeats, has been shown to be associated with progression of various types of human diseases, including some cancers. In some diseases in humans and possibly other animals, the number of repeats at a locus increases during disease progression.

Are Levels of Polymorphisms at Microsatellite Loci Predictable? Within a taxon, microsatellite loci differ greatly in their molecular characteristics, and it is not clear what effects these have on their levels or patterns of variation. Characteristics in which individual microsatellite loci vary include the following: the number of bases in the core tandem repeat unit (2, 3, or 4), the number of tandem repeats at an individual locus (5 to 100), the actual DNA sequence of the core nucleotide repeat motif, and the DNA sequence in single-copy regions that flank the microsatellite loci. Tandem repeats at a locus maybe perfect (CTCTCT), imperfect (CTCTACT)., or compound (CTCTGAGA), characteristics that also may affect their levels of variation. Recombination rate, transcription rate, age and sex, and efficiency of mismatch DNA repair probably all contribute to the differing rates and patterns of variation among individual microsatellite loci. Although it is impossible to predict which loci will exhibit high or low levels of allelic diversity *a priori* based on sequence, studies in yeast suggest that longer, perfect repeats provide higher levels of allelic diversity than loci with shorter, imperfect repeats, but that has not been empirically verified for fishes.

Applications of Microsatellites: They are very abundant, so sufficient markers can be readily developed for any research objective. Some microsatellite exhibit high levels of allelic variation, thus can be used for species that show low overall level of variation with other markers like allozymes or mitochondrial DNA and populations that are inbred or have experienced several bottlenecks and recently derived or geographically proximate populations where genetic differentiation may be limited. They are also used in pedigree analysis. Not all microsatellites display extremely high levels of allelic variation. It may be from di-allelic to more than a dozen. Thus selection of microsatellite markers can be done for any given research problem e.g. low number of alleles i.e. 3 to 5 for population studies whereas with more alleles for aquaculture genetic studies.

Expressed sequence tags (ESTs): Expressed sequence tags (ESTs) are single-pass sequences generated from random sequencing of cDNA clones. The EST approach is an efficient way to identify genes and analyze their expression by means of expression profiling. It offers a rapid and valuable first look at genes expressed in specific tissue types, under specific physiological conditions, or during specific developmental stages. ESTs are useful for the development of cDNA microarrays that allow analysis of differentially expressed genes to be determined in a systematic way, in addition to their great value in genome mapping. For genome mapping, ESTs are most useful for linkage mapping and physical mapping in animal genomics such as those of cattle and swine, where radiation hybrid panels are available for mapping non-polymorphic DNA markers. A radiation panel is composed of lines of hybrid cells, with each hybrid cell containing small fragments of irradiated chromosomes of the species of interest. Typically, the cells from species of interest are radiated to break chromosomes into small fragments. The radiated cells are unable to survive by themselves. However,

the radiated cells can be fused with recipient cells to form hybrid cells retaining a short segment of the radiated chromosome. Characterization of the chromosomal break points within many hybrid cell lines would allow linkage and physical mapping of markers and genes. In spite of its popularity in mammalian genome mapping, radiation hybrid panels are not yet available for any aquaculture species. Development of radiation hybrid panels from aquaculture species is not expected in the near future, given the fact that physical mapping using BAC libraries can provide even higher resolution and the fact that BAC libraries are already available from several aquaculture species. Therefore, ESTs are useful for mapping in aquaculture species only if polymorphic ESTs are identified. Additionally, ESTs can be mapped to physical maps by hybridization, and integration of physical and genetic linkage maps would in turn anchor the ESTs to the linkage maps. Likewise, ESTs can be mapped to genetic linkage maps if they are found to be associated with microsatellites. In this context, microsatellite-containing ESTs are rich resources of type I markers.

Single nucleotide polymorphism (SNP): Single nucleotide polymorphisms or SNPs (pronounced "snips") are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered. Such sequence differences due to base substitutions have been well characterized since the beginning of DNA sequencing in 1977, but the ability to genotype SNPs rapidly in large numbers of samples was not possible until the application of gene chip technology in the late 1990s. SNPs are again becoming a focal point in molecular marker development since they are the most abundant polymorphism in any organism, adaptable to automation, and reveal hidden polymorphism not detected with other markers and methods. For example a SNP might change the DNA sequence A**A**GGCTAA to A**T**GGCTAA. For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs, which make up about 90% of all human genetic variation, occur every 100 to 300 bases along the 3-billion-base human genome. Two of every three SNPs involve the replacement of cytosine (C) with thymine (T). SNPs can occur in both coding (gene) and noncoding regions of the genome. Many SNPs have no effect on cell function, but scientists believe others could predispose people to disease or influence their response to a drug. Theoretically, a SNP within a locus can produce as many as four alleles, each containing one of four bases at the SNP site: A, T, C, and G. Practically, however, most SNPs are usually restricted to one of two alleles (most often either the two pyrimidines C/ T or the two purines A/G) and have been regarded as bi-allelic. Obviously, their PIC is not as high as multi-allele microsatellites, but this shortcoming is balanced by their great abundance. SNP markers are inherited as co-dominant markers. Rates of SNP mutations in most taxa are on the order of 10^{-8} to 10^{-10} per locus per generation. The occurrence of SNPs is quite frequent, approximately 0.1% between any two randomly selected humans and even higher in some other taxa. The differences between taxa reflect the age of the species and, thus, comparisons in humans may present a conservative estimation of nucleotide diversity. Todate, about six million SNPs have been revealed in human genome, which means that SNPs occur approximately every 500bp.

SNPs have properties and a density in the human genome that makes them attractive as markers or tools for identification of genes in as yet uncharacterized parts of the genome that may have some relation to a specific disease. There are great expectations that SNPs will be useful in identifying candidate genes that contribute to population-wide, polygenic diseases. At present several initiatives are ongoing to exploit the information contents of genetic variability. Their purpose is to (1)

identify genes that contribute to disease, (2) identify gene targets for development of new therapeutic principles and (3) identify genes that may predict outcome from therapy.

DNA sequencing has been the most accurate and most-used approach for SNP discovery and genotyping. Random shotgun sequencing, amplicon sequencing using PCR, pyrosequencing and comparative EST analysis are among the most popular sequencing methods for SNP discovery. Each approach has its advantages and limitations, but all are still useful for SNP genotyping, especially in small laboratories limited by budget and labor constraints. Despite technological advances, SNP genotyping is still a challenging endeavor and large-scale analysis however, depends on the availability of specialized, expensive and cutting-edge equipments. Another consideration is the expense of genotyping in relation to sample sizes. Microarray (gene chip) technology and quantitative PCR are particularly useful in medical and clinical settings where large numbers of samples (thousands of individuals per locus) are involved and that can justify the cost involved in the development of the gene chips and hybridization probes.

The exploitation of SNPs is likely to proceed in several phases: In the first phase a sufficiently dense map of SNPs will be created which will eventually cover the entire genome. The physical location of SNPs will be determined in a similar way to micro-satellite markers. SNPs will, at least initially, be selected based on how informative they might be as genetic markers. SNPs in low proportions (<10 %) will be less informative than SNPs at higher frequency (30-50 %) in a given population. In a second phase, the relative frequencies of SNPs covering a large portion of the human genome will be correlated to specific diseases by comparing allelic frequencies in healthy and diseased populations. This information will focus further analysis to a smaller part of the genome thus continuously increasing the resolution of useful SNPs. In a third phase genetic variability will be studied in more detail utilizing SNPs. Typically, a limited number of genes (10-100) which link with specific diseases will be investigated for genetic variability. In this phase genetic variability present at a lower frequency is likely to be more informative. Genes contributing to disease are expected to be identified in an iterative process. The majority of SNPs are likely to be located in non-coding regions (SNP) but SNPs located in the coding regions (cSNP) will also be detected. The identification of SNPs located in coding regions of genes (cSNPs) will be particularly important since they may represent a genotype/phenotype relationship in specific diseases.

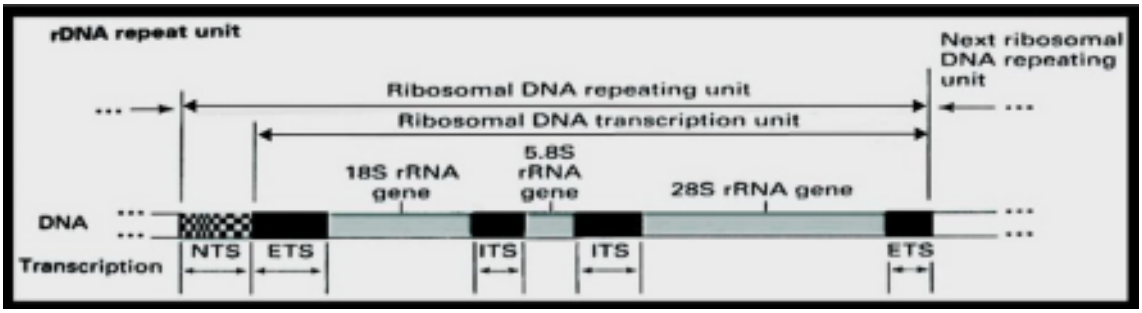
Single-Copy Coding Nuclear DNA: In recent years, the use of single-copy nDNA (scnDNA) has received attention in population studies of fishes or other organisms. In taxa with genomes that are non-polyploid, genes that encode for protein products are usually, although not always, single-copy genes. The exons, introns, and 5' and 3' regions immediately flanking genes can serve as single-copy, coding nDNA targets for population genetic analysis. However, there are advantages to their use not enjoyed by repetitive DNA markers (microsatellites), namely, that *de novo* generation of

mutants, SNPs, is much easier to understand than that of repetitive DNA. This allows for more certainty in the models upon which much data analysis is based. Also, compared to microsatellites, the rate of mutational changes (substitutions per site) across the genome may be, far more constant for single-copy sequences. Five single-copy proto-oncogene (genes that are highly conserved in animal taxa and are important in cancer development) probes such as K-*ras* and *C-abl* have been used to distinguish among all four North American fish species of *Morone* (striped bass, white bass, white perch, and yellow bass) and fixed differences were found among the species at nine genetic loci, ensuring a high degree of certainty (97%) in distinguishing among parental species, F_1 hybrids, and later generation hybrids. This type of nDNA also has been used to identify differences among salmonid stocks. **Pantophysin** (*PanI*) locus is identified as a scnDNA marker, involved in a variety of shuttling, secretory, and endocytotic recycling pathways. Population diagnostic polymorphisms at the *PanI* locus have been described in fish such as Atlantic cod from the northeastern and northwestern Atlantic due to prolonged natural selection and have provided exciting results both in terms of stock identification and in understanding those evolutionary processes that shape their population structure.

A primary concern with the use of single-copy coding nDNA sequences in population studies was that they do not exhibit levels of allelic diversity/polymorphism comparable to repetitive DNA loci such as microsatellites or minisatellites and, in fact, polymorphic single-copy loci are often, although not always, biallelic. Yet, it has been estimated that the average gene contains approximate four SNPs with frequencies of at least a few percent in human population, with about 240,000 to 400,000 common SNPs across the entire genome. Also, it has been estimated that approximately three times more SNP loci than microsatellites are needed to estimate population genetic parameters with statistical confidence. However, data from human and other genome sequence projects along with increasingly automated and sophisticated means of their analyses suggest that these polymorphisms could yield a rich harvest of informative markers [in humans, any two copies of the human genome will differ by about 3 million nucleotides or about one variant per 1,000 bases on average]. Thus, there are almost certainly sufficient levels of diversity at single-copy loci to serve as sensitive targets for stock identification studies. Comparative sequence data from these fish genome projects will allow for easier design of PCR primers at individual coding-gene loci. Multiplexing of PCRs from several single-copy loci and automated methods, such as pyrosequencing for screening multiple SNP loci simultaneously for allelic variants at previously defined informative polymorphic loci will allow for rapid multi-loci surveys of many individuals. Additionally; duplication of individual loci can and does occur, thus confounding results from analysis of single-copy genes. For example, pseudogenes frequently arise that have lost their coding function, but whose sequences are very similar to those of the actual gene from which they were derived. Similarly, polyploidy genomes, such as those that occur in sturgeon species, can also introduce problems in data analysis of single-copy and repetitive nDNA sequences.

ITS (Internal Transcribed Spacers): Eukaryotic nuclear ribosomal RNA genes (known as ribosomal DNA or rDNA) are found as parts of repeat units that are arranged in tandem arrays of thousands of copies long, located at the chromosomal sites known as nucleolar organizing regions (NORs). Each repeat unit consists of a transcribed region (having slowly evolving genes for 18S, 5.8S and 28S rRNAs) and the external transcribed spacers *i.e.* ETS1 and ETS2). Each repeat unit is separated by

regions of non-transcribed DNA termed *intergenic spacer* (IGS) or *non-transcribed spacer* (NTS). In the transcribed region, pieces of non-functional RNA, called internal transcribed spacers (ITS) are found on either side of 5.8S rRNA gene and these are described as ITS1 and ITS2. During rRNA maturation, ETS and ITS pieces are excised and as non-functional maturation by-products rapidly degraded. The length and sequences of ITS and external transcribed spacer regions (ITS:-1, ITS-2, 5'ETS) as well as portions of 28S rDNA regions are believed to be fast evolving and therefore may vary within species. The intergenic spacer region (IGS) between the genes evolves most rapidly. Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 bp) enable easy amplification of ITS region due to high copy number (up to-30000 per cell) of rDNA repeats. This makes the ITS and 5' ETS regions an interesting subject for evolutionary/phylogenetic investigations as well as biogeographic investigations.



Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it a) is (due to the high copy number of rRNA genes) easy to amplify even from small quantities of DNA, and b) has a high degree of variation even between closely related species. This can be explained by the relatively low evolutionary pressure acting on such non-functional sequences. For example, ITS has proven especially useful for elucidating relationships among congeneric species and closely related genera as well as clinically important yeast species. The ITS region is now perhaps the most widely sequenced DNA region in fungi. ITS 1 that separates the 18S and 5.8S nuclear ribosomal RNA gene is frequently used as a molecular marker for phylogenetic and population analyses in various groups of animals such as hard corals, mollusks, marine benthic copepods, insects, freshwater crayfish, *Sacculina* (Cirripedia) and teleosts. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. ITS sequences are nowadays being used to know the genetic diversity among different strains of bacteria also. Most ITS sequences reported in animals are about 200-400 bp as is evident from the GenBank database and ITS-1 of crayfish and spiny lobster are among the longest ITS-1 sequences (~760bp) reported.

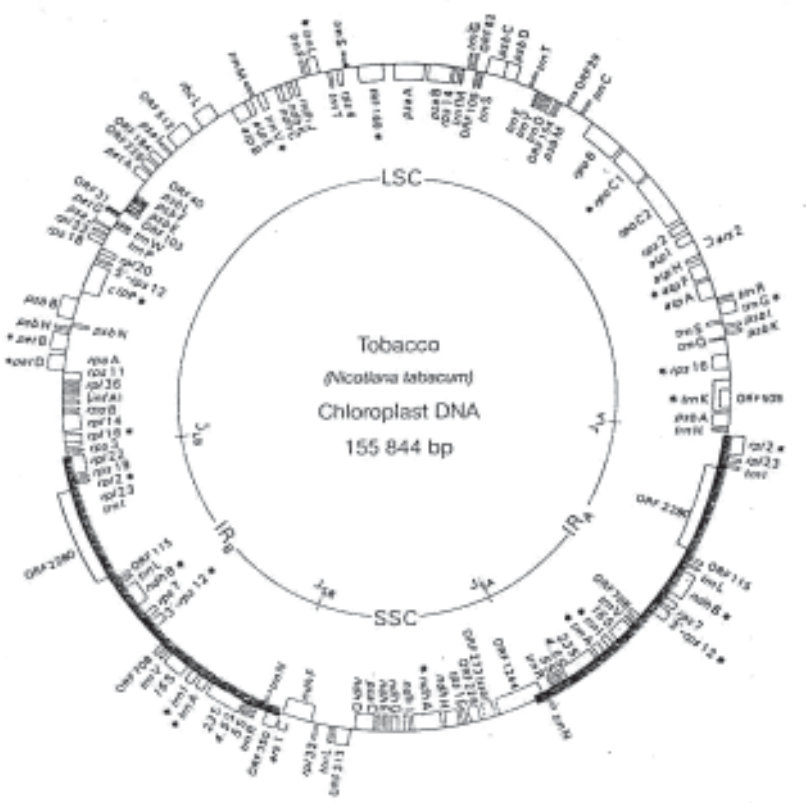
Chloroplast DNA (cp DNA):

Chloroplasts are replicative organelles and contain a number of copies of a double-stranded circular DNA chromosome which is also known as **cpDNA.** The number of copies of this chromosome in each chloroplast varies between cells; 20 – 30 in old leaves to 100 in young leaves. Chloroplast chromosomes lie within the stroma and a number of features of their structure resemble prokaryotic

chromosomes. They are circular DNA molecules which, unlike nuclear chromosomes, are not complexed with histones. Replication of the chloroplast genome and its distribution between daughter proplastids is a complex and ill-defined process. Chloroplasts genomes have been classified into 3 types. Two groups of land plants namely the gymnosperms, Pinaceae and a group of legumes (including peas and broad bean) have chloroplast chromosomes without an inverted repeat – IR (Group I). Most land plants, including all other angiosperms have chloroplast genomes containing a large (6 – 76 kb) inverted repeat; these are Group II genomes. The alga *Euglena* has three tandem repeats in its Group III chloroplast genome.

Chloroplasts are maternally inherited, that is there is essentially no transmission of chloroplasts through the male pollen gamete; thus the mode of inheritance of cpDNA is uniparental and the genome is typically non-recombinant and effectively haploid. The cpDNA of plants has been a focus of research in plant molecular evolution and systematics. Several features of this genome have facilitated molecular evolutionary analyses. First, the genome is small and constitutes an abundant component of cellular DNA. Second, the chloroplast genome has been extensively characterized at the molecular level providing the basic information to support comparative evolutionary research. And third, rates of nucleotide substitution are relatively slow and therefore provide the appropriate window of resolution to study plant phylogeny at deep levels of evolution. Chloroplast genomes contain between 120 – 140 genes and of ~160 kbp size (120 x 10^3 to 200 x 10^3 base pairs in higher plants and 180 x 10^3 base pairs in green algae). It is identified as one of the relatively stable genome with marked conservation of gene content and a substantial conservation of structural organization. Conservation of gene content and a relatively slow rate of nucleotide substitution in protein-coding genes have made the chloroplast genome an ideal focus for studies of plant evolutionary history. This has led to determining the DNA sequence of the cpDNA gene *rbcL* encoding the large subunit of ribulose-1, 5-bisphosphate carboxylase (RuBisCo). Unlike the animal mitochondrial genome, cpDNA of several plants contain introns. The functional categories of cpDNA are (i) DNA regions that do not code for tRNA, ribosomal RNA (rRNA) or protein (referred to as “noncoding DNA”); (ii) protein-coding genes; and (iii) chloroplast introns. The chloroplast is highly condensed compared with eukaryotic genomes; for example, only 32% of the rice genome is non-coding. Most of this non-coding DNA is found in very short segments separating the functional genes. The genetic information for the synthesis of much of chloroplast encoded proteins is present in the chloroplast genome, with four genes for rRNA present in the large inverted repeat (IR). The other cp protein synthesis genes identified include genes for ribosomal proteins, 30tRNA genes and a RNA polymerase gene together with protein synthesis coupling and elongation factors.

The chloroplast genome shares many features with animal mtDNA and the two have been referred to as ‘natural counterparts’. Its conserved gene order, the widespread availability of primers and a general lack of heteroplasmy and recombination, have made the chloroplast genome an attractive tool for phylogenetic studies of plants. Furthermore, its uniparental mode of inheritance (usually maternal in angiosperms and paternal in gymnosperms) makes it possible to elucidate the relative contributions of seed and pollen flow to the genetic structure of natural populations by comparing nuclear and chloroplast markers. Regions in cpDNA such as *trH-psbA* spacer, *rbcL*, *matK*, *rpoC*1, *rpoB*, *accD* and *YCF*5 are identified as the most promising regions in the cpDNA for DNA barcoding in plants and universal primer pairs for these regions have been developed



Gene map of the tobacco chloroplast genome. Genes shown inside the circle are transcribed clockwise, genes on the outside are transcribed anticlockwise. Asterisks denote split genes. The major open reading frames are included. IRF, intron-containing reading frame; IR, inverted repeat; LSC, large single-copy region; SSC, small single-copy region; J, junctions between IR and LSC and SSC. (From Sugiura, M. (1992) The chloroplast genome. *Plant Molecular Biology*, **19**, 149–168.)

(barcoding@kew.org). An interesting feature of cpDNA is the occurrence of polymorphic mononucleotide microsatellites (cpSSR) that are increasingly used in population genetics and understanding crop plant evolution and domestication. Unlike, nuclear microsatellites, cpSSRs are uniparentally inherited (some species have maternal inheritance of the chloroplast and others paternal), nonrecombinant and all loci are linked. The genotyping of cpSSRs will result in haplotypes that will be composed of the combination of alleles found at each cpSSR locus. Chloroplast microsatellites are fast evolving and typically consist of mononucleotide motifs that are repeated 8 to 15 times. Levels of polymorphism in cpSSRs are quite variable across loci and across species, and some loci have been found to be monomorphic in all species study. Many studies have demonstrated high levels of intraspecific variability of cpSSRs; hence, they represent potentially useful markers at the population level in plants.

Fatty Acid Profiles in the cell membrane phospholipids as Natural Markers for Stock Identification:

In the lipids of animals, about 20 fatty acids appear in relative amounts of more than 1%. A larger number are present in minor concentrations. Different species of fish have characteristic fatty acid profiles, but the variability in the composition of the tissue fatty acids of fish is very large.

Different tissues have different fatty acid profiles. Within each type of tissue, the fatty acids are bound in a number of different lipid classes, phospholipids, triacylglycerides, wax esters, cholesterol esters, and so on, all with different profiles. On top of these variations, the composition of tissue fatty acids may be influenced by factors such as age, maturity; condition, and reproductive cycle of the fish. External factors, such as water temperature and salinity; and probably a pressure, have been shown to have an effect on fatty acid composition. Numerous investigations have shown that the diet of an organism has an impact on the fatty acid profile [triacylglycerides in storage lipids] in its tissue. On the contrary, the composition of fatty acids in membrane phospholipids is genetically controlled and stable over time. The phospholipid fatty acids may therefore be used as a natural mark over a longer timescale and may serve as a natural mark for identification of stocks. The investigations in fish, *e.g.,* Faroe stocks of cod showed that the between-stock difference in fatty acid composition in heart tissue, in which the lipids were made up of between 80% and 90% phospholipids, was caused by inborn factors. Compared with the composition of the fatty acids in triacylglycerides, the composition of fatty acids in phospholipids in phospholipid-rich tissue, such as heart, is generally expected to be less sensitive to diet. In-depth studies are being undertaken in different organisms to see how stable the fatty acid profile is under shifting dietary regimens; during changes in other environmental factors, such as temperature and changing physiological conditions.

8

Recombinant DNA Technology and Molecular Cloning

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Recombinant DNA is any artificially created DNA molecule which brings together DNA sequences that are not usually found together in nature. The production and use of recombinant DNA molecule to produce new genetic combinations that are of value to science, medicine, agriculture, and industry is termed as recombinant DNA technology. The propagation of recombinant DNA molecule inside a particular host cell so that many copies of the same sequence are produced is known as molecular cloning.

The entire procedure for molecular cloning may be classified into the following five steps

1. Identification and isolation of the desired gene or DNA fragment to be cloned
2. Insertion of the isolated gene in a suitable vector
3. Introduction of this vector into a suitable host
4. Selection of the transformed cells
5. Multiplication of the introduced DNA molecule in the host

Two major classes of enzymes are important tools in the preparation of recombinant DNA: restriction endonucleases and DNA ligases.

Restriction endonucleases along with methylases are part of a defense mechanism known as restriction modification system (RM). The RM system is used by bacteria, and perhaps other prokaryotic organisms as a defense mechanism that protects bacterial cells against invasion by foreign DNA molecules such as those contained in viruses. Restriction endonucleases work by cutting the invading DNA into small, nonthreatening pieces, crucial to this protective device is the ability of the nuclease to discriminate between its own DNA and the invading DNA; this is done by methylases, they modify bacterial DNA to protect it from its own restriction endonucleases. There are three kinds of RM system: type I, type II and type III, the name given in order of discovery. Type II restriction endonucleases is the one employed in recombinant DNA technology. The cuts made by these enzymes often leave single DNA strands with sticky ends due to the asymmetry of the cut (made to a double-stranded molecule). Type II restriction endonucleases serve as a powerful tool for manipulating DNA in a controlled way, there are several hundreds of them and a hundred different specific recognition sequences. The recognition sequences are symmetric; the same sequence of four to eight nucleotides is found on both strands, but run in opposite directions. Restriction enzymes

usually cut phosphodiester bonds of both strands in a staggered manner, so that the resulting double-stranded DNA fragments have single-stranded ends, called sticky ends. Blunt end cutting endonucleases are rather rare. The single-stranded short extensions form hydrogen-bonded base pairs with complementary single-stranded stretches on other DNA molecules. These unions are temporary since they are only held by a few hydrogen bonds. These unions can be made permanent by adding the enzyme DNA ligase. DNA ligase joins two pieces of DNA by forming covalent phosphodiester bonds.

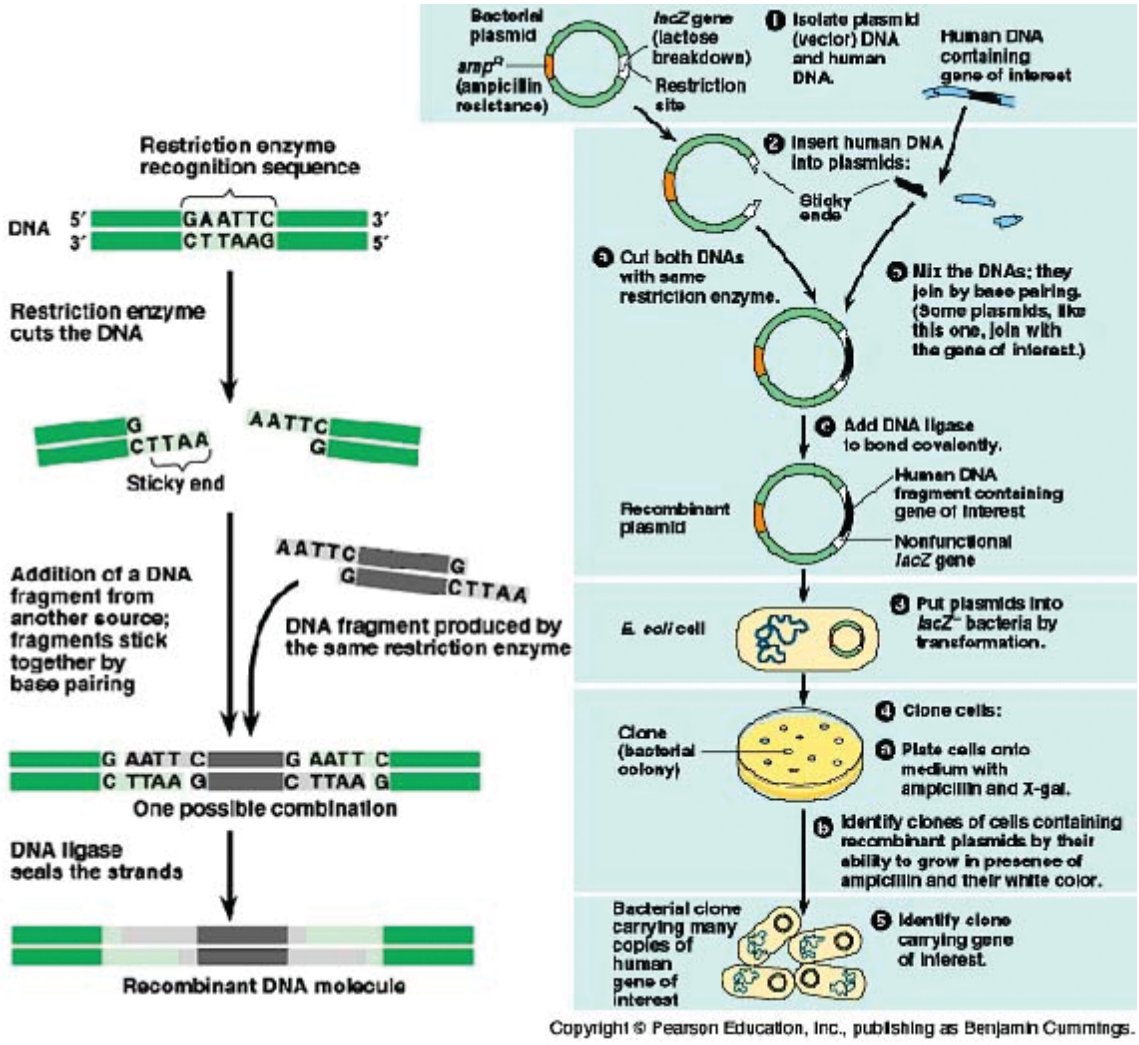
For molecular cloning of DNA molecules of interest they have to be inserted into a DNA molecule that has the capability to replicate autonomously in the host cells. Such DNA molecules are called as cloning vectors. The most commonly used cloning vectors are plasmids, they are covalently closed circular (CCC) extra chromosomal DNA havig the chareteristic sequence of orgin of replication found in most gram negative and gram positive bacteria, and also in some yeasts. A plasmid used for recombinant cloning should ideally posses certain properties like:

- Ability to replicate autonomously in bacterial cells
- Must possess selectable markers
- Should contain a unique spectrum of restriction endonucleases cleavage sites
- Should not be transmissible nor mobilisable
- Molecular weight of the plasmids should be as low as possible, because the transformation efficiency decreases as the size increases
- Should be able to maintain multiple copies per cell.

But naturally occurring plasmids may not possess all the essential properties suitable to be an ideal cloning vector, so they are modified/engineered for incorporating the above mentioned characteristics. Plasmids can normally incorporate foreign inserts of up to 10kb. Other commonly used cloning vectors are phages, and cosmids; they can accommodate 20kb and 45kb, respectively. For cloning of larger fragments of DNA molecules, new generation of artificial chromosomes vectors are employed namely bacterial artificial chromosomes (BAC), yeast artificial chromosome (YAC), and mammalian artificial chromosome (MAC), which can accommodate 300kb, 1000kb, and more than 1000kb, respectively. There is another class of vectors called as expression vectors that is used to produce a recombinant protein from a desired gene. Expression vectors apart from the ability of self replication in the host should have a regulatory region called as promoter for efficient transcription of the recombinant gene resulting in large number of stable mRNA, and thus proteins.

A recombinant vector is produced by first digesting both the DNA molecule of interest and the cloning vector using the same pair of restriction enzymes, the resulting fragments will be complimentary to each other. The DNA fragments thus generated is then ligated together using DNA ligase. For multiplication and propagation of the recombinant vector thus produced, they should be transferred to a suitable host cell; this process is known as transformation/transfection. Foreign DNA cannot be readily sent across the membrane, this is achieved using any of the following methods like,

- Heat shock- the recombinant plasmid is co incubated with calcium chloride treated cells known as competent cells, and a heat shock is given for 1-2 minutes at 42°C, the host cell membranes become permeable to recombinant plasmid which passes into the cell.



Panel on left depicts production of recombinant molecule using restriction enzyme and DNA ligase, panel on right illustrates the various steps involved in molecular cloning

- Electroporation- the host cells are subjected to a high voltage pulse which temporarily disrupts the membrane allows the vector to enter.
- Viruses- since viruses have mechanism to infect susceptible cell and replicate themselves a genetically engineered virus can deliver the desired DNA sequence into the target host cell.
- Gene gun- gold particles coated with foreign DNA segments are fired into the host cell.
- Microinjection- a cell is held in place with a pipette under a microscope and foreign DNA injected directly into the nucleus using fine needle.
- Liposome- vectors can be enclosed in a liposome, which are small membrane bound vesicles; liposome's fuse with the cell membrane or nuclear membrane and deliver the DNA into the cytoplasm/nucleus.

For distinguishing transformed and non transformed cells the cloning vector contains a selectable marker, selectable marker can be for antibiotic resistance, substrate utilization or any other characteristics which can distinguish transformed hosts from untransformed hosts. The transformed cells are selected and further cultured, within the transformed cell the recombinant DNA molecule replicates, producing dozens of identical copies known as clones. Further as the host cell replicate, the recombinant DNA is passed on to all progeny cells, creating a population of identical cells, all carrying the cloned sequence. The cloned recombinant can be recovered from host cell, purified and used for various applications.

A collection of clones that contains all the genetic information in an individual is called a genomic library. When the clone contains a reverse transcribed DNA from m-RNA, it is alled as a cDNA clone. Such clones representing all the reverse transribed m-RNA of a particular organism or tissue, it is called a cDNA library. cDNA clones lack intron sequences, making them the clones of choice for expressing and characterizing the protein product of a gene.

Recombinant DNA research is an exciting and challenging field, which holds great promises. It finds application in various fields of science like;

- Gene isolation/purification/synthesis
- Sequencing/Genomics/Proteomics
- Expression analysis (transcriptional and translational levels)
- Restriction fragment length polymorphisms (RFLPs)
- Gene therapy
- Recombinant Vaccines/ Monoclonal antibodies
- Genetically modified organisms/ Xenotransplantation
- Molecular modeling
- Bioremediation
- Forensics/Bioterrorism detection

In the near future recombinant DNA technologies will play a key role in preventing genetic diseases, producing targeted and personalized medicines, provide effective and less toxic pharmaceuticals. It will also have a great impact on agriculture and livestock through genetically modified organisms that possess traits for increased productivity and disease resistance.

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DNA barcoding and molecular taxonomy of marine organisms

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Taxonomical awareness on species, the intraspecific and interspecific relations etc, are the necessary pre requisites in different fields of applied biology, such as in biological resources management and conservation, in mariculture ventures, in pest/pathogen control and identification, etc. Generally, there are two contexts in which significant problems arise in species identification, where the molecular approaches are applicable. The first concerns the very fundamentals of taxonomy, in identifying the species, the phylogenetic status and the identification at subspecies and hybrids level. The second concerns to ecologists are the issue of identifying the species sex, or identity of individuals under circumstances where simple morphology cannot be relied upon. Biodiversity studies require species level analyses to assess the community structures. In conventional taxonomic practice, organisms were classified based on their fine morphological characters, comparative osteology, etc., which are more time consuming, laborious and expensive. Moreover, in some situations, morphological variation arises due to the environmental factors rather than genetic causes and is therefore not heritable. In this context, classification of organisms based on the molecular variations using the modern molecular tools has got a wide acceptance globally.

In molecular taxonomy, variations in protein profile and the nucleic acid structure among organisms has been utilised in determining the evolutionary relationship of the species. Applications of molecular taxonomy and molecular systematics were pioneered by Charles G. Sibley (birds), Herbert C. Dessauer (herpetology), and Morris Goodman (primates), followed by Allan C. Wilson, Robert K. Selander and John C. Avise (who studied in various groups) during 1960's and 70's. Allozymes, RFLP, RAPD, microsatellite, mitochondrial DNA sequence analysis, etc. are the most popular genetic markers commonly used in molecular taxonomic studies. Eventhough, proteins and mitochondrial, nuclear and chloroplast DNA are used for molecular taxonomic analyses, applications involving the protein are far fewer than those with DNA markers due to several reasons. Most importantly, protein polymorphism is usually much less than that detectable in DNA, thus greatly limiting the resolving power of protein methods. Protein analysis generally requires relatively large amounts of tissue, which will be a limiting factor when working with small organisms. Proteins are also often differentially expressed both in space (tissue specificity) and time (developmental regulation), which limits its availability. Further, proteins are difficult to store in non-denatured state than DNA under field conditions, which may or may not matter according to the type of analysis involved.

Despite the limitations recounted above, protein can be a useful marker in certain circumstances. 'Protein profiling' using the sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

is an established molecular tool in identifying/differentiating organisms. This is a simple procedure and protein sample can be collected and stored in ethanol, just like DNA, because denaturation is inherent in the protocol. Allozyme analysis is rarely used in identification studies because of its low resolving power. Allozyme study requires non-denatured protein (usually by deep freezing) and also more expensive and time consuming to generate the results. This situation paved the way to establish the DNA sequencing as a reliable and acceptable tool in recent times. Hence, these are considered as a superior marker for evolutionary studies, because the actions of evolution are ultimately reflected in DNA sequences. As there are millions of species and life stage transformations, DNA based identification aid in resolution and strengthen the classical taxonomical identification system.

DNA barcoding is a taxonomic method that uses a short DNA sequence of the organism's gene to identify the species. In 2003, Paul Hebert, researcher at the University of Guelph in Ontario, Canada, proposed "DNA barcoding" as a way to identify species. DNA barcoding uses a short genetic sequence from a standard part of the organism's genome as the way a supermarket scanner distinguishes products using the black stripes of the Universal Product Code (UPC). Partial sequence of the mitochondrial cytochrome c oxidase subunit I (*cox1*- usually referred to as COI in barcoding studies) gene are considered as a potential 'barcode'. The intent of DNA barcoding is to use large-scale screening of one or a few reference genes in order to (i) assign unknown individuals to species and (ii) enhance discovery of new species. The purpose of DNA barcoding is to identify a species with a piece of DNA with which a biologist could run several biotic surveys without the need of morphological keys.

What is the need for DNA barcoding?

Until now, biological specimens were identified using morphological features like the shape, size and color of body parts. In some cases a trained individual could make routine identifications using morphological "keys" but in most cases an experienced professional taxonomist is needed. If a specimen is damaged or is in an immature stage of development, even taxonomists may be unable to make identifications. Molecular markers can be used to solve these problems which offer a wide range of options for identifying species, but the question arises as to whether a general, universally applicable method might be found. Now comes the role of DNA barcoding, could provide just such an approach. The idea here is to select one or a few genes that are shared by most, if not all organisms on earth and which show large interspecific but small intraspecific levels of variation. The sequences of such genes could then become the equivalent of species-specific barcodes.

A DNA barcode is not just any DNA sequence, it is a rigorously standardized sequence of a minimum length and quality from an agreed-upon gene, deposited in a major sequence database, and attached to a voucher specimen whose origins and current status are recorded. The primary goal is to develop an accurate, rapid, cost-effective, and universally accessible DNA-based system for species identification. It could also be applied where traditional methods are unrevealing, for instance identification of eggs and immature forms, and analysis of stomach contents or excreta to determine food webs. The Barcode of Life Data System (BoLD), provides an online interface (http://www.BOLDsystems.org) allowing the scientists and researchers to work together and share information on a global scale. Hundreds of different barcoding projects around the world are contributing to a barcode library that contains hundreds of thousands of specimen barcodes from around 70,000 species. DNA barcoding has the potential to increase access to taxonomic knowledge

in all regions of the world. Databases of reference barcodes are connecting specimens to their correct species names, providing a direct route to species information associated with those names. The Consortium for the Barcode of Life (CBOL) is working with GenBank and its partner DNA repositories (European Molecular Biology Laboratory (EMBL) and DNA Data Bank of Japan (DDBJ)) to construct a global library of reference barcode sequences. Once fully implemented, this system will revolutionize access to biological information and will exert broad impacts on research, policy, pest and disease control, food safety, resource management, conservation, and many other areas in which society interacts with wild biodiversity.

A DNA barcoding system will enable the prompt diagnosis of invasive species, thereby allowing quarantine and eradication efforts to begin years earlier with massive reductions in cost and increased chances of success. The same strategy extends to the selection of optimal control strategies for pest/ pathogen species impacting the varied natural resource sectors. Similarly, it can, as well, play a critical role in regulating trade in endangered or protected species or products. DNA barcoding can also assist with one of the most intractable practical problems in ecology, notably the study of predator–prey interactions. Particularly with small organisms, identifying prey and especially prey range can be a daunting task. Mostly this has to be done by the manual examination of gut contents. Unfortunately digestive processes rapidly destroy many morphological clues to prey identity, but molecules or fragments of them may persist for longer. DNA-based methods offer a useful alternative and further the barcoding gene is from mtDNA has the advantage in this kind of study of high copy number, thus maximizing the chances of detection by PCR before its complete destruction by digestive enzymes. DNA barcoding seeks merely to aid in delimiting species - to highlight genetically distinct groups exhibiting levels of sequence divergence suggestive of species status – but are never sufficient to describe new species.

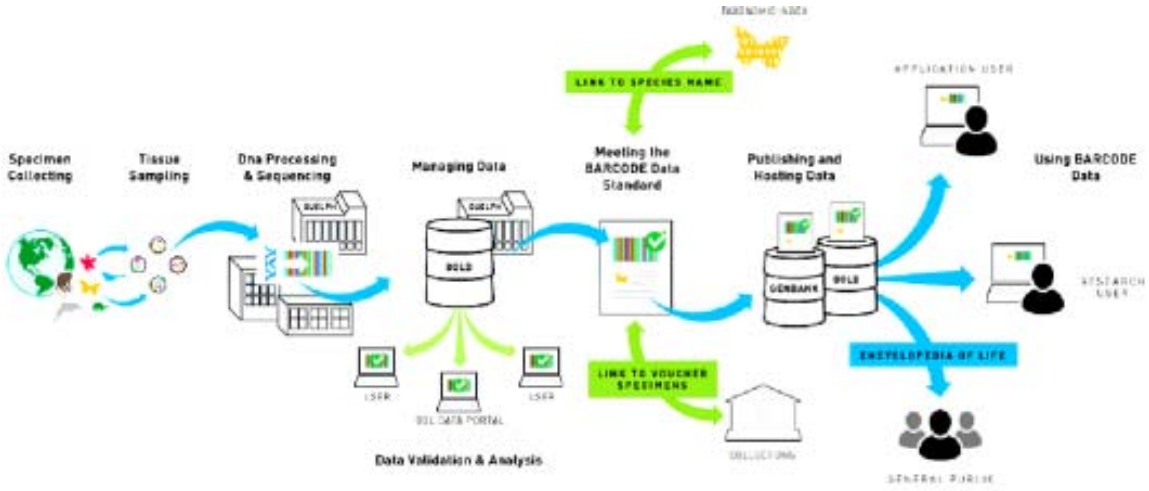

Fig. 1. The barcoding pipeline (source: CBOL website)

Marker of Choice:

DNA barcoding is based on a relatively simple concept. Most eukaryote cells contain mitochondria and mitochondrial DNA (mtDNA), therein observed relatively higher mutation rate. This results in significant variation in mtDNA sequences even between the species and in principle, a comparatively less variation within species. Desirable locus for DNA barcoding should be standardized, preferably

present in all taxa of interest and sequenceable without species specific PCR primers, short enough to be easily sequenced with the available technology and expected to provide large variation between species and a relatively less variation within the species. Although several loci have been suggested, for animals and many other eukaryotes, partial region of the mitochondrial cytochrome c oxidase subunit I (COI) gene was proposed as a potential 'barcode' and widely accepted internationally.

Even though the mitochondrial genes encoding ribosomal DNA (12S, 16S) are widely used as marker in phylogenetic analysis, their utility in taxonomic analyses are found to be limited due to the prevalence of insertions and deletions (indels) which in turn complicate sequence alignments. There are different protein-coding genes in the animal mitochondrial genome which are better target due to the rarity/absence of indels as this will lead to frame shift. The important advantages of COI as the gene for DNA barcoding are the availability of universal primers for amplifying this gene in different animal phyla and it possesses a greater range in phylogenetic signal than the other mitochondrial gene. This gene has some other desirable properties too, to be used as the gene for barcoding. The COI gene being mitochondrial it is usually present in high copy number per cell and is a necessary gene in all aerobic organisms. Partial COI sequence of about 650 bp has turned out to have, in a wide variety of organisms from insects to birds, high interspecific but low intraspecific variation. However, there are some significant difficulties with this locus. Anaerobic organisms are excluded and interspecific variation of mtDNA (including COI) in plants other than algae is often too low to be useful. Prokaryotes, which include most of the earth's biodiversity, are essentially excluded. There is also a risk of errors with any single locus from lineage sorting in recently diverged species where reciprocal monophyly has not yet been achieved. It looks as if at the very least barcoding will have to include sequences from several different genes to be universally applicable. In the case of plants, plastid loci such as ribulose bisphosphate carboxylase (*rbcL*) and megakatyocyte-associated tyrosine kinase (*matK*) have been proposed. Nuclear ribosomal DNA intragenic spacer (ITS) regions have also been proposed for species identification in several other eukaryotes such as protozoa.

The selection of COI as a target gene for DNA barcoding is supported by published and ongoing work, which demonstrates that barcoding via COI, will meet the goals for a wide diversity of animal taxa (http: //www.barcodinglife.com//). A model COI profile, based upon the analysis of a single individual from each of 200 closely allied species of lepidopterans, was 100% successful in correctly identifying subsequent specimens (Hebert *et al*., 2003a). Two hundred and seven species of fish, mostly Australian marine fish, were sequenced (barcoded) for a 655 bp region of the mitochondrial cytochrome oxidase subunit I gene (cox1) as reported by Ward *et al*. (2005). Spies *et al*. (2006) examined the variation at the mtDNA COI gene in 15 species of North Pacific skates and indicated that, a DNA-based barcoding approach may be useful for species identification. DNA barcoding reveals a likely second species of Asian sea bass (barramundi) (*Lates calcarifer*); Ward *et al*., (2008a) strongly suggest that barramundi from Australia and from Myanmar are different species based on the sequencing of 650 base pair region of the mitochondrial COI gene. Out of fifteen fish species barcoded from Northern (Atlantic and Mediterranean) and Southern (Australasian) Hemisphere waters using COI sequences, Ward *et al*. (2008b) observed significant evidence of spatial genetic differentiation for this gene in two fishes; the silver scabbardfish (*Lepidopus caudatus*) and John dory (*Zeus faber*). These observations further supported the scope of barcoding in identifying species and also the geographical variations expected within species.

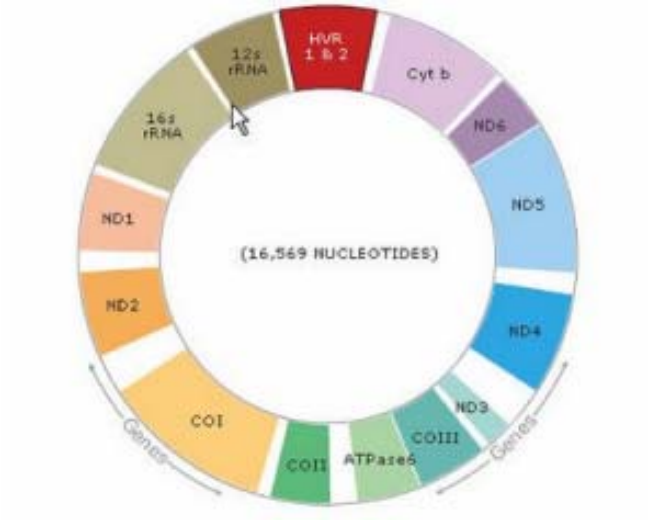

Fig. 2. Diagrammatic representation of vertebrate mitochondrial genome

Components of Barcoding:

Any barcoding project has 4 components

1. The Specimen which forms the treasure trove for DNA barcoding.
2. DNA analysis involving standard laboratory protocol.
3. Database to assign unknown specimens to known ones. The main data base is Barcode of Life database (FISH BOL, BOLD) created and maintained by University of Guelph, Ontario, Canada which offers researchers to collect analyze and manage barcode data. It's an international collaboration which helps in assembling the CO1 sequences of different species. The FISH BOL helps in curating the barcode sequence, electropherograms, voucher specimens, GIS data of different collection sites and aids in interpretation of data.
4. Data analysis which helps in identifying the unknown species with closely matching species in the data base.

Steps in DNA barcoding:

The main steps involved in DNA barcoding of samples are,

1. Collection and field identification of specimens and its taxonomic data.
2. Maintaining voucher specimen with voucher data (catalogue number and institution storing), collection records (collector, collection date and location with GPS coordinates) and photographs of the specimen.
3. Extraction of DNA from tissue samples using standardized protocol.
4. Amplification of mtDNA genes like COI by polymerase chain reaction (PCR).
5. Qualification and quantification of PCR products through Agarose gel electrophoresis.
6. Purification of PCR products and sequencing.

7. Analysis and validation of sequence data.
8. Submission of morphological data, specimen voucher data, sequence information and photographs in FISH BOL database.

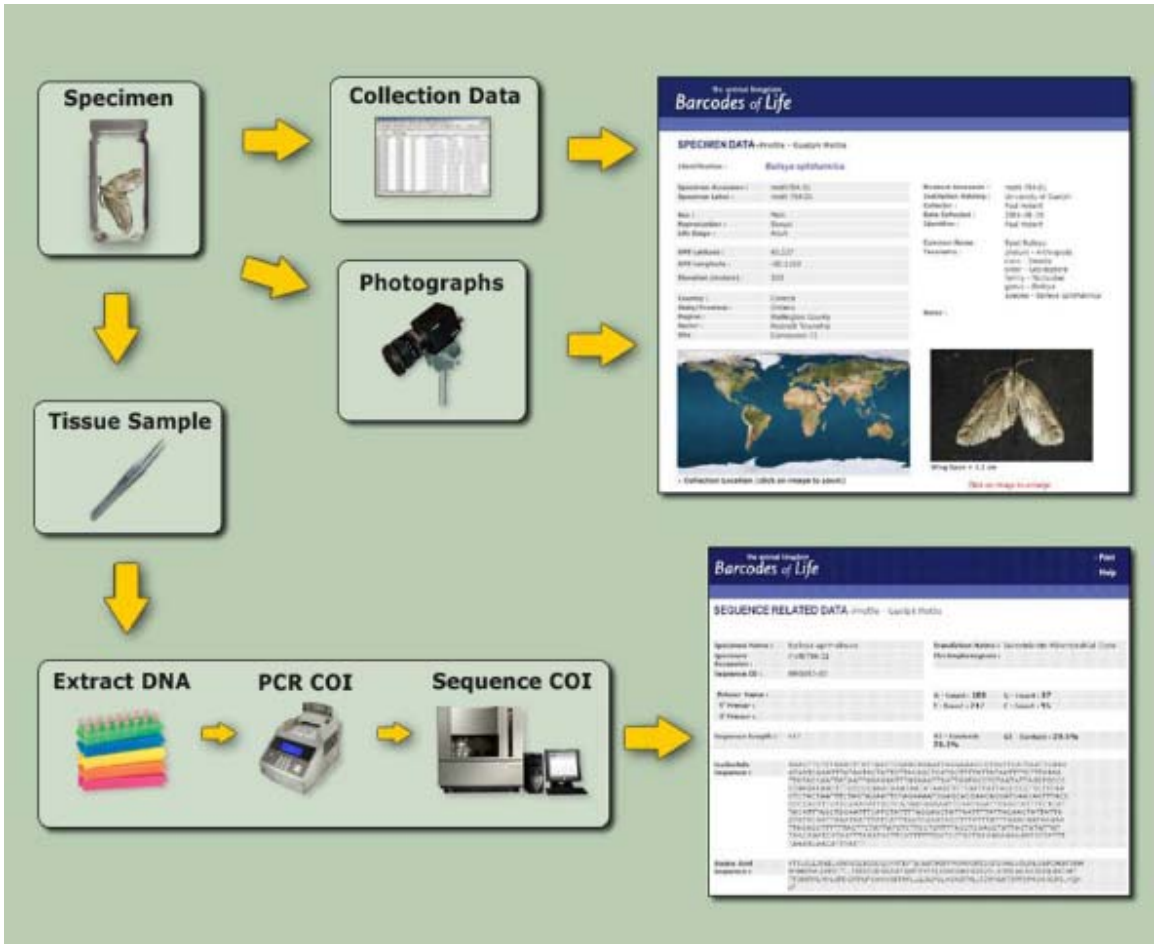

Fig 3. Barcoding: Process & database (Source: www.barcodinglife.org)

Utilities of DNA barcoding:

1. *Consumer protection*: DNA barcoding mainly involve consumer, agricultural, health and environmental protection. Apart from, protection of ecosystem, DNA barcoding also helps in identification of catch and by-catch on commercial vessels and at the dock, better understanding of the food chain through analysis of gut contents and improved fish stock assessments, based on identification of larvae as well as juveniles and adults. The Consortium for the Barcode of Life (CBOL) generates barcodes for economically relevant and potentially hazardous fish species. The barcoding method would improve species identification, which is essential in determining associated hazards, addressing economic fraud issues and aiding in food-borne illness outbreak investigations.

2. *Conservation of Biodiversity*: A library of barcodes will enable researchers to identify species, about its nativity, abundance and endangering status both locally and globally. The molecular taxonomy is very critical in developing strategies to preserve different genetic entities or species to enable the species identification of eggs, larvae and tissues. DNA barcoding is a combination of molecular techniques and traditional taxonomy, cost-effective to investigate the unexplored species and biodiversity.

3. *Cataloguing of extinct species*: Molecular taxonomy can identify species even with tiny, damaged, old and ancient specimens. DNA barcoding helps in identifying illegally obtained wildlife species and poorly preserved samples. Barcoding forms a bridge between creating a reference library of barcodes of species already known to science which in turn will help us to understand the molecular pattern of speciation.

Limitations of DNA barcodes:

DNA barcodes increase our ability to identify species accurately. But the major draw back is that, only a representative sample from a population of a particular species is taken for study. This sample may not be reflecting the entire diversity present in a particular species leading to inaccurate identification of a species with unusual genetic variation patterns. Barcoding only identify species but hardly throws light on the evolutionary pattern of a species. The mtDNA would not reflect upon the evolutionary relationships between different groups and will not provide sufficient information on the taxa of species leading to flaws in identification. As the mtDNA is inherited maternally, hybrids cannot be identified using COI based DNA barcodes. Occurrence of nuclear mitochondrial pseudogenes (numts), which are nonfunctional copies of mtDNA in the nucleus that have been found in major clades of eukaryotic organisms can be easily co-amplified with orthologous mtDNA by using conserved universal primers forms another major limiting factor for DNA barcoding. Many of which are highly divergent from orthologous mtDNA sequences, and DNA barcoding analysis may incorrectly overestimates the number of unique species based on this and can introduce serious ambiguity into DNA barcoding.

Conclusion:

The barcoding research not only provides an increased understanding of biodiversity but also helps to understand the basic evolutionary process. The DNA barcoding would not replace the Linnaean system of classification. The large scale sequencing when integrated with the traditional taxonomical identification will contribute to the challenge of identifying species and enhance the rate of discovering the biological diversity. The real challenge lies in defining the boundaries of using barcoding technique with a large phylogeographic structure. DNA barcoding generates a plethora of information which does not compete with the traditional taxonomy but in turn supplements the taxonomists for accurate identification of species using both morphological keys and molecular markers. If mtDNA alone is used for barcoding a species, then we may end up with identifying only a few species in such a large global diversified conditions. So, an integrated approach using different markers should be carried out while using molecular taxonomy for categorizing a species to particular taxa.

Suggested Readings:

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10

Bioinformatics Applications in Biotechnology

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Bioinformatics refers to the creation and advancement of algorithms, computational and statistical techniques, and useful to solve formal and practical problems arising from the management and analysis of biological data. Bioinformatics is considered as amalgam of biological sciences especially biotechnology with computer science and information technology. It is the application of computer technology to the management of biological information. Genomes of several model organisms and microbes are completely sequenced recently. Affordable access and availability to the technology that supports such investigations has led to a significant increase in the amount of data generated. The astronomical growth of biological information through large scale sequencing of genomes has resulted in an unprecedented synergism between information technology and molecular biology. Constraints of cost, time and uncertainty that is associated with 'wet' molecular biology studies and data-intensive techniques like X-ray crystallography has made in-silico approaches more and more popular among biologists today. Present scope of Bioinformatics encompass the wide area of data acquisition, storage, retrieval, analysis and interpretation along with parallel development of application tools in terms of computational infrastructure, software, databases and so on.

A brief history

The history of bioinformatics began with the discovery of digital code of life by Watson and Crick in 1953. Exactly after fifty years the announcement of the complete human genome happened. Dr. Margaret Oakley Dayhoff (1925-1983) was a pioneer in the use of computers in chemistry and biology, beginning with her PhD thesis project in 1948. She is credited today as a founder of the field of Bioinformatics. The advances in sequencing technologies and instrumentation are generating unprecedented digital data now. This sea of data is too much for the human brain to process and hence computational methods are increasingly used wherever they are applicable. Major milestones in the development of bioinformatics are listed below.

1953 - Watson and Crick propose the double helix model for DNA

1965 - Margaret Dayhoff's Atlas of Protein Sequences

1970 - Needleman-Wunsch algorithm

1977 - DNA sequencing and software to analyze it

1980 - World's first nucleotide sequence database, European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Data Library, opens

1981 - Smith-Waterman algorithm developed

1982 - GenBank Release 3 made public

1983 - Sequence database searching algorithm (Wilbur-Lipman)

1985 - Lipman and Pearson publish rapid sequence database search tool, FASTA

1986 - Applied Biosystems launches first commercial DNA sequencer

- Swiss Institute for Bioinformatics (SIB), European Institute for Bioinformatics establish curated protein sequence database, SwissProt

1988 - National Center for Biotechnology Information (NCBI) created at NIH/N

- CLUSTAL, multiple sequence alignment program for proteins and DNA, released

1990 - BLAST: fast sequence similarity searching from NCBI

- SIB founds Prosite, database of protein families and domains

1991 - EST: expressed sequence tag sequencing

- Oak Ridge National Laboratory team develops gene recognition and assembly Internet link (GRAIL) software package for genome annotation

1993 - Sanger Centre, Hinxton, UK was established

1994 - EMBL European Bioinformatics Institute, Hinxton, UK

1995 - First bacterial genome, *Haemophilus influenzae* completely sequenced

1996 - Yeast genome completely sequenced

1997 - PSI-BLAST

1998 - *Caenorhabditis elegans*, the worm (multicellular) genome completely sequenced

1999 - Fly (*Drosophila*) genome completely sequenced

1999 - NCBI releases RefSeq, non-redundant set of sequences for major research organisms including DNA, RNA and protein products

2000 - EMBL/EBI and Sanger Institute establish Ensembl, an automatic annotation system for selected eukaryotic genomes

2001 - International Human Genome Sequencing Consortium and Celera Genomics publish first drafts of the human genome

- Genome Bioinformatics Group at UC Santa Cruz releases first genome browser

2003 - Human Genome Project completed, Golden Jubilee of DNA double helix

2006 - Swiss-Prot, the manually curated section of the UniProt Knowledge base, celebrating its 20 years of service to the scientific community in 2006

Development of databases and software tools

The first challenge facing the bioinformatics community today is the intelligent and efficient storage of mass of data produced by the genome sequencing projects. It is then their responsibility to provide easy and reliable access to this data. The data itself is meaningless before analysis and the sheer volume present makes it impossible for even a trained biologist to begin to interpret it manually. Therefore, incisive computer tools must be developed to allow the extraction of meaningful biological information.

Biological databases are archives of consistent data that are stored in a uniform and efficient manner. These databases contain data from a broad spectrum of molecular biology areas. Primary or archived databases contain information and annotation of DNA and protein sequences, DNA and protein structures and DNA and protein expression profiles. Secondary or derived databases are so called because they contain the results of analysis on the primary resources including information on sequence patterns or motifs, variants and mutations and evolutionary relationships. A list of major databases is given in Table 1.

Bioinformatics tools are software programs that are designed to extract meaningful information from the mass of data stored in databases. A large number of software (many of them free) is available through Internet servers and from other sources. Proven software for a particular application can be obtained from reliable web resources that link to computational biology software sites. Most of the major biological databases (PDB, TIGR, NCBI etc.), research organizations and groups provide web implementations of their software tools. The source code of these is often shared freely. But often these programmes lack ease of use and integration with other functions. Commercial software companies address this problem by adding much greater usability and integration. Unique programs that commercial packages never offer you, are available free of cost. If you are good in programming, you can develop scripts that allow you to hit a web server with multiple requests without entering them manually into a form. By this you will be able to download a local copy of the software and run it on your own machine so that you avoid slow data transfer to and from remote sites. A list of such useful tools is provided in Table 2.

Applications of Bioinformatics

Present day applications of Bioinformatics are diverse. They range from studies of evolution of life on earth to generation of designer drugs. Sequence analysis focus upon the finding of new genes, analyze structure of the gene to determine its function and correlate how an altered structure of a gene can be linked with diseases. Molecular modeling studies attempts to understand how the three-dimensional topography of a protein is related to its function. Other complex applications include modeling of cell signaling and metabolic pathways, studying protein-protein interaction, understand mechanisms how protein families evolve and map the expression pattern of a plethora of genes in different cells and tissues. In short, the applications of Bioinformatics can be seen in three major areas viz. sequence analysis, structure analysis and function analysis.

1. Sequence analysis

Comparing sequences provides a foundation for many bioinformatics tools and allows inference of the function, structure and evolution of genes and genomes. Statistically significant similarities are found between homologous sequences that are related by divergence from a common ancestor. Thus the degree of similarity between two sequences can be measured while their homology is a case of being either true of false. This set of tools can be used to identify similarities between novel query sequences of unknown structure and function and database sequences whose structure and function have been elucidated. Sequence comparison methods can be grouped into pair-wise, sequence-profile and profile-profile comparison. FASTA and BLAST are the popular tools for pair-wise comparison while PSI-BLAST is a popular example of a sequence-profile comparison method. Phylogenetic analysis can be used in comparative genomics. A multiple alignment using tools like

ClustalW is the first step in this direction. Two widely used packages for phylogenetic analysis are PAUP and PHYLIP.

Several genomes have been fully sequenced and in several cases sequencing is in progress. It is bioinformatics that turned the dream of sequencing the human genome into reality. There are number of tools for sequence assembly like Arachne, GAP4, AMOS and so on. Dozens of computer programs for identifying genes are available like Genscan, GeneMarkHMM, GRAIL, Genie and Glimmer. Genome annotation tools including tools for analysis of repetitive DNAs such as RepeatMasker, RepeatFinder and RECON are widely used. Several sequence based methods are developed for proteins which includes Pfam, ProDom and COG. Other methods are based on 'fingerprints' of small conserved motifs in sequences as with PROSITE, PRINTS and BLOCKS. InterPro is a database that integrates domain information from multiple protein domain databases. Another set of tools allows you to carry out further, more detailed analysis on your query sequence including evolutionary analysis, identification of mutations, hydropathy regions, CpG islands and compositional biases. The identification of these and other biological properties are all clues that aid the search to elucidate the specific function of your sequence.

2. Structural Analysis

The function of a protein is more directly a consequence of its structure rather than its sequence with structural homologs tending to share functions. Predicting the structure of a protein from the sequence information is one of the most exciting areas of Bioinformatics. The crux of the process lies in the assignment of folds and domains in the primary structure, thereby developing an acceptable model of tertiary and quaternary level structures. Lately, several methods are available e.g. comparative modeling, threading methods, ab initio methods and use of genetic algorithms. SCOP and CATH are the two well known structure based family resources.

Protein functions can be analyzed by comparing your protein sequence to the secondary (or derived) protein databases that contain information on motifs, signatures and protein domains. Highly significant hits against these different pattern databases allow you to approximate the biochemical function of your query protein. ENZYME is a typical example of a function family.

3. Functional Analysis

Transcriptome analysis: The primary goal of transcriptome analysis is to learn how changes in transcript abundance control growth and development of an organism and its response to the environment. DNA microarray technology is a powerful tool for this. Many tools are available that perform a variety of analysis on large microarray data sets. Gene Traffic, GeneSpring, GCOS (Affymetrix) etc. are some commercial software. Some of the public software for microarray data analysis are Cluster, CaARRAY, BASE and Bioconductor. Microarray data are also combined with other information such as regulatory sequence analysis, gene ontology and pathway information to infer coregulated processes. RSAT is a oligonucleotide frequency-based method for regulatory gene analysis while AlignACE is probabilistic-based method. Whole genome tiled arrays are the latest in the series which are used to detect transcription without bias towards known or predicted gene structures and alternative splice variants.

Proteomics: Proteomics is a leading technology for the qualitative and quantitative characterization of proteins and their interactions on a genome scale. Electrophoresis, mass spectrometry (MS) including peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS) are some advanced techniques used for this. The data generated through these techniques are often complicated and computational analyses are essential for interpreting them. Several bioinformatics tools have been developed in these fields. Examples are SWISS-2DPAGE, Melanie, Flicker, PDQuest for 2-D electrophoresis analysis; Emowse, MS-Fit and Mascot for PMF protein identification; SEQUEST and Mascot for MS/MS-based peptide/protein identification. For the de novo sequencing approach using MS/MS spectra the two popular software packages are Lutefisk and PEAKS.

Metabolomics: Metabolomics is the analysis of the complete pool of small metabolites in a cell at a given time. Standards and databases are being developed in this area. As more reliable data are collected, one can integrate all these data to develop dynamic simulations of metabolic networks and perform analysis and simulation in a cellular modeling environment like E-Cell or CellDesigner.

Bioinformatics for next generation sequencing

Next generation sequencing is currently revolutionizing genome sequencing. Bioinformatics has brought down the heavy costs of genome sequencing by way of longer read sizes that has accelerated the data generated. Bioinformatics takes care of the handling and storage of large amounts of such raw sequence data. The diversity and rapid evolution of next-generation sequencing technology is posing challenges for bioinformatics in sequence quality scoring, alignment, assembly and data release. A variety of software tools are available for analyzing next-generation sequencing data. They are useful for de novo assembly, genome browsing and annotation, alignment of sequence reads to a reference and base calling and/or polymorphism detection. SSAKE (http://www.bcgsc.ca/platform/bioinfo/software/ssake), VELVET (http://www.ebi.ac.uk/ %7Ezerbino/velvet), Edena (http://www.genomic.ch/edena) and ALLPATHS are some of the new algorithms available for processing of short reads.

Genome annotation

Genome annotation is the process of attaching biological information to sequences. There are two types of genome annotations, structural and functional. Structural annotation consists of identifying ORF and their localization, gene structure, coding region and location of regulatory motifs. Functional annotation consists of elucidating the biological, biochemical, regulatory and expression profiles of genetic elements.

Genome mapping

Genome mapping means assembling reads against an existing backbone sequence. New generation sequencers like Illumina and Applied Biosystems generate short reads of 35 and 50 bp, respectively, and mapping such short read tags to a reference genome can pose several challenges. The alignment programmes such as Genome Analyzer (Illumina) and SOLiD (Applied Biosystems) deal with these new challenges. Some other useful tools are MAQ (http://maq.sourceforge.net), RMAP (http://rulai.cshl.edu/rmap), SOAP (http://soap.genomics.org.cn), SOCS (http://www.appliedbiosystems.com) etc.

Conclusion

The ultimate goal of the field is to enable the discovery of new biological insights as well as to create a global perspective from which unifying principles in biology can be discerned. Ultimately, all of this information must be combined to form a comprehensive picture of normal cellular activities so that researchers may study how these activities are altered in different disease states. Thus, fundamental aspects of biological science such as molecular sequence homology, protein structure engineering and design, species phylogeny and taxonomy and the analysis and simulation of molecular networks have all been addressed and explored by bioinformatics.

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Marine fish breeding and larviculture

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Introduction

As the yield from the capture fisheries stagnates and population growth rate in the world, the requirement or protein is expected to come from increased aquaculture production. In order to meet the demand for more food fish and to develop new products for the export market, the most important component of any culture system must be met – that of adequate supply of fry and juveniles for culture. Most of the worlds fish aquaculture still depend on the fry almost comes exclusively from wild. Seed supply from the wild is often unpredictable and seasonal. Controlled hatchery production of seeds of economically important finfish ensures a steady supply of quality seeds for aquaculture operations.

Broodstock Development

An adequate supply of broodfish is essential for successful induced-breeding operations or artificial propagation, especially of the most important cultured species. There are two sources of finfish broodstock: wild-caught adults and those reared in ponds or cages. Most marine fish (groupers, seabass, snappers etc.) broodstock are obtained as wild adults. The disadvantages of using wild stock are uncertainty of capturing them, the relatively large expenditure needed for their capture and transport, and the limited opportunities of obtaining good quality eggs.

In certain cases, it is also difficult to obtain adults from the wild. Thus developing breeders in ponds/cages is another option. Fishes domesticated for few years attains sexual maturity in captive conditions. It is advantageous to use pond or cage-reared broodstock as they are already used to culture conditions and are thus easier to develop into broodfish. In general, fish selected for broodstock should be fast-growing, lively fish, among the largest and strongest members of their age group and free from parasites and diseases.

Transporting Spawners

There are several ways of transporting spawners: from the most simple receptacle, such as plastic bags, to the most sophisticated like special transport vehicles. Containers vary with the size, species and number of fish to be transported and the location of the collecting grounds. A combination of pre-transport starvation, rapid anaesthetization at capture, cool transport water and anesthetization at transport were also used for long distance transport.

Age at maturity

The age at maturity varies for different species of fishes. Knowledge about the age at which the species matures is useful in the selection of right sized brooders for breeding purpose. Rabbitfish begins sexual maturation and spawning in one year of captivity. As Protandrous hermaphrodites, the seabass are mature males on the third year of captivity and became females on the following year. On the other hand, groupers, being protogynous hermaphrodites, are mature females after four years of its growth. It takes longer for them to be transformed to mature males. Both milkfish and snappers take 5 years to attain sexual maturity.

Determination of Sex and Maturity of Spawners

Two common aspects in the artificial propagation of finfish are the determination of sex and the maturity of spawners. Often, it is difficult to determine the sex of spawners through examining the external morphology of the fish. In some species, a gravid female exhibits a fuller profile than the ripe male; its abdomen is distended. Ripe males are easy to distinguish during the spawning season since milt oozes out from the urogenital pore as its abdomen is pressed. If the degree of maturity is right, the milt will be white and creamy; poor milt is watery and curdled. Milt which is not ripe will demand strong pressure and will be mixed with blood.

Assessment of gonadal maturation of broodstock is still a major difficulty in the artificial propagation of finfish. The commonly-used method to assess sexual development is through gonadal biopsy. Gametes are removed from either an anaesthetized or unanaesthetized fish by using a polyethylene cannula. The inner diameter of the cannula to be used varies with the size of eggs to be sampled. The cannula is inserted 4–15 cm into the ovary or testis and gametes are drawn into the cannula by aspiration as the cannula is slowly withdrawn. The distance to which the cannula is inserted varies with the length of the ovary or testis. Samples from the middle portion, especially of the ovary, are generally considered to be the most representative.

The eggs collected are removed from the cannula by blowing them into a Petri dish. They are preserved in 1% formalin in 0.9% NaCl. The average egg diameter is determined from a batch of 50–10 by using a micrometer and their developmental stage is assessed under the microscope. Gonadal maturation is then expressed in terms of average egg diameter and the developmental stage of the eggs.

The milt collected is removed from the cannula by blowing it onto a clean dry Petri dish. A small portion of this is mixed with a drop of seawater or brackishwater, depending upon the species, and examined immediately under the microscope. Sperm motility and vitality are then assessed.

Factors Affecting Gonad Development

Nutrition

Poor nutrition can result in poor or no reproductive performance and that lack of vitamin supplement could affect sperm quality. Mere reliance on natural food may lead to poor or variable reproductive performance. Fish broodstock diets are now formulated to include high levels of n-3 fatty acids which include enhanced levels of both decosahexaenoic acid and eicosapentaenoic acid. Eggs considered to be of better quality have higher content of these fatty acids. Furthermore,

successful embryonic development in fish has been shown to be dependant on the balance of aminoacids present in the egg. Results of other studies indicate that reduced feed levels may adversely affect fecundity and composition of ova. Deficiency of Vitamin C in the diet results in eggs that show considerably higher mortalities than eggs. However broodstock fed on 'natural diet/s' often produce eggs of better quality than those on formulated commercial diets. Thus it appears that different fish species may have different dietary requirements and that diets of broodstock should be tailor made to ensure good egg quality.

Environment

Photoperiod

One of the factors considered being of great importance to the inducement of sexual maturation and spawning is photoperiod. Photoperiod manipulation is now being employed to alter the normal reproduction of a few cultured species, for example, mullet, rabbitfish, rainbow trout, tilapia, carp and catfish. The greatest advantage of altering the spawning time of the cultured species is the availability of fry for stocking in ponds, pens and cages throughout the year.

Temperature

Water temperature is another important factor which influences the maturation and spawning of fish. In some species of fish functional maturity is directly controlled by temperature; in others, the time of spawning is regulated by the day-length cycle such that it occurs when the temperature is optimum for survival and the food supply is adequate.

Salinity

Some species of fish, e.g., salmon, migrate from the marine to the freshwater environment in order to spawn, while other species, such as eels, migrate from freshwater to the marine environment to complete their reproductive cycle. This confirms that salinity is somehow related to maturation and spawning.

Other environmental factors

In addition to photoperiod, temperature and salinity, there are other less obvious factors which may affect the maturation and spawning of broodstock. There is, however, paucity of information regarding the effects of these less obvious factors, which include rainfall, stress, sex ratios, stocking density, isolation from human disturbance, dissolved oxygen, social behaviour of fish, heavy metals, pesticides, and irradiation. Furthermore, the design of holding systems for broodstock such as ponds, tanks and cages is largely unknown.

Spawning and Fertilization

Selection of Spawners : The selection of spawners from the broodstock should be done months before the beginning of natural spawning to allow ample time for the fish to be conditioned to environmental and diet controls. Spawners are normally selected based on the following criteria:

- fish should be active
- fins and scales should be complete

- fish should be free from disease and parasites
- fish should be free from injury or wounds
- males and females of similar size are preferred

Spawning

Presently, two major techniques are employed in the mass production of fish seeds: artificial fertilization and induced spawning.

1. Artificial Fertilization

Spawners are caught in natural spawning grounds near the mouth of the river or in saltwater lakes. Normally, the fishermen will net the fish during spring tide 2–3 days before the new moon or full moon, up to 5–6 days after the new moon or full moon.

The degree of maturity of the collected spawners should be immediately checked. If the female has ripe eggs and the milt of the male is at the running stage, stripping is done in the boat. The fertilized eggs can then be transported to the hatchery for subsequent hatching. In cases where only the male is caught, the milt is collected by stripping into a dry glass container and is then stored in an ice box or refrigerator. The milt can maintain its viability after a week in cold storage (5°–15°C). The preserved milt should be made available for immediate use when a ripe female is caught.

The dry method of fertilization is normally used in this case. The eggs are stripped directly from the female into a dry and clean container where the milt is added. A feather is used in mixing the milt and eggs for about 5 min. Filtered seawater are added to the mixture while stirring and then allowed to stand undisturbed for 5 min.

2. Induced Spawning

All of the cultured species exhibit spontaneous spawning but this is seasonal and at times unpredictable. Thus induced spawning to ensure availability of eggs, to meet fry demand and as a supplement to natural spawning may be undertaken.

Manipulations of various environmental parameters, such as temperature, photoperiod, salinity, tank volume and depth, substrate vegetation, etc. can often improve the reliability of spawning. However, in some species hormonal treatments are the only means of controlling reproduction reliably. Over the years, a variety of hormonal approaches have been used successfully. These methods began with the crude use of ground pituitaries from mature fish—containing gonadotropin (GtH) which were injected into broodstock to induce spawning. Today, various synthetic, highly potent agonists of the gonadotropin-releasing hormone (GnRHa) are available as well as sustained-release delivery systems for their controlled administration These methods have contributed significantly to the development of more reliable, less species-specific methods for the control of reproduction of captive broodstocks.

A. Hormonal induction of ovulation and spawning

Most research and development efforts on the use of hormones to control finfish reproductive cycles in aquaculture have focused on the induction of Final Oocyte Maturation (FOM), ovulation, spermiation and spawning in fish that do not complete these processes in captivity.

However, hormonal manipulations have important applications in commercial aquaculture, even for fish that do undergo FOM and spermiation spontaneously in captivity. In many fish hatcheries, ovulation is induced with hormones in order to synchronize and optimize egg collection and fry production, thereby minimizing the handling and stress to the fish, and reducing labor requirements

SPH - acetone-dried pituitary gland homogenate

Hypophysation, the use of ground pituitaries and pituitary extracts to induce spawning in fish, started in the late 1930s in Brazil. Collection of pituitaries for hypophysation was done from reproductively mature broodstock, either males or females. It was found that pituitaries collected during the spawning season were more efficacious in inducing spawning. Use of ground pituitaries, however, is associated with various drawbacks, the most important ones being (a) the great variability in pituitary LH content; (b) the administration of additional hormones present in the pituitary that may adversely affect thephysiology of the treated fish, and the potential for transmission of diseases from donor fish to recipient broodstocks.

Human chorionic gonadotropin (hCG)

Unlike LH preparations of piscine origin, hCG is often given in a single dose, which ranges between 100 and 4000 international units (IU) per kg body weight. There is one situation in which hCG be preferred over GnRHa. The advantage of hCG is that it acts directly at the level of the gonad and does not require the existence of LH stores or activation of the pituitary gonadotropes. hCG may be more appropriate because it acts much faster, via direct stimulation of the gonad, in inducing FOM, spermiation and spawning

Use of gonadotropin-releasing hormone (GnRH) and agonists (GnRHa)

Studies in female broodstocks indicated that GnRH and GnRHa were effective in inducing ovarian development, FOM and ovulation in doses ranging from 1 to 15 mg GnRH $^{kg-1}$ or 1 to 100 mg GnRHa kg^{-1}. The use of GnRH peptides for spawning induction therapies has important advantages over the use of GtH preparations. First, GnRH and its agonists are small decapeptides that do not trigger an immune response and can be used again in subsequent spawning seasons with no reduction in their efficacy. Second, by inducing the release of the endogenous LH, the GnRH repairs the endocrine disruption that results in the failure of captive fish to undergo FOM, ovulation and spawning. Also, GnRH acts at a higher level of the hypothalamus–pituitary–gonad axis. Consequently, GnRH can provide a more balanced stimulation of reproductive events and, presumably, a better integration of these events with other physiological functions, by directly or indirectly affecting the release of other hormones necessary for successful FOM, spermiation and spawning. A third advantage of GnRHa, is that it can be synthesized and obtained in pure form, and thus does not carry the risk of transmitting diseases. Finally, because of the structural similarity of the GnRHs among many fish species the use of GnRHs, unlike the use of gonadotropins, is generic and the same GnRHa has been successfully applied to a wide range of fish species.

Sustained-release delivery systems for GnRHa

Almost from the first experiments using pituitary extracts for spawning induction, it was recognized that administration of the hormone in a sustained fashion would improve the efficacy of the procedure. The multiple treatments those are often necessary for a successful response present various problems to the hatchery manager. First, repetitive handling of broodstock requires substantial labor, time and monitoring. Especially in situations where the broodfish are kept outdoors, in ponds or cages, it is difficult, very time consuming, and labor intensive to crowd, capture, anaesthetize and inject the fish with hormones, frequently while hatchery personnel are exposed to the elements of nature. Secondly, repetitive handling is stressful to the fish and can often result in pre-spawning mortalities, or at the very least it can adversely affect the progression of FOM.

Over the last 20 years, a variety of GnRHa-delivery systems have been developed and tested in cultured fishes for the control of FOM, ovulation and spermiation. The first such delivery system was prepared using cholesterol and was tested in Atlantic salmon. Cholesterol implants are prepared as solid, cylindrical pellets (3 mm in diameter) and are implanted intramuscularly using an implanter or a scalpel. This GnRHa-delivery system is easy to fabricate and relatively inexpensive, but the GnRHa release from the pellets seems to be extremely variable probably because each implant is prepared individually. The next type of GnRHa-delivery system was fabricated in the form of microspheres (5–200 mm in diameter), using co-polymers of lactic acid and glycolic acid (LGA). The greatest advantage of biodegradable, microspheric delivery systems is that the same preparation can be used to treat fish varying in size from a few grams to many kg. This can be done because the microspheres are suspended in vehicle and are administered on a volume to weight basis. The last type of GnRHa-delivery system used for spawning induction is prepared in the form of a solid, monolithic implant, using a non-degradable co-polymer of Ethylene and Vinyl Acetate (EVAc. Unlike the biodegradable microspheres and similar to the cholesterol pellets, EVAc delivery systems have a long shelf-life and can maintain their effectiveness for up to 3 years if stored desiccated at 20 ºC.

Fishes having eggs with an average diameter equal to or greater than 0.65 mm are induced to spawn by injecting hormones intramuscularly a few centimetres below the dorsal fin. In the first injection the fish is given a combination of 10 mg SPH/kg body weight + 1 000–10 000 IU HCG/kg body weight. In the second injection, the fish is given a combination of 10 mg SPH/kg body weight + 2 000–20 000 IU HCG/kg body weight. Injections are administered intramuscularly a few centimetres below the dorsal fin after which the fish is completely anaesthetized by immersing it in seawater containing 100 ppm 2-phenoxyethanol. The time interval between injections is 24 hours for most marine fish. This interval was selected to ensure that final maturation of eggs is completed before the fish dies or before the eyes of the breeders are completely covered with a white opaque substance.

Milkfish can be induced to spawn when females possess oocytes of 0.67mm in average diameter. The females and the males with milt are injected with either 1000 IU of Human Chorionic Gonadotropin (HCG) or 100µg of LHRHa per kg of body weight. Similar spaning agent and dosage was used for snapper, but he minimum oocyte diameter is only 0.42mm as spawned eggs of snapper (0.80mm) was smaller than that of milkfish (1.20mm). the effectiveness of LHRHa, administered by injection or pellet implantation has been demonstrated to induce spawning of seabass. A single injection of 100µg/kg BW induced spawning of seabass with an initial oocyte diameter of 0.40mm and above. A single injection of hCG (2IU/g BW) or implantation of LHRHa can induce spawning of the rabbitifsh.

Usually, only two injections are needed to induce both captive and wild adult fish to spawn as long as the dosage and time-interval mentioned above are followed; however, badly injured fish may need a third injection. In such cases, the dosage of the third injection is that of the second injection. When a third injection is necessary, usually the fertilization and hatching rates are very low.

B. Induced Spawning by Environmental Manipulation

The method involves the simulation of the natural spawning environment in which temperature, artificial rainfall and tidal fluctuation are manipulated.

At the beginning of the new moon or full moon, the water temperature in the spawning tank is manipulated by reducing the water level in the tank to 30 cm deep at noon and exposing to the sun for 2–3 hours. This procedure increases water temperature in the spawning tank to 31°–32°C. Filtered seawater is then rapidly added to the tank to simulate the rising tide. In effect, the water temperature is drastically decreased to 27°–28°C.

The fish spawn immediately the night after manipulation (18.00–20.00 h) or, if no spawning occurs, manipulation is repeated for 2–3 more days until spawning is achieved.

Whether the fish are induced to spawn by hormone treatment or environmental manipulation, they would continue to spawn for 3–5 days after the first spawning provided the environmental factors that stimulate spawning are present, e.g., new or full moon, changes in salinity and temperature, etc.

Fertilization and Incubation

The fish that are induced to spawn by hormone injection will be ready to spawn within 9–12 hours after the final injection. The schedule of injections for subsequent spawning must be synchronized with the natural spawning time of the fish which occurs in late evening between 18.00 and 24.00 h. On the other hand, in the stripping method, it is still necessary to sample the eggs from gonads by cannulation and examine them under the microscope. The fish has spawned only if at least 40% of the eggs are transparent.

Determination of egg and larval quality

Several parameters are used to assess fish egg and larval quality. These include the rates of egg viability, hatching and normal larvae. Chemical composition of eggs are also analysed and of the egg chemical constituents, fatty acids, amino acids, ascorbic acid, yolk protein and DNA and RNA have been reported to have an influence on egg and larval quality.

Larvae-rearing

The rearing tanks are usually made of plastic, fibreglass or concrete. The shape of the tanks can be retangular or circular. Volume ranges from 1 to 10m^3. The tanks are usually protected from sunshine and heavy rain.

Five hours before hatching, the developing eggs are transferred to larvae-rearing tanks. The tanks are provided with mild aeration. The larvae start to hatch 16–25 h after fertilization depending on temperature and species. The usual stocking density of developing eggs is 100–200 eggs/l.

Factors Affecting Mass-rearing of Marine Finfish Larvae

- Type of food
- Food density
- Water quality
- Environmental factors

The most important environmental factors affecting larval growth and survival are: (1) light, (2) temperature, and (3) salinity.

(1) Light. The effect of light intensity and photoperiod on the growth and survival of larvae has received little attention in the past. Generally, fish larvae are reared either under continuous light or under day and night conditions.

Light is of primary importance since most marine fish larvae are visual feeders. Nevertheless, the larval eye at first feeding is very simple, with no capabilities of distinguishing between different illuminations. High light intensities of about 1000–2000 lx at the water surface are commonly used in hatcheries. Illumination in first feeding tanks for marine fish larvae. The reflections from surfaces in a tank are very important for the light distribution in the water body. Black tanks are best suited to reproduce natural illumination conditions. White-walled tanks should be avoided since they would be a perfect wall trap due to the phototaxis of the larvae. Green water and dark walled tanks seems to be beneficial, as growth, survival and nutritional condition are usually enhanced.

(2) Temperature. Temperature can be either beneficial or detrimental to fish larvae. Temperature regimes outside the tolerance limits of a particular species will cause mortality of larvae while temperature regimes within the range that give good survival may be used to accelerate or even maximize growth of the larvae. High temperatures will shorten the time from hatching to metamorphosis, and consequently, mortality may be reduced.

The effects of temperature on the growth and survival of fish larvae must be determined for each species. Apparently, the eggs and larvae of tropical and subtropical species are generally stenothermal.

(3) Salinity. The effect of salinity on the growth and survival of fish larvae is primarily on larval osmoregulation. Survival of larvae of many species may be better at low salinities than higher salinities since low salinities are isosmotic to body fluids.

Rearing Environment

Good quality seawater at 30–31 ppt is required for larvae rearing. Water temperature is also important and should range from 26° to 28°C to promote fast growth of larvae.

Larval tanks are prepared one to two days prior to the transfer of newly-hatched larvae. Filtered seawater is added to the tanks and very mild aeration is provided. After stocking, unicellular algae (*Tetraselmis* sp. or *Chlorella* spp.) are added to the tank and maintained at a density of 8–10 × 10 or 3–4 × 10 per ml for *Tetraselmis* sp. and *Chlorella* spp., respectively. These algae serve a dual purpose: as a direct food to the larvae and rotifer and as a water conditioner in the rearing tank.

Green water and clear water

Microalgae affect the microbiology, nutrition, feeding and behaviour of larvae. The addition of microalgae to the tanks during early rearing of the larvae may affect rearing performance. Microalgae addition rapidly affects the biochemical composition of the rotifers in the larval tanks. Larvae from green water tanks showed higher survival and growth, and less gut contents than larvae reared in clear water. In the former, the ingested rotifers had higher energy and protein content, suggesting that these variables are important for achieving high growth and survival in the larvae.

The growth and survival of fish larvae can also be affected by the type of microalgae used. Interactions between algae and bacteria in the larval tanks might be more important than the nutritional value of the algae. Dead or dying algae would increase the bacterial substrate.

Fish larvae can be reared under stagnant or open-system conditions. Generally, partial water changes are provided and microalgae are supplied to the rearing tanks during the initial stages of culture. Low exchange rates of water may affect the retention time of prey in the larval tanks and changes may occur in the biochemical composition of the prey before being consumed by the larvae. Algal addition is advantageous since the prey can continue feeding. Consequently, in clear water systems, there is a progressive decrease with time in prey quality. This loss of prey quality can be partially avoided by reduction of the prey residence time through an adequate adjustment of the prey density and the prey/larvae ratio.

The day following stocking, the bottom of the larvae-rearing tank should be cleaned and every day thereafter. This is done by siphoning off unfertilized eggs, faeces, dead larvae and uneaten food accumulating on the bottom of the tank. About 20% of the tank water is changed daily for the first 25 days of the rearing period, then increased to 40–60% per day for the remaining culture period. Since seabass can also be cultured in freshwater, it is recommended to reduce the salinity of rearing water when the larvae are still in the hatchery, before transfer to a freshwater environment. Beginning from the twentieth day, salinity can be gradually lowered until freshwater condition is reached on the twenty-fifth day.

Food and feeding

Prey size

Prey size may affect the prey ingestion by early fish larvae. It has been reported that the use of small sized rotifers significantly improves the initial feeding performance of fish larvae at the earlier developmental stages. The effect on feeding of using small sized rotifers is mainly due to an increase in feeding incidence rather than in ingestion rates. Therefore, small rotifer supply would improve the incorporation of the larvae to the exogenous feeding from mouth opening. In spite of this, only large rotifers are commonly used in hatcheries for some species. Small sized nauplii of various copepod species were found to very useful for the larval rearing of marine finfishes especially for the species with small larval mouth openings.

Prey density

Maintenance of appropriate feed density in the larval tanks is most important. Since the marine finfish larvae are visual feeders, availability of the prey in the vicinity increases the chances of feeding and saves energy of larvae used for searching the prey.

Larval diets

Most species of marine fish that have been cultured are reared on a sequential diet of rotifers, brine shrimp nauplii and dry supplemental diets.

Microalgae are the customary food given to zooplankton that will be fed to larval fish. The type of culture, temperature, nutrients, other conditions and growth phase all can affect the nutritional value of microalgae to zooplankton and to the fish larvae eating them.

Rotifers

The rotifers are considered as an important live feed in hatchery operation due to their planktonic nature, tolerance to a wide range of environmental conditions, high reproduction rate (0.7-1.4 offspring/ female/day), small size and slow swimming nature. More over the filter-feeding nature of the rotifers facilitates the inclusion of specific nutrients essential for the larval predators through bioencapsulation into their body tissues. As a result it became a suitable prey for fish larvae that have just resorbed their yolk sac. The availability of large quantities of this live food source has contributed to the successful hatchery production of more than 60 marine finfish species and 18 species of crustaceans worldwide.

Two main species of rotifer have been used are *Brachionus plicatilis* (large size) and *Brachionus rotundiformis* (small size).

Health and nutritional quality of rotifers depends on several culture factors: type of culture, water quality, temperature, foods, rotifer density and age of culture. Rotifers are also cultured in many species of algae. These algae should contain significant amounts of DHA and EPA because one or both of theses are essential fatty acids in the diet of marine fish. The ability of rotifers to synthesize these fatty acids is limited and their diet must include a generous portion of these if the requirements of marine fish larvae eating the rotifers are to be met.

The rotifer diet has little effect on the rotifer size and the use of different strains/species of rotifers is required to provide optimal prey size to the larvae.

Artemia

Among the live diets used in the larviculture of fish and shellfish, nauplii of the brine shrimp *Artemia* constitute the most widely used food item. the unique property of the small branchiopod crustacean *Artemia* to form dormant embryos, so-called 'cysts', may account to a great extent to the designation of a convenient, suitable, or excellent larval food source that it has been credited with. In marine finfish larval rearing, artemia feeding is done when larvae is big enough to capture larger preys. Artemia is usually given after 5-10 days of initial rotifer feeding.

Artemia nauplii are maintained in the larval culture tank at densities of 0.5 to 2 per ml for most species of finfish. To estimate the amount of *Artemia* required one must consider both the volume of the tank and the expected number of *Artemia* the larvae will consume. Based on the stage or the age of the larvae, estimate a daily *Artemia* requirement per ml. The total requirement is calculated by multiplying the predicted requirement per ml by the total volume of the rearing tanks. Each gram of cysts contains approximately 200,000 to 300,000 cysts. *Artemia* generally have at least a 50 percent hatch.

Copepods

Copepods were found to be best alternative and most appropriatefor marine fish larvae in which rotifers are an unsuitable first feed. Copepod nauplii are a common natural feed for marine fish larvae species. Small size of copepod nauplii make them suitable for small marie fish larvae at first feeding. Copepods has been used in successful production of marine fish larvae of groupers, snappers, etc. However, the ability to produce copepod nauplii on a large scale has yet to be accomplished as successfully as it has been for rotifers.

Trocophores of bivalves have been found to be a good supplement starter food if given with small rotifers and then replaced with rotifers as soon as the fish are ready. Wild planktons can collected with various nets and traps and can be used for feeding larvae. Nutritional quality is likely to be very high but the appreciable chance of introducing pathogens or pests into the system. Another alternative is extensive culture of zooplankton in ponds and impoundments.

Enrichment of live feeds

To reduce uncertainty concerning lipid quality of zooplankton, they can be enriched. Some marine oils are reliable sources of EPA and DHA, and mixtures of purified oils are also used. Because rotifers cultured in baker's yeast alone are deficient in DHA and EPA, they can be enriched upto 48hrs with yeast enriched with oil and sometimes vitamin or with other materials. Addition of cuttle liver oil to bakers yeast fed to rotifers increased both EPA nad DHA levels. Brine shrimp nauplii can also be enriched with emulsified marine oil or a micronutrient-fortified marine oil emulsion. A variety of commercial enrichment media for rotifers and artemia are available to improve the nutritional quality of these organisms.

Compound larval feeds

The three main types are microencapsulated, microbound and microcoated diets. early marine fish larvae have difficultuy in accepting and digesting microcapsules and microparticulates. Early weaning was the orinal goal of supplementing with compounded feed, but co feeding of compounded feeds with live feeds can at least reduce the live food requirement. Microencapulsted eeds provide ad alternative way to administer vaccines and therapeutic agents to larvae. During early stages, larvae have difficulty in recognizing inert particles as feed. Typically, early marine larvae probably depend on to a greater degree on small colloidal proteins in zooplankton because they donot have the enzymes necessary for digesting and absorbing larger protein molecules. Older larvae have greater capabilities to make more kinds of enzymes and to adjust enzyme production according to the type of food.

Feed management

Newly hatched larvae are usually not given food on the first day because they derived their nourishment from the yolk and the eyes and mouth are still non functional. During the initial days the larvae were given enriched rotifers at a density of 5-20 rotifers/ml depnding upon the species and age of the larvae. As the larvae grows bigger, freshly hatched brine shrimp nauplii at a density of 1-10 1induviduals /ml depending upon the species and age of the larvae. As the feeding of brine shrimp progress the rotifer density is slowly decreased and finally stopped. As the larvae grow bigger, compounded feeds were given to larvae at a rate of 1-4g/t.

Water management

Siphonoing of the tank bottom to remove dirt, dead larvae, wastes and decaying uneaten food should be done everyday starting from the second day of rearing . Daily water exchange from as high as 70% of the tank volume to as low as 30% is undertaken prior to feeding. The percentage of water exchange is dependant on the age of the larvae.

Fry harvest/packing /transport

At the end of larviculture, fry can be harvested and transported to fish farms. Transport is usually done in cool periods of the day. Fishes are transported in oxygenated bags places inside carton boxes lined with thermocole sheets. The transport densities depend upon the size of the fish, species of the fish, distance to be traveled etc. Reducing the temperature and salinity during transport help to improve the survival.

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Marine Metagenomics

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Metagenomics is defined as function-based or sequence-based cultivation-independent analysis of the collective microbial genomes present in a given habitat. Metagenomics combines the power of genomics, bioinformatics, and systems biology (holistic approach to understanding biology). It involves extraction of the DNA directly from all the microbes living in a particular environment, and this mixed sample of DNA is cloned in suitable vectors in the laboratory, creating a library that contains the genomes of all the microbes found in that environment. The library is then studied in several ways, either by analyzing the nucleotide sequence of the cloned DNA or determining what the cloned genes express. Metagenomics has been applied for understanding the genetic diversity, population structure, and ecology of complex microbial assemblages and enzyme and natural product discovery. The most dramatic discovery from metagenomics to date include the discovery of bacteriorhodopsin genes in seawater, and the discovery of *Pelagibacter*, which represents more than one-third of the prokaryotic cells in the surface of the ocean. Metagenomic analysis of viral assemblages in major oceanic regions has revealed that the global marine viral diversity is very high and presumably comprises several hundred thousands of species. Cyanophages and a newly discovered clade of single-stranded DNA phages dominate the Sargasso Sea, whereas prophage-like sequences are most common in the Arctic. Metagenomic technology has been successfully used to study single genes such as cellulases, pathways of antibiotic synthesis, decipher genomes of organisms such as archaea, anammox bacteria. Metagenomic analysis of sponge microbial communities have shown to contain genes and gene clusters typical for the biosynthesis of biologically active natural products. An array of novel bioactive compounds such as terragines,violacein, indirubin and turbomycins have been discovered through metagenomic cloning and expression. Metagenomics promises to provide new molecules with diverse functions, but ultimately, expression systems are required for any new enzymes and bioactive molecules to become an economic success.

In 1923 the *Bergey's Manual* stated categorically that no organism could be classified without being cultured. However, by the mid-1980s, it was realised that there existed a vast unknown majority of microbes that could not be uncultured *in vitro*. The term "the great plate count anomaly" was coined by Staley and Konopka in 1985 to describe the difference in orders of magnitude between the numbers of cells from natural environments that form colonies on agar media and the numbers countable by microscopic examination. Only 0.01 to 0.1% of oceanic marine bacterial cells produce colonies by standard plating techniques. Bacteria may be recalcitrant to culturing for diverse reasons such as lack of necessary symbionts, nutrients, or surfaces, excess inhibitory compounds, incorrect

combinations of temperature, pressure, or atmospheric gas composition, accumulation of toxic waste products from their own metabolism, and intrinsically slow growth rate or rapid dispersion from colonies (Simu and Hagstrom, 2004). However, under certain conditions, bacteria may be viable but not culturable, as was demonstrated by the work of Colwell and colleagues, who showed that strains of *Vibrio cholerae* were indeed alive and virulent when isolated from aquatic environments (Colwell et al, 1985).

The realisation that the vast majority of the microbes found in nature could not be cultured in the laboratory has spawned various techniques, heralding initiation of a new era of microbial ecology. The first such development is the sequencing of ribosomal RNAs and the genes encoding them to describe uncultured bacteria in the environment. Woese (1987) originally proposed a 16S rRNA-based phylogeny, and using this technique, described 12 bacterial phyla, each with cultured representatives. The concept of cloning DNA directly from an environment was initially suggested by Pace (1985). Schmidt and co-workers (1991) constructed a *ë* phage library from a seawater sample and screened it for 16S rRNA genes. The most startling results of many microbial diversity studies that have employed 16S rRNA culture-independent methods is the revelation of the richness of the uncultured microbial world.

Biotechnology has a continuous demand for novel genes and enzymes and compounds. Soil microbial diversity is estimated to range from about 3,000 and 11,000 genomes per gram of soil with less than 1% being accessible through cultivation techniques (Torsvik et al. 2002). Pure culture analysis of soil microorganisms has revealed that they are a rich source of novel therapeutic compounds such as antibiotics, anticancer agents and immunosuppressants, as well as a wide range of biotechnologically valuable products (Schmeisser et al, 2007). However, the cultivation-dependent approach is limited by the fact that the overwhelming majority of microorganisms present in the environment cannot be cultured under laboratory conditions. Direct genomic cloning offers the opportunity to capture operons or genes encoding pathways that may direct the synthesis of complex molecules of biotechnological potential. There is a vast amount of information held within the genomes of uncultured microorganisms, and metagenomics is one of the key technologies used to access and investigate this potential (Handelsman 2004).

What is Metagenomics?

The term "*Genome"* was first proposed by Hans Winkler, a professor of botany at the University of Hamburg, Germany, in 1920 to describe the total hereditary material contained in an organism. Today *genome* is used to describe the entire DNA present in a haploid set of chromosomes in eukaryotes, in a single chromosome in bacteria, or all the DNA or RNA in viruses. The suffix "*ome"* is derived from the Greek for *"all"* or *"every."* In Greek, 'meta' means *"transcendent."* 'Meta' in the first sense means that this new science seeks to understand biology at the aggregate level, transcending the individual organism to focus on the genes in the community. Operationally 'Metagenomics' is novel, in that, it involves functional and sequence-based analysis of all the collective microbial genomes simultaneously in an environmental sample. Metagenomics has helped to address some of the most complex medical, environmental, agricultural and economic challenges during recent years.

Construction and analysis of metagenomic libraries

Two approaches, the sequence-driven analysis and the function-driven analysis have emerged to extract biological information from metagenomic libraries (Fig 1). Sequence-driven metagenomic analysis relies on the use of conserved DNA sequences to design hybridization probes or PCR primers to screen metagenomic libraries for clones that contain sequences of interest. Sequence driven metagenomcs will be particularly useful in understanding the biology of uncultured microbes such as *Acidobacterium*, which occur ubiquitously in nature and abundant across the globe.

The function-driven analysis is involves identification of clones that express a desired trait, followed by characterization of the active clones by sequence and biochemical analysis. This approach identifies clones that have potential applications in medicine, agriculture or industry by focusing on natural products or proteins that have useful activities. Function-driven screening of metagenomic libraries is not dependent on sequence information or sequence similarity to known genes. Thus, this approach has the potential to discover new classes of genes that encode either known or new functions. A significant limitation of this technique is the dependence on expression of the target genes and production of functional gene products in a foreign host, which is in most studies, *Escherichia coli*. Thus, the incapability to discover functional gene products or a low detection frequency during function-based screens of metagenomic libraries might be a result of the inability of the host to express the foreign genes and to form active recombinant proteins. The major advantage of a function-based screening approach is that only full-length genes and functional gene products are detected. To identify enzymatic functions of individual clones, chemical dyes and insoluble or chromophore-containing derivatives of enzyme substrates can be incorporated into the growth medium. Examples for this simple activity-based approach are the detection of recombinant *E. coli* clones exhibiting protease activity on indicator agar containing skimmed milk as protease substrate, or the detection of lipolytic activity by employing indicator agar containing tributyrin or tricaprylin as enzyme substrates (Waschkowitz et al. 2009 ;Heath et al. 2009)

One of the constraints with analysis of metagenomic libraries is the low frequency of clones of a desired nature. To increase the proportion of active clones in a library, several strategies have been designed to enrich for the sequences of interest before cloning. The potential power of this strategy is evident in the elucidation of genomes of uncultured bacteria and archaea that are highly enriched in associations with their hosts. The first complete genome sequence obtained for an uncultured bacterium is for *Buchnera aphidicola*, an obligate symbiont of aphids. A similar example of deciphering genomes of enrichment cultures is that of anaerobic ammonium oxidation (anammox). It has now been recognized that the anammox reaction has great ecological significance, as it is responsible for removing up to 50% of fixed nitrogen from the oceans (Strous and Jetten, 2004). Anammox bacteria grow very slowly and are not available in pure culture. For genome analysis an inoculum of wastewater sludge was grown in a bioreactor for one year, clocking up 10–15 generations. Since the dominant bacteria from the community could not be isolated in pure culture, enrichment cultures and purified cells from a complex bioreactor community were used for deciphering the genome of the anammox bacterium *Kuenenia stuttgartiensis* by metagenomics (Strous et al, 2006). This strategy could be used to enrich for organisms that grow on xenobiotics or on substrates such as starch, cellulose and proteins to find amylases, cellulases and proteases, respectively, or other enzymes of interest in metagenomic libraries.

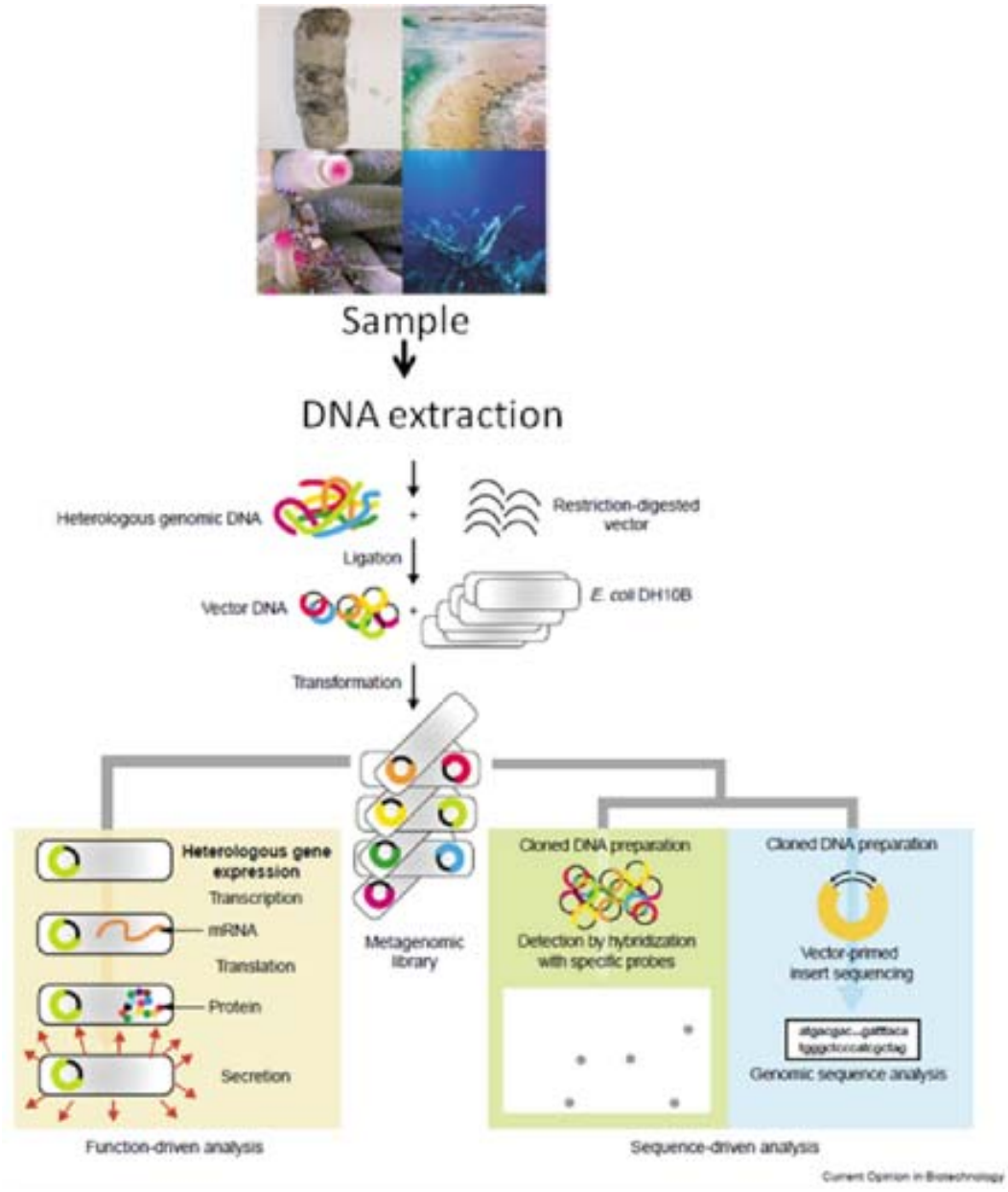

Fig 1. Construction and analysis of metagenomic libraries (Schloss and Handelsman, 2003)

Metagenomic analysis of marine microbial communities

Approximately 71% of the earth's surface i.e., about 361 million square kilometers is covered by the ocean. When we think of ocean life, we tend to think of sharks and squid and sea turtles and such large animals. Underpinning these large life forms is a massive but much less conspicuous world of microscopic organisms that include bacteria, archaea and viruses. Marine environments are believed to contain a total of approximately 3.67×10^{30} microorganisms, about densities of up to 10^6 per ml of seawater, and account for most oceanic biomass and metabolism. Marine microbes

are thought to act as part of the biological conduit that transports carbon dioxide from the surface to the deep oceanic realms. By removing carbon from the atmosphere and sequestering it in the form of organic matter, marine microorganisms may significantly affect the biogeochemistry of the planet and macro-organism health (Newman and Banfield, 2002). Since only a small fraction of microbial species have been cultured and studied, we don't even understand what sorts of metabolic processes and organisms exist and how they relate to the carbon, nitrogen, and sulfur cycles in the environment. Realisation of the importance of microbes in biogeochemistry and productivity of the oceans and the advancements in throughput and cost-reduction of sequencing technologies have increased the number of metagenomic sequencing projects, such as the Sorcerer II Global Ocean Sampling (GOS) (Biers et al., 2009; Rusch et al., 2007), or the metagenomic comparison of 45 distinct microbiomes and 42 viromes (Dinsdale et al., 2008). In a classical metagenomic study of genome fragments from a BAC library of marine picoplankton, Beja *et al*. (2000) identified a new class of genes of the rhodopsin family, named proteorhodopsin, from an uncultivated alphaproteobacterium SAR86. At that time, this rhodopsin family was known to exist only in extremely halophilic archaea and had never before been observed in cultured bacteria. Bacteriorhodopsin is an integral membrane protein usually found in archaea, most notably halobacteria. It acts as a proton pump, *i.e*., it captures light energy and uses it to move protons across the membrane out of the cell. The resulting proton gradient is subsequently converted into chemical energy. One of the most extensive microbial metagenomic studies in the ocean was the shotgun sequencing of microorganisms of size ranges from 0.1 to 3.0 mm in the Sargasso Sea in the Atlantic Ocean near Bermuda (Venter *et al*. 2004). Their study generated almost 2 million sequence reads, yielding over 1.6 billion base pairs of raw DNA sequence. Based on sequence relatedness and unique rRNA gene counts, the analysis suggested that these DNA fragments were derived from at least 1800 genomic species including 148 previously unknown bacterial phylogenetic types. Their analysis also identified spatial variation in species richness and relative abundance.

Direct analysis of rRNA gene sequences had shown that the vast majority of microorganisms present in the environment had not been captured by culture-dependent methods (Handelsman, 2004). Culturing efforts have intensified recently, and successes have included pure cultures of members, now termed the genus *Pelagibacter*, which represents more than one-third of the prokaryotic cells in the surface of the ocean but was known only by its 16S rRNA signature until 2002 (Cho and Giovannoni, 2004). A similar finding is the finding of abundance of *Acidobacteria* in the terrestrial environments, typically representing 20 to 30% of the 16S rRNA sequences amplified by PCR from soil DNA, and until recently only three members have been cultured (Smit et al, 2001; Janssen et al, 2002).

Diversity of viruses in marine ecosystem

Viruses are the most common biological entities in the marine environment. Viruses are 10 times more abundant than bacteria and account for 94% of nucleic acid containing particles in the marine environment. Metagenomic approaches to understand viral diversity in the seawater started the landmark paper by Breitbart et al. (2002). Extrapolating from <1000 sequenced sub-clones and the number of sequences with overlap, the number of identifiable viral sequences in seawater was estimated to number between 300–7000 new viral types depending on contig assembly stringency. Thirty to forty per cent of the significant tBLASTx hits were for phage sequences, followed by repeat

and mobile elements and bacteria, archaea and eukarya sequences based on Genbank annotations. A similar study of dsDNA viruses in near shore sediments indicated much phylogenetic overlap with seawater bacteriophages and the presence of at least 104 distinct genotypes per kilogram of sediments (Breitbart et al., 2004). Subsequently, the Global Ocean Sampling (GOS) Expedition, by Craig Venter in 2003, was one of the important projects exploring the marine metagenome on a larger scale. Angly et al (2006) reported 60%–80% of sequences without sequence similarities in Genbank and among those with tBLASTx, 98% were related to positive strand ssRNA viruses. No RNA bacteriophages were detected indicating that most marine bacteriophages have DNA genomes and that most hosts of marine RNA viruses may be eukaryotes. Metagenomic analyses of 184 viral assemblages collected over a decade and representing 68 sites in four major oceanic regions showed that most of the viral sequences were not similar to those in the current databases. There was a distinct ‘‘marine-ness’’ quality to the viral assemblages. Global diversity was very high, presumably several hundred thousand of species, and regional richness varied on a North-South latitudinal gradient. The marine regions had different assemblages of viruses. Cyanophages and a newly discovered clade of single-stranded DNA phages dominated the Sargasso Sea, whereas prophage-like sequences were most common in the Arctic. Most viral species were found to be widespread and the difference between viral assemblages was attributed to variation in the occurrence of the most common viral species and not by exclusion of different viral genomes (Angly et al. 2006). Studies on the RNA viruses in the ocean are relatively scanty. RNA viruses are known to infect marine organisms from bacteria to whales, but RNA virus communities in the sea remain essentially unknown. Culley et al. (2006) using reverse-transcribed whole-genome shotgun sequencing reported a diverse assemblage of previously unknown RNA viruses including a broad group of marine picorna-like viruses, and distant relatives of viruses infecting arthropods and higher plants.

Biotechnological Applications

With the advent of efficient cloning vectors such as bacterial artificial chromosomes (BACs) and cosmids, together with improved DNA isolation techniques and advanced screening methodologies using robotic instrumentation, it has now become possible to express large fragments of DNA and subsequently screen large clone libraries for functional activities. Most biocatalysts employed for biotechnological or industrial purposes are of microbial origin. The cloning of metagenomic DNA and the subsequent screening of the constructed complex environmental libraries have shown the potential to encounter entirely new classes of genes such as lipases, antibiotics, antibiotic resistance genes, oxidoreductases, catabolic enzymes, and biotin synthesis (Table 1). Screens to identify such molecules have been based either on sequence-driven approach or on function-driven approach. The sequence-based screening approach is limited to the identification of new members of known gene families. Several novel functional enzymes such as chitinases, alcohol oxidoreductases, diol dehydratases, and enzymes conferring antibiotic resistance have been recovered by employing sequence driven approaches (Simon *&* Daniel, 2009). A number of novel hydrolytic enzymes have recently been cloned from Antarctic sea water bacterial metagenomic DNA, while a novel low-temperature-active lipase has also recently been isolated from a metagenomic library of Baltic Sea marine sediment bacteria (Acevedo et al, 2008).

Table 1. Metagenomically derived industrial enzymes (modified from Singh et al, 2009)

Functional genes/ enzymes identified	Industrial application
Decarboxylase	Biosynthesis of coenzyme A and development of new antibacterial agents
Dehydratase	Biosynthesis of bulk chemicals, *e.g.*, 1,3-propanediol 1,3-propanediol
Lipases/esterases	As detergent additives, in food processing, in environmental bioremediation, and in biomass and plant waste degradation for the production of useful organocompounds
Nitrilases	for the production of fine chemicals and pharmaceutical intermediates; Commercial production of (*R*)-mandelic acid from mandelonitrile
Proteases	Bio-detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes
Alcohol oxidoreductases, di-keto-D-gluconic acid reductases, 4-hydroxy-butyrate dehydrogenases	Synthesis of carbonyl compounds, hydroxy acids, and amino acids, Chiral alcohols, Production of dihydroxyacetone textile industry as softening agents for starched clothes, bread
a-Amylases	making to break down complex sugars
a-1,4-Glucan branching enzymes	in starch industries
Chitinases	efficient production of specific chitin products, Protection against fungal pathogens, Potential insecticides
Cellulases	Pulp and paper industry, Food, brewing, wine, animal feed, textile industry
Pectinase	Pectin degradation
Alkane hydroxylase	secondary metabolites (steroids, polyketides, terpenes, etc.) and pharmaceutical and agrochemical intermediates

The metagenomic approaches applied to isolate novel compounds from marine environments have resulted in the identification of a biosynthesis gene cluster for the antibiotic violacein (Brady et al, 2001), novel *N*-acyltyrosine antibiotics (Brady et al, 2002), the novel antibiotic turbomycins (Gillespie et al, 2002), antibiotic compoundsrelated to indirubin MacNeil et al, 2001) and a family of novel natural products, the terragenines (Wang et al, 2000). Marine invertebrates, particularly sponges and ascidians, are well known for their production of bioactive natural products. Successes in the marine environment include identification of the biosynthetic machinery for the cytotoxic peptide patellamide from the cyanobacterial symbiont, *Prochloron*, of a marine didemnid ascidian.

Marine invertebrates, such as sponges, have proven to be a rich source of biologically active and pharmacologically valuable natural products, with a high potential to become effective drugs for therapeutic use (Sipkema et al. 2005). Metagenomes of sponge microbial communities have been shown to contain genes and gene clusters typical for the biosynthesis of biologically active natural products (Kennedy et al, 2007). Vast majority of bacteria associated with sponges have, to date, not successfully been cultured. Construction of a sponge metagenomic library and subsequent screening has revealed presence of gene clusters such as polyketide synthase and gene clusters that code

for biosynthetic pathways involved in the production of bioactive bacterial secondary metabolites (Piel et al, 2004). A novel compound, salinosporamide, isolated from *Salinospora* sp. strains, having a strong anti-tumor activity has been also discovered using metagenomics (Feling et al, 2003).

Sequencing and Bioinformatics tools for metagenomics

Most large-scale metagenomic studies have relied on pyrosequencing approaches, such as Roche's 454 platform. Pyrosequencing can deliver high throughput (several hundred thousand reactions in a single instrument run) along with the longest reads of all currently available next-generation sequencing platforms (around 450 bp). This longer fragment length is essential when attempting to classify unknown sequences from many different organisms in a mixed sample, since shorter fragments may lack enough sequence-specific information to be attributed to one particular organism. Other next-generation sequencing-by-synthesis technologies-including Illumina's Genome Analyzer and Applied Biosystem's SOLiD platform-have emerged in recent years to help in metagenomics studies. To make sense of the tremendous numbers of new sequences from the ocean, Wu et al (2009) developed ways to sort each into its proper phylogenic family, known as "binning", which is essentially, putting each sequence into the right category, using methods such as genome assembly, reference genome alignment, phylogenetic analysis etc (Table 2).

Table 2. Methods of Binning (Eisen, 2007)

Method	Description
Genome assembly	Identify regions of overlap between different fragments from the same organism to build larger contiguous pieces (contigs).
Reference genome alignment	Identify ESS fragments or contigs that are very similar to already assembled sections of the genome of single microbial types.
Phylogenetic analysis	Build evolutionary trees of genes encoded by ESS fragments or contigs. Assign fragments or contigs to taxonomic groups based on nearest neighbor(s) in trees.
Word frequency and nucleotide composition analysis	Measure word frequency and composition of each fragment. Group by clustering algorithms or principal component analysis.
Population genetics	Build alignments of fragments or contigs with similarity to each other (but not as much as needed for assembly). Examine haplotype structure, predicted effective population size, and synonymous and non synonymous substitution patterns.

Craig Venter Institute's Global Ocean Sampling (GOS) expedition has revealed a remarkable breadth and depth of microbial diversity in the oceans. There is a need for a systematic way to explore the structure and function of ocean ecosystems, and their impact on global carbon processing and climate. Metagenomics has the potential to shed light on the genetic controls of these processes by investigating the key players, their roles, and community compositions that may change as a function of time, climate, nutrients, carbon dioxide, and anthropogenic factors. These studies include a substantial informatics component, requiring researchers to take on complex computational and mathematical challenges. The Community Cyber infrastructure for Advanced Marine Microbial Ecology Research and Analysis (CAMERA) project (http://camera.calit2.net.) was established for

attempting to bridge these gaps and in developing global methods for monitoring microbial communities in the ocean and their response to environmental changes. The objective of CAMERA is to create a rich, distinctive data repository and bioinformatics tools resource that will address many of the unique challenges of metagenomics and enable researchers to unravel the biology of environmental microorganisms. CAMERA's database includes environmental metagenomic and genomic sequence data, associated environmental parameters ("metadata"), pre-computed search results, and software tools to support powerful cross-analysis of environmental samples.

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13

Quantitative Genetic Tools for Development of Superior Brood Stock

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Success in aquaculture depends on the use of animals with high genetic potential and application of sound management techniques. Though one can bring about improvement in the production performance of a population by environmental manipulations as well as through genetic manipulations, any improvement from the former can not be transmitted to the next generation. The genetic improvement, on the other hand, is inherited by the next generation, and therefore, more important. Any of the different genetic manipulation techniques available can be employed for producing genetically improved brood stock.

In addition to the conventional quantitative genetic techniques like selection and breeding, modern tools like chromosomal engineering (induction of polyploidy, gynogenesis and androgenesis) can also be employed. The most modern technique is genetic engineering where in a desirable gene or set of genes from any source can be transferred into a host animal for producing a transgenic animal with desired characteristics. In farm animals and plants, quantitative genetic techniques like selection and breeding have played an important role in their increased productivity. Although, the plant and animal breeders have conducted scientific breeding programmes on crops and livestock for thousands of years, fish farmers are only beginning to use selection, hybridization and other breeding programmes to improve aquaculture species. Of late, genetics has acquired an important place in aquaculture for producing high yielding strains of fish and shellfish, and for development of disease resistant strains.

Most of the economically important traits in plants and animals are quantitative traits. Quantitative genetics deals with the inheritance of quantitative traits. Quantitative traits are those which are measurable/ quantifiable, and since metric units used for measurement they are also called as Metric traits. They are controlled by many genes which are mostly additive in nature. (In contrast, qualitative traits are controlled by one or a few genes which are non-additive)

In case of additive genes, contribution of each allele (of the same locus or different locus) is additive or cumulative. Contribution of individual gene can vary, and the individual contributions cannot be noticed. Continuous variation of phenotypes is characteristics of quantitative traits. There will be overlapping phenotypes with no sharp distinction between the phenotypes of different genotypes. Genotypes of individuals are indistinguishable from phenotype and hence individuals can not be can be classified into distinct phenotypes as per Mendelian ratios. Instead, many gradations of phenotypes are available leading to continuous variation and bell shaped cure of distribution. In

contrast, qualitative traits show discrete, discontinuous variation, and so individuals can be can be classified into distinct phenotypes as per Mendelian ratios. Study of quantitative traits are made on population basis and quantitative genetic analysis involves description of genetic architecture of the population using statistical procedures. Phenotypic expression of the additive genes of the quantitative traits are very much modified by environment. Genotype sets the maximum limit to which the individual can express the character.

Quantitative genetics is the theoretical basis for all the breeding programmes in animals and plants. It is the logical development of the basic principles of inheritance.

Tools available to the breeder for genetic improvement

Variation

Variation among individuals within the population is the basic and most important pre- requisite for any genetic improvement programme. With out variation no improvement is possible.

In order to study the genetic properties of the population we have to partition the phenotypic variation into component parts attributable to different causes. The phenotypic variation can be divided in to components attributable to the genotype and to the influence of environment. The genotype is the particular assemblage of genes possessed by the individual and the environment is the non-genetic factors affecting it.

Variation is quantified and expressed as variance ($ó^2$). Variance is defined as the average of the squared deviations of individual's value from the population's mean value. The two components of the total variance (phenotypic variance) are the genotypic variance and environmental variance.

$V_P = V_G + V_E + V_{G-E}$

Where,

V_P = Phenotypic variance

V_G = Genotypic variance

V_E = Environmental variance

V_{G-E} = Genotype-environmental interaction variance

The partition of the variance in to its components formulates the question of importance of individual portion in determining its phenotype. The relative importance of the cause of variation means the amount of variation it contributes to the total variation.

Genotype variance: The variance in the population which is due to the difference in the genotype of its members is termed as genotypic variance. The relative importance the genotype as the determinant of phenotype is the ratio of genotypic variance to phenotypic variance (V_G / V_P) is termed as the heritability (h^2).

Environmental variance: It is the variance due to all non-genetic effects starting from feeding to environmental conditions. Environmental variance cannot be eliminated in a population. It plays a major role in determining the phenotypic variance of the population.

Genotype and environmental interaction variance: Apart from the above two components, variance can arise from the interaction between the genotype and environment also. A single genotype

may perform differently in two different environments. It may show good performance in one environment but may be poor in second. The performance of a population may be reduced or increased in different environments.

Genotypic variance consists of additive genetic variance (breeding value) and non-additive genetic variance (Dominance and interaction deviations).

$V_G = V_A + V_D + V_I$

Where,

V_A = Additive genetic variance

V_D = Dominace variance

V_I = Interactions (Epistatic) variance

Additive genetic variance: It is the sum of the effects of all the additively acting alleles that help to produce a phenotype. It is the breeding value. It is an important component because it is the chief cause of resemblances between the relatives, especially with respect to the quantitative traits. This is the portion of the variance which is definitely and surely being transmitted to the offsprings from the parents.

Non-additive genetic variance: These variations are due to the dominance and epistatic alleles, and therefore, consists of the following.

Dominance variance: This is due to the dominance effects of the alleles at the loci. V_D may not be transmitted to the next generation from the parents since the alleles are disrupted during the meiosis.

Interaction (Epistatic) variance: It occurs due to the interaction between the alleles two or more genes. These interactions may be two factor or three factor or so on. The amount of variation due to interaction is rather small; therefore, the breeder can ignore it.

Heritability (h^2)

The heritability is one of the most important properties of the economically important quantitative traits. It expresses the proportion of the total variance attributable to genetic causes.

Heritability is defined as the ratio of genetic variance to phenotypic variance in a broad sense.

$$\text{Heritability } (H^2) = \frac{\text{Genotypic Variance}}{\text{Phenotypic Variance}}$$

It in narrow sense is defined as the ratio of additive genetic variance to the phenotypic variance.

$$\text{Heritability } (h^2) = \frac{\text{Additive genetic Variance}}{\text{Phenotypic Variance}}$$

Heritability ranges from 0-1 or expressed in percentage. It plays an important role in selection programmes for genetic improvement. An important function of heritability in quantitative genetics / animal or plant breeding is for indicating the reliability of using the phenotypic value as a guide to the breeding value. It can be used to predict the genetic gain from selective breeding. It is the property of the population for the trait under consideration, and not of an individual. It is valid for only the given population in a given environment.

Genetic correlations

Economic traits are usually correlated. Because of this, selection for one trait may lead to simultaneous increase or decrease in the other traits. These are called correlated responses and the traits are called correlated traits. This is expressed in terms of correlation co-efficient (r) which ranges from -1 to +1. Correlated response may be positive or negative. It is due to the pleotrophic effect of the alleles. This plays an important role in trait selection for improvement.

Selective Breeding for fish stock improvement:

Breeding is the practical aspect of the genetics. Clear and thorough understanding of the genetic rationale and genetic principles will avoid the inadvertent mistakes during the breeding. Therefore, the breeder should have clear understanding of its pedigree records, genetic parameters like heritability, correlations and of course reproductive biology and behavior of the species before planning a breeding programme. Therefore, breeding studies for the evaluation of the genetic and phenotypic parameters are the essential pre-mediated step. Planning for the breeding programme could be made only after careful consideration of the above parameters. As for example, when there is relatively larger additive genetic variance, simple selection methods like individual/mass selection should yield good progress. On the other hand if non additive genetic variance is predominant, special selective breeding schemes are to be formulated to exploit them. If heterosis is found to be high, cross breeding programmes could be given priority. When 'over dominance' is important for a trait, reciprocal or recurrent reciprocal selection needs to be employed. Genotype environmental interactions of high magnitude calls for developing different strains to suit each of the environments.

Selective breeding means careful selection of superior individuals from the population as parents for the next generation. This is based on the Robert Backwell theory of "like begets like". The breeder selects (saves) the individuals that possess certain desired phenotypes and culls (removes) those that do not in the hope that the selected individuals will be able to transmit their superiority to their offspring, thereby creating a genetically improved population. Thus two elite/best performing individuals are selected and bred to produce the best performing progeny. In animals and plants this lead to production of pure strains and also improvement in production. Selective breeding of fishes is a very useful approach for genetic improvement of cultured fish populations. There are a number of methods, which can be used for selection of superior genotypes for scientific breeding. Careful consideration of the species and trait to be improved are before taking up the selective breeding programme.

Selection of species

Selection of the species is very important aspect of selective breeding programme. While selecting a species the breeder should consider two things, the first is that it should have well established breeding and seed production technology and the second is its economic importance.

Selection of trait

The trait or traits under selection should be economically important, highly heritable, positively correlated and it should be easily measurable. Some economically important traits in fishes are growth rate, body size & shape, meat quality and disease resistance.

Methods of selection :

Selection of the parents for next generation is based on its additive genetic makeup or breeding value. The genetic potential of an individual is judged by the phenotypic performance of the individual in several ways.

Individual selection/ Mass selection

In this method the individuals are selected based on their own performance, viz. on the basis of their phenotypic value. This selection method is very effective when the h^2 of the trait is high. It is usually simple to operate and it yields the rapid response.

Pedigree selection

In this method individuals are selected based on the performance of their parents or grand parents. Pedigree records of the ancestors are considered for selection of parents.

Family selection

Families are selected or rejected as a unit according to the mean phenotypic value of the family. Within the family, variations are not considered. The accuracy of the family selection depends on the heritability of the trait, family size, family type and the variation due to the environment. This is more useful when selection is practiced for less heritable traits like reproductive traits, carcass quality and disease resistance.

With in family selection

In this method, families are considered as a sub population and individuals are selected based on their performance in relation to the mean performance of their family. Individuals with better performance from good families are selected and the others are culled. This method is more efficient than individual selection and family selection. Selection within families would eliminate the non-genetic variation.

Progeny testing

Parents are selected based on the performance of the progeny. This method is reliable, and practicable if cryopreservation of the gametes is possible.

Selection programme for improvement of more than one trait:

Tandem selection

In this method selection is aimed at improving one trait at a time for generations till the goal is reached. Then other trait is selected and it continued for all the traits. It is simple but time consuming, it is rarely practiced because it takes long time and there is a chance of negative correlation between traits which decreases the overall performance.

Independent culling levels

Independent culling level is a selection programme employed when two are more traits are considered for simultaneous improvement. In this, the breeder has to fix the minimum performance level for each trait, and the individual must perform above the minimum level to get selected. Disadvantage of this method is that an individual should be outstanding in all the traits to get selected.

An individual with best performance in one trait and average performance in the other is liable for rejection. The breeder may end up with only a few individuals which satisfy the set performance levels of all the traits leading to inbreeding depression.

Selection index

Index selection is the best and most efficient method of selection progrmme. In this method all the traits considered for selection are given scores, in every individual, according to their performance. An index is made for each animal based on the scores of the different traits considered for selection, giving weightage to each trait according to their relative economic importance using linear regression equation.

Breeding plans :

Selective breeding programme intends to exploit the genetic variations existing in the population, so there should be wide genetic base in the foundation stock. For this, different stocks may be inducted from different sources, making sure the presence wide genetic base.

Superior individuals selected from the population are *inter se* mated to produce the next generation. Continuous selection eliminates the undesirable alleles present in the population. It makes the population homozygous. This is very much useful in evolution of different strains and lines. Mating of related individuals can lead to inbreeding depression which a breeder should be concerned about. To overcome this, crossing between the strains or inbred lines is employed. Out crossing is very useful method in selective breeding wherein unrelated individuals of same species are bred to minimize the effect of inbreeding depression. Crossing of two inbredlines increases the performance because of heterosis. Heterosis is the observed superiority of the hybrid progeny over the parental mean. Hybridization is the rapid method of bringing about genetic modification. Species hybridization is the inter specific crossing.

The salient results of a selective breeding programme carried out in Artemia at CMFRI, Cochin to study the quantitative genetic parameters as well as the response to selection are presented briefly below.

Methodology:

Artemia franciscana (Kellogs1906) from Great Salt Lake, Utah was used for the study. Method of selection followed was Mass selection (individual selection) and the trait under selection was the naupilar size (length in ìm). Bi-directional mass selection was practiced in two sub-populations derived from the base generation viz., SNS line & BNS line with the aim of developing two divergent stocks. While selection for reducing the naupliar size was practiced in SNS line, BNS was selected for bigger naupliar size. Six selected generations were raised. Intensity of selection (i) common for male and female together was estimated as the mean of two sexes i.e. i = ½ (im + if) (Falconer, 1981). Intensity of selection for male and female separately calculated as ratio of effective selection differential to the phenotypic standard deviation. The heritability values of the selected trait (naupliar size) were estimated from full sib data and from regression of offspring on parent as per the procedure given by Becker (1975).The predicted genetic response per generation was calculated for each line separately within sex as per the procedure described by Falconer (1960).

Results :

Heritability of naupilar length:

The heritability estimates of naupilar length, from the regression of progeny on parents, pooled over generations, were 0.2123 ± 0.0766 and 0.3885 ± 0.1108 for males and females respectively in SNS line. The corresponding estimates in BNS were 0.5777 ± 0.1154 and 0.3364 ± 0.1176 respectively. The heritability estimates from full sib data, pooled over generations, were 1.3256 ± 0.0474 and 1.1004 ± 0.0522 for males and females respectively in SNS line, whereas, the corresponding estimates in BNS were 1.2580 ± 0.0583 and 1.4221 ± 0.0479 respectively. While the moderate values of heritability estimated from b_{op} indicated existence of fairly good amount of additive genetic variance which can be exploited through simple selective breeding techniques, the very high estimates of heritability from full sib analysis indicated existence of non-additive genetic variances also.

Selection differential:

Selection differentials, averaged over generations, were slightly higher in females of both SNS and BNS lines. Their mean values were -16.6780 ìm and -16. 3966 ìm in SNS males, -19.9266 ìm and -22.3101 ìm in SNS females, 16.2308 ì m and 15.8700 ì m in BNS males and 17.1180 ìm and 17.0019 ì min BNS females.

Phenotypic responses :

Phenotypic responses for naupliar length from selection was quite substantial. The naupliar size in SNS line, from six generations of selection for smaller size, could be reduced from 486.99 ìm and 490.58 ìm in males and females respectively to 441.67 ìm and 453.05 ìm. The cumulative gain for males and females were -44.32 ìm and -37.52 ìm respectively with average gain per generation being -5.76 ìm and -4.96 ìm. In the BNS line, the naupliar size could be increased to 495.58 ìm and 529.37 ìm in males and females from 486.99 ìm and 490.58 ìm in the base generation, through five generations of selection for bigger naupliar size. The total gain worked out in males and females were 8.59 ìm and 38.80 ìm with mean gain of 0.39 ìm and 5.52 ìm respectively. The mean phenotypic responses were statistically significant except for BNS males.

Realised genetic gain:

The observed phenotypic response is the combined effect of both genetic and environmental factors. Since the environment rarely remains the same over the period of selection, separating out these effects becomes rather difficult. One of the most commonly used methods for removing environmental effect from the phenotypic gains and for determining genetic gain is the use of an unselected control population. Such a control line was used in the present study. Most of the phenotypic responses realized from selection were due to genetic gains. In the SNS line, total genetic gain realized from six generations of individual selection for reduction of the naupliar length was -41.7244 ìm in males and -38.7585 ìm in females. Whereas in BNS line, the total genetic gain from five generations of selection were 12.6427 ìm and 39.4836 ìm in males and females respectively.

The realized mean genetic gain per generation, estimated from regression of control corrected generation means on generation numbers was -5.2585 ìm in males and -5.2289 ìm in females of

SNS, and 0.9338 ìm in males and 5.3493 ìm in females of BNS line. The mean genetic gains were fairly high and statistically significant except in BNS males.

Expected genetic gains :

Expected responses were calculated using heritability estimated from regression of offspring on parent (bop) and also full sib heritability. While, estimates as per former were close to realized genetic gains, those from latter were on the higher side. This result indicates that heritability estimates from full sibs are indeed inflated by non-additive genetic variance , unlike the bop which includes only additive genetic variance.

Generation wise phenotypic response in naupliar length realized from bi-directional selection for reducing naupliar length in SNS line and for increasing naupliar length in BNS line showed that the response in both the lines were in the desired direction. It can be seen that while in SNS line, both the sexes readily responded to selection for decrease in naupliar size, there was a differential response to selection for the higher size in BNS line. In BNS line, the females showed 14.5 times higher response than males, while in the SNS line both sexes showed comparable response. It is rather difficult to explain whether this low response in males was due to attainment of the genetically pre-set maximum size for that sex or due to any other reasons.

The realized response calculated by subtracting the mean control values of each generations from the corresponding selected generation mean is free of environmental effects and therefore, gives the true genetic gain from selection. Comparison of the genetic and phenotypic gains realized in this study point towards the fact that though the environment had played a role in deviating the phenotypic response from the genetic response, its effect was comparatively low and that the genetic gain was quite substantial. Most of the documented selection studies in the aquatic species have reported the response to selection with out considering the environmental effects and therefore, represents only the phenotypic response and not the genetic response. The substantial genetic gains realized in this study indicate the usefulness of selective breeding for developing genetically altered lines.

Genetic improvement need not be restricted to maximizing growth and feed conversion, but can be in survivability and disease resistance. Like any other biological traits there is naturally occurring genetic diversity in disease resistance also. Selective breeding for disease resistance, supported by marker assisted selection is need of the hour which calls for investigations in the field of genetic markers of enhanced disease resistance.

The decision to conduct a selective breeding programme is a decision that must be made on a case-by-case basis. Because selective breeding programmes require dedication, a certain level of sophistication, record keeping and the investment of extra labour. Additionally, selective breeding programmes are not free; they also require the investment of money. Finally, these programmes usually do not produce immediate improvements. Improvements are usually not seen for at least one growing season, so a breeder must be able to incorporate long-term planning into his programme, and he must be patient. A final requirement that must be met for conducting a selective breeding programme is the existence of proper facilities.

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14

Reproductive endocrinology in aquaculture: transition from hormone to gene

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Sea food (fish and shellfish harvested from capture fisheries and aquaculture production in marine and aquatic environment) is the most traded food producing industry. It provides essential local food, livelihood and export earnings (Smith et al 2010). More than one billion people rely on fish as their main protein source. Capture fisheries, aquatic counter part, for the terrestrial hunting supports the livelihood of more than 35 million people around the world. The international trade in fish product in 2002 was $ US 58.2 billion (FAO 2007), and it was greater than the gross domestic product of 70 % of world nations. However, fisheries cannot expand much further without becoming a real danger for aquatic ecosystem. Aquaculture has often been suggested as an alternative to enhance the global fish production, and many government and international agencies stressed the development of aquaculture. With an average annual growth rate of 8.9% since 1970, aquaculture is now considered to be the fastest growing food producing sector in the world (Bondad – Reantago and Subasinghe, 2005). Aquaculture production is rapidly increasing, and more than 48% of world's food fish supply for consumption is supplied by aquaculture.

Aquaculture has emerged as a revolution in agriculture, and aquaculture is supposed to replace fisheries or at least to enhance fish supply. It is once thought that it would replace fisheries as animal husbandry replaced hunting on land. Although it seems to be highly ambitious, it would certainly help to release the pressure on increasingly depleting natural fish supply. These changes will modify employment and livelihood pattern of those investors in the industry (Durate, et al 2007).

Husbandry of aquatic organism is an ancient enterprise, and roots of aquaculture dates back to more than 4000 years ago when Asian emperors maintained stocks of their favorite fishes in ponds. Until late nineteenth centaury aquaculture research mainly consisted of domestication and rearing of wild stocks in captivity (Overtuf, 2009). It is only since 1970s the truly world wide aquaculture industry has developed (Kirk, 1987). Systems of aquaculture range from less technology involved wild accumulation to a high-tech intensive system. The most extensive way of culture is wild accumulation where species are held and protected for a short period of time prior to sale in order to accumulate sufficient number or attaining optimum marketable characteristics (e. g. hard or soft shelled crabs, gravid crabs etc.) In ultra intensive culture, hatchery production and subsequent grow out cultivation are carried out in strictly ensured biosecured culture environment with controls on all wastes access and decontamination measures (Martin, 2007).

In aquaculture, there are certain areas where science can play crucial roles. For example: reproduction, early development, nutrition, health and genetics (Donaldson, 1996). The most essential

characteristic of an aquaculture species is the ability to control reproduction and produce viable offsprings from captive broodstock. Many aquaculture species, however, faces some form of reproductive dysfunction in captivity (Vijayan et al. 2003). This dysfunction is largely due to dramatic difference in the environmental conditions faced by animal in natural environmental conditions and captivity. The captive environmental conditions, which lack the natural stimuli fail to induce appropriate endogenous response in fish. It is almost impossible to simulate the natural conditions for breeding under the captivity for many aquacultured species.

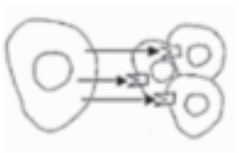
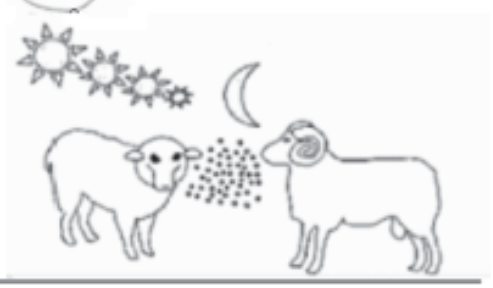
Complete control over the reproduction is the most elusive goals of almost all form of aquaculture. It is crucial for seed production, selective breeding, growth rate, feed efficiency, meat quality and biosecurity (Weber, 2009). Among all, seed production is the primary need of development of any form of aquaculture. Sustainability of any aquaculture, therefore, largely depends on how efficiently aquaculturists can mange reproduction under captivity. Some species reproduce more easily than others. For example, Indian white shrimp, *Fenneropenaeus indicus*, is relatively easy to breed species when compare to tiger shrimp, *Penaeus monodon*. However, this relative easiness in breeding may not be a desirable quality in grow out production system, where reproductive growth adversely affect the somatic growth of the animal. Conversely, delayed maturation in *P monodon* is a desirable characteristic in grow out production system. However, delayed maturation of a species hampers the seed stock production and selective breeding. These diverse needs illustrate that each species require different levels of control over reproduction, and even within the same segment of the industry. Whatever the goal of aquaculturist to control or manipulate reproduction, the progress in gaining control over reproduction process is dependant on our greater understanding of mechanism controlling these processes. This chapter summarizes the present understanding on control of reproduction in aquaculture with special reference to crustacean reproductive control. The first part of this chapter is a preamble to the general endocrinology providing the general concepts and principles in endocrinology. The second part deals with history of endocrinological applications of aquaculture, present understanding of crustacean and finfish endocrinology, endocrine methodologies and future perspective for the application of endocrinology

Endocrinology: a preamble

When the life began as a unicellular organism, about three billion years ago, the time was simpler and communication between cells (organisms) were modest than that those required to maintain multicellular organisms. During the course of evolution the multicellular organisms developed a communication system through chemical signals that coordinate multiple organic functions. The substances that provide a chemical basis for communication between cells are called hormones. The investigation of these chemicals in early years of twentieth century gave birth to a new discipline, the endocrinology (Fingerman, 1997). In strict sense, endocrine glands are specialized organ that delivers their products, the hormones, directly into the interstitial spaces and enter circulatory system. In early years, the endocrinology is confined to the study of these specialized cells. Later, it was demonstrated that nervous system is also closely associated with the functioning of endocrine system in order to respond to varieties of stimulus and contingencies in their internal and external environment. These two systems have been evolved in animals to initiate and coordinate appropriate responses. These stimuli may either be an active danger posed by the predator or may be a gradual threat posed by seasonal environmental change. Although these two systems are morphologically different,

both the system will operate fundamentally in similar ways. The nervous system is organized as cellular net work, where axons direct information via chemical messengers called neurotransmitters, and these neurotransmitters are secreted into synapses and act on target located on the other side. Neurotransmitters can also diffuse into circulatory system and act on distantly located target tissue as hormones do. These neurotransmitters that act outside the synaptic cleft are called neurohormones. In addition to endocrine and neurohormones, hormones have a broader usage in recent years (Table 1).

Table 1 Terminology of different hormone producing organ

No	Hormone type	Action	
1	Endocrine	Signals are released into the blood and exert action at different site	
2	Paracrine	Signals diffuse from one cell type to target cell located in the proximity within the same organ.	
3	Neurocrine	Signals are exerted either as neurotransmitter or neurohormone	
4	Autocrine	Signals produced by one cell population and regulate the same population	
5	Pheromone and photocrine	Pheromones are signals are secreted in to the envrionment targeting individuals of same species. In photocrine system day light affects structure within the eye and signals transmitted to the brain and influences reproduction	

Method of hormone delivery: The specific receptors in the target cells determine the selectivity hormone action. Receptors are protein present in the target cells and bind a particular hormone and initiate a response. There are two types of receptors: cell surface receptors and intra cellular receptors (Fig 1). Protein/peptide hormones use cell surface receptors whereas steroid hormone uses intracellular receptors.

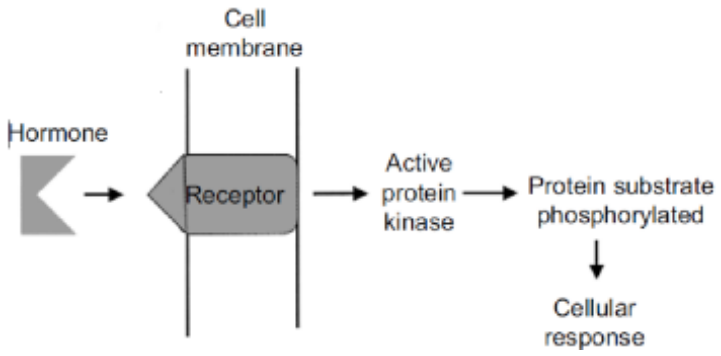


Figure 1 Action of peptide hormone on target tissue

How do hormones function: Hormones modulate the activity of target tissues, and effects of hormones are found even long after the levels of hormones returned to the basal levels. They are usually present in tracer levels ranging from 10^{-6} g/ml to 10^{-9} g/ml. Combined effect of more than one hormone on a biological response will be in number of different ways (Fig 2). The action of these hormones may be additive, if they cause same response. Thus the combined effect is simply sum of the response of two individual hormones. Some times two hormones have same biological effect, but the combined effect may be non- additive. In still different way the combined effect will be more than the additive effect of individual hormone effect, and it is known as synergistic. In some cases some hormones will not have effect on their own but must be present for another hormone to have an effect

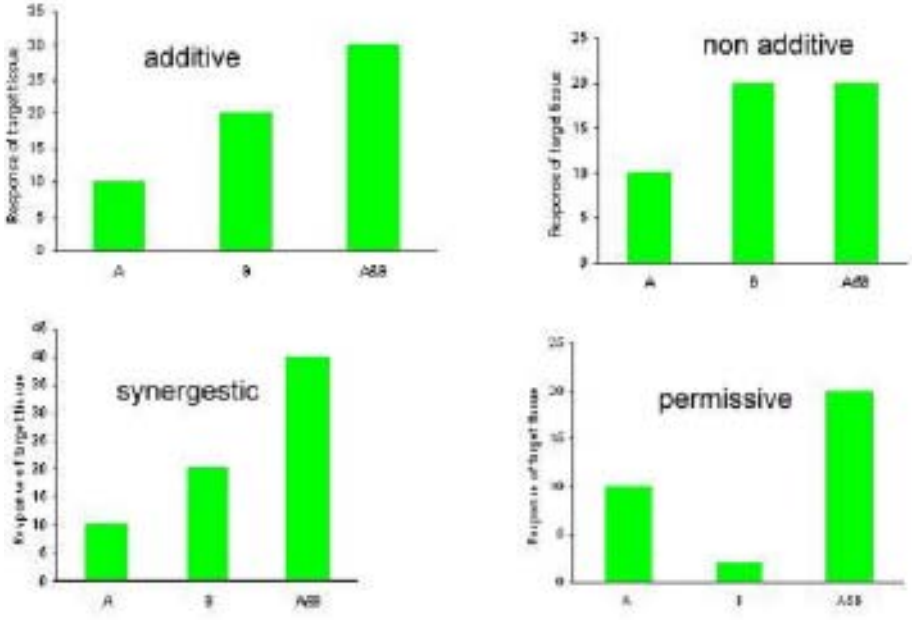


Figure 2 Various forms action of hormones on target tissue results different types of responses

Hormones and aquaculture

Husbandry of aquatic organisms is an ancient enterprise. However, the modern science and technology applied to it is only in the past quarter of the centaury. Fish endocrinology has been developed as a basic science with little applied interest. The past fish endocrinologists, largely confined the physiological and evolutionary aspects and not with fish production. Only recently, the endocrinologists have become concerned on aquaculture. Now aquaculture and ranching of salmon are considered to be the domain of fish endocrinologists. Hormonal application in induced breeding started in Brazil, when fish pituitary glands were injected to induce maturation. This is originally described by Argentinean physiologist, B. A. Houssay who recorded precocious spawning in viviparous fishes when they administered with crude extract of the pituitary gland. The role of pituitary gland (hypophysis) in the reproduction of vertebrates may considered to have commenced three years before, when S. Aschheim and B. Zondek found that pituitary implants accelerated the sexual development and cycle of female mice, and P. E Smith for the first time unequivocally demonstrated the effect of hypophysectomy and subsequent replacement therapy on the gonads of the laboratory rats (Allen,1939). This new finding came to the attention of R. von Ihering, who was the Director of the *Comissao Tecnica de Piscicultura do Nordeste at Ceara,* Brazil, who had been facing the problems of failure in spawning in native fresh water food fish. Even when caught in nearly ripe condition these species rarely completed the maturation. In nature, spawning precipitously followed the first storms of rainy season, but these environmental cues could not be duplicated in the captivity. By 1934, von Ihering had developed successful techniques to induce ovulation using fish pituitary- this is generally known as hypophysation. During almost similar period, Russians were also discovered successful spawning and breeding of sturgeon using freshly removed pituitary gland extract. These findings were in fact a fortunate discovery as hydroelectric stations and dams were built in rivers and it prevents the upstream spawning migration of sturgeon.

Although hypophysation revolutionized fish culture, and contributed enormously towards the development of finfish aquaculture, it suffers from number of limitations. Crude pituitary extract vary in their content of gonadotropin depending upon state of maturity of donor fish, and in addition to the gonadotropin it contain a range of other hormones which may have a synergistic and antagonistic effect on reproductive process. It is extremely difficult to quantify the gonadotropin content of pituitary extract and standardize the dosage (Zohar, 1989). The chances to the transmission of pathogen via pituitary extract are another threat of the use of pituitary extract. Although not totally reliable, hypophysation was not abandoned.

This first generation technology paved the way for the second generation technology, which involve the purified human chorionic gonadotropin (hCG). Although it has been proved to be success in many species, owing to its species specificity it did not work in many species (Zohar, 1989). This hormone has high molecular weight and when injected these hormones stimulate the immune system of fish and adversely affect the health of the animals. The discovery of the portal (closed) circulation between pituitary and brain initiated the discovery of the gonadotropin releasing hormone (GnRH) (Bowers et al. 1970). These discoveries radically changed our thoughts on the organization of the hormonal system, but some time passed before they affected aquaculture practices. Bretton and Well (1973) used the brain hormone for the first time to release gonadotropic hormones from the pituitary in fresh water carps. Later many workers used the GnRH for inducement of spawning in

fishes. The advantages of use of brain hormones are obvious: pituitary gonadotropins differ in species used for aquaculture in their molecular structure and success rate is unpredictable in many cases. Brain hormones have undergone little change during evolution (McCeery et al 1982), and thus same peptide molecule may well activate the gonadotrophs of pituitary of any vertebrates. Further, being low molecular weight compound, the immunogenitic potential of GnRH is far less than the pituitary hormones. GnRH molecules can be modified by replacing amino acids, and the resulted products in vivo degraded far slower than the native forms. The use of GnRH in the induced breeding of finfish is the third generation technology and it has attracted significant interest in recent years especially: the discovery of new natural forms of GnRH, the development and testing of potent GnRH analogue and the development of novel means of administration of hormones (Donaldson, 1996). In some teleosts gonadotrophs of pituitary are innervated by nerve fibers producing the dopamine, the neurotransmitter inhibit the production of gonadotropin 2. A combination of therapy with dopamine antagonists have been found successful in some cases, where GnRH alone does not provide successful spawning. The most recent development in induced maturation of fin fishes is the incorporation of GnRH analogue into a polymeric sustained-release delivery system, which releases the hormone over a period of days. The current model for the control of reproduction of fin fishes is presented in the figure 3

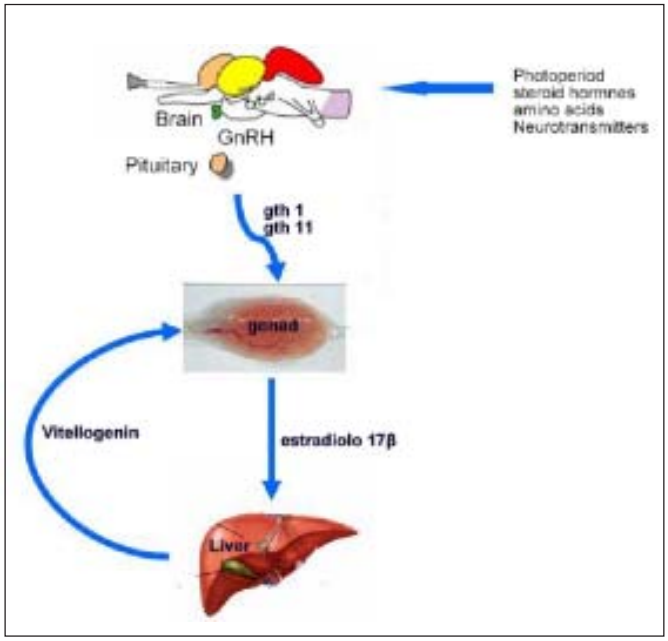


Figure 3 Schematic representation of hypothalamus-pituitary-gonad-liver axis in finfish

Reproductive endocrinology of penaeid shrimp and shrimp aquaculture

Generally, in invertebrates, oogenesis can be stimulated by a release from inhibition or by a secretion of a stimulator (Schuetz, 1969). Ovarian maturation (oogenesis) in Crustacea is said to be stimulated by the gonad stimulating hormones secreted by the brain and thoracic ganglia and inhibited by Gonad Inhibiting hormones (GIH) of the eyestalk (Adiyodi and Adiyodi, 1970). The antagonism of eyestalk may be reduced by a decline in the titre of the GIH as the shrimp grows and moves into an environment suitable for spawning. Final spawning act may, in fact, be triggered by a stimulus, either visual or hormonal originating in the eyestalk (Muthu and Laxmynarayan, 1982). Panouse (1943) demonstrated that the removal of the eyestalk of palaemonid shrimp would lead to ovarian development and spawning. However, this technique was first performed in penaeids only in 1970 in *P. duorarum* by Caillouet (1970). It had far reaching impact on crustacean aquaculture in general and penaeid shrimp farming in particular. The first successful maturation and spawning of *P. monodon* was achieved by Santiago (1977), although Alikunhi et al (1975) achieved maturation with unviable spawning. The great majority of the captive maturation has been from ablated females, although

few workers have reported maturation in unablated females (Santiago, 1977, Primavera, 1978. Emmerson, 1983), only Emmerson (1983) was successful in obtaining viable spawning (16.7 to 82% hatch rate). Although eyestalk ablation started as a stop- gap procedure to induce maturation and spawning in penaeid shrimp in early 1970s, this procedure has been continuing in commercial seed production industry across the world.

The most acknowledged consensus of crustacean reproductive endocrinology is that reproduction is controlled by two antagonistic hormones, one inhibits (Vitellogenin inhibiting hormone or Gonad inhibiting hormone, V/GIH) and other stimulates (Vitellogenin stimulating hormone, Gonad stimulating hormone, V/GSH). This simple endocrine axis has been questioned by many recent researchers and postulated a multi hormonal system involving several neuroenodcrine and endocrine pathways, involving neurotransmittes (serotonin or 5 hydroxytreptamine), steroids (progensterone, estradiol), terpenoids(methyl farnesoate) and vertebrate peptide hormones (GnRH). Additionally the role of other neurohormones in the CHH family (i.e. MIH and CHH) in reproduction in penaeid shrimps has also been reported. Thus crustacean reproduction is an end result of multiple vitellogenic related endocrine cascades (Figure 4). Nevertheless the bihormonal axis is still central to the shrimp reproductive endocrinology.

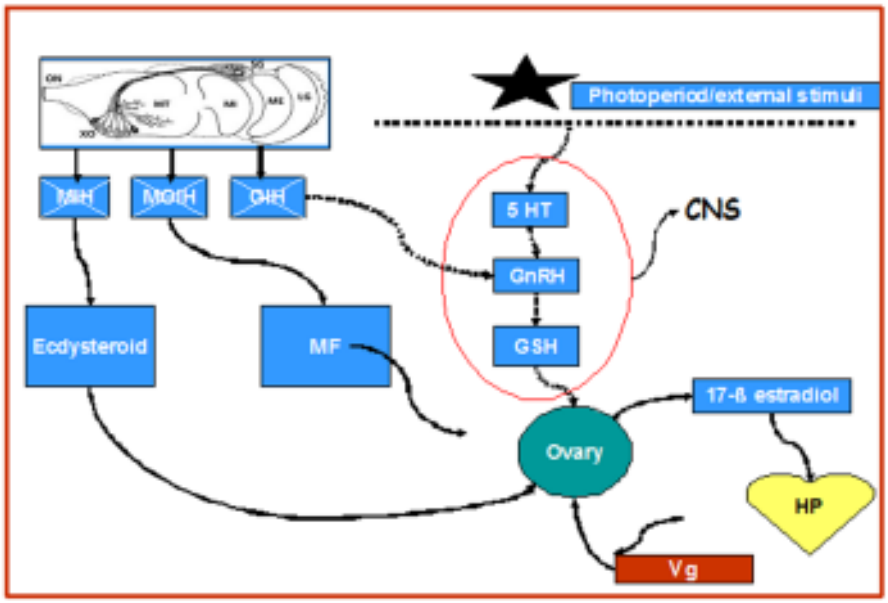


Figure 4 Schematic representation of shrimp reproduction and various factors controlling reproduction. CNS: central nervous system; GIH: Gonad inhibiting hormone; GnRH: Gonadotropic releasing hormone; GSH: Gonad stimulating hormone; 5HT: 5 hydroxy tryptamine; HP: hepatopancreas; LG: Lamia ganglioris, MF: methyl farnesoate; MT: Medulla terminalis; ME: Medulla externa: MI: Medulla interna; MIH: Molt inhibiting hormone, MOIH: Mandibular organ inhibiting hormone; Vg: vitellogenin

Gonad inhibiting hormone or vitellogenin inhibiting hormone (G/VIH):

Although the existence of GIH has been proposed as early as 1940s (Panouse, 1943), this hormone is purified and characterized in late 1980s only (Soyez, et al., 1987). Initially, this hormone is thought to be a female hormone, as it inhibits vitellogenesis (accumulation of yolk protein, the most crucial step in the female maturation). Later, however, it was also reported in the male

crustaceans, and now more appropriately it is called as gonad inhibiting hormone. Owing to the difficulties in purifying and isolating this hormone, only few crustacean GIH has been studied so far. Recently, our laboratory has sequenced GIH from the eyestalk of *Penaeus monodon* (Vijayan et al ms). A 633 bp sequence was cloned from the eyestalk of *P.monodon* by RT PCR. Protein alignment with all known crustacean GIH revealed that homology of *P.monodon* GIH shares 61, 46, 46, 44, 45 and 35 percent identity with *Metapenaeus ensis, Rimicaris kairei, Nephrops norvegicus, Homarus gammarus, H. americanus,* and *Armadillidium vulgarae* respectively (Figure 5). Interestingly, aminoacid identity of *P. monodon* with MIH of other penaeid shrimps is relatively high (56-74%). On the basis of aminoacid conservation Lacombe et al proposed to subdivide the V/GIH group into sequence involved in reproduction and sequence for molting.

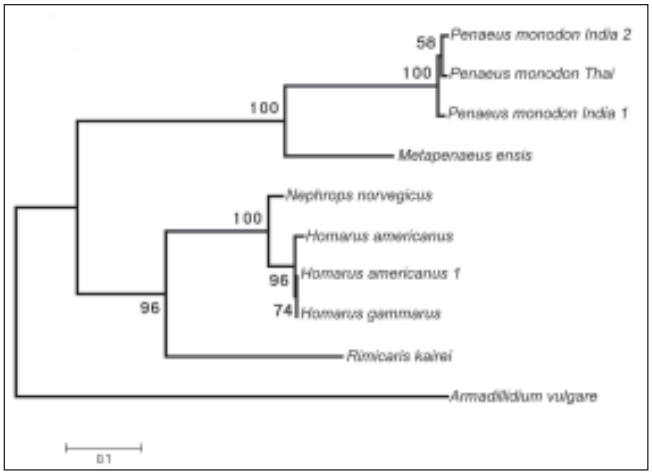


Figure 5 Phylogeny of GIH in decapod crustaceans based on maximum likelihood analysis of aminoacid dataset. Numbers at nodes are bootstrap values based on 100 replicates (Vijayan et al ms on review).

Gonad stimulating hormones/gonadotropins

It is generally accepted that identification of gonad stimulating hormones (GSH) in crustaceans would greatly expedite domestication and closing of life cycle of commercially important aquacultured crustaceans that do not reproduce readily in captivity. Research on GSH has been started in 1960s. A substance known to stimulate vitellogenesis has been found in brain and thoracic ganglion of several crustacean species (Otsu, 1963; Gomez, 1965). But the chemistry of this hormone is not yet elucidated. De Kleijn and Van Herp (1998) suggested that this hormone might be a crustacean hyper glycemic hormone.

Methyl Farnesoate: Parallel to the search for GSH, studies on other gonad stimulating hormones (gonadotropins) have been carried out by several researchers. These investigations results in the discovery of a crustacean hormone, Methyl farnesoate (MF) (Laufer, 1987). MF is an intermediate compound produced during the juvenile hormone biosynthetic pathway (Fig 6) juvenile hormone is well known and extensively studied in insects where they play several regulatory roles both as gonadotropin in adults and morphogens during development. Juvenile hormone as such is not found in crustaceans, but MF is isolated from mandibular organs of crustaceans. Mandibular organ actively synthesize MF during vitellogenesis and become less active during non-reproductive periods. The

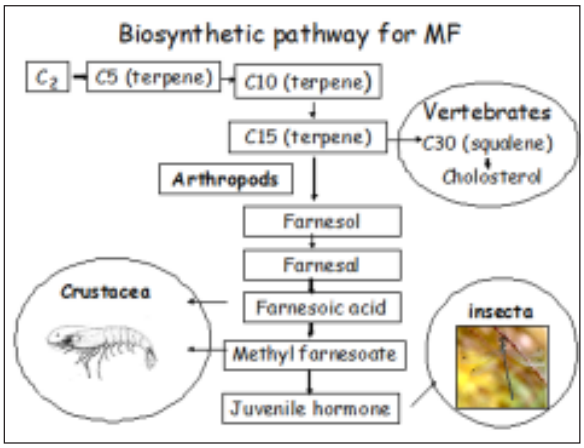


Figure 6 Biosynthetic pathway for methyl farnesoate

secretion of MF is tissue specific and circulated through the blood, and circulating levels are positively correlated with the reproductive state of females. It has also been shown that mandibular organ is negatively regulated by an eyestalk neuro hormone, mandibular organ inhibiting hormones (MOIH). The inhibition is artificially reversed *in vivo* by eyestalk ablation. However, MF can be applied directly, and in many cases successful ovarian maturation and spawning have been achieved as well. Table 2 shows that experimental results of MF treatment to study the effect of this hormone on reproduction of females.

Table 2 Effect of methyl farnesoate on crustacean reproduction

Species	Mode of study	Nature of response	Reference
Triops longicudatus	*In vitro*	No effect	Riley and Tsukimura 1998
Triops longicudatus	*In vivo*	Inhibition	Tsukimura et al 2006
Litopenaeus vannamei	*In vitro*	Reproductive stimulation	Tsukimura and Kamemoto 1991
Litopenaeus vannamei	*In vivo*	Improvement of reproductive performance	Laufer 1992
Sicyonia ingentis	*In vivo*	Reproductive stimulation	Balasubramanian et al 2010
Penaeus monodon	*In vivo*	Improvement of reproductive performance	Hall et al 1999
Penaeus monodon	*In vivo*	Reproductive inhibition	Marsden et al 2008
Macrobrachium rosenbergii	*In vivo*	No effect	Wilder et al 1994
Homarus americanus		No effect	Tsukimura et al 1993
Procambarus clarkii	*In vivo*	Reproductive stimulation	Laufer et al 1998

Ecdysteroid: Ecdysteroid is a polyhydrated keto steroid, found in most arthropods and have a primary function as molting hormone. In crustacea ecdysteroids are synthesized in 'Y' organ (=ecdysal gland), and the alternative sources of Ecdysteroid is the epidermis, and ovary. It has been reported that Ecdysteroid has a possible role in reproduction and maturation as in the case of insects (Subramoniam 2000). It has been suggested that crustacean hormones are multifunctional in nature, a single hormone can mediate different functions. Ecdysteroid mediate as hormone that promotes protective membrane in embryos, then they function as molting hormone from larvae to adult life. In adult they function as gonadotropin. Chang (2001) called it as an 'amazing economy of nature'

Vertebrate-like steroids: Several decapod crustaceans have the ability to synthesize the vertebrate-type steroids (e. g. progesterone, 17-β- estradiol, and testosterone). Some of them fluctuate during the reproductive cycles, and therefore, indicates their role in the reproduction. Many crustaceans have been found to be responding to the injections of these steroids. In additions to the steroids, biogenic amines such as, 5 hydroxy tryptamine (serotonin) are also found to have ovarian stimulatory effect. Recently Wongprasert (2006) reported that serotonin injected *P. monodon* had ovarian maturation and spawning similar to that of unilateral eyestalk ablated females. Although the mode of action of these hormones/neuro transmitters is not properly understood, there seems to be a tremendous potential in using these hormones to stimulate gonadal maturation in the aquacultured species.

In summary, the crustacean egg production is controlled by a cascade of hormonal activities which is triggered by environmental and nutrional factors There fore, all these factors should be considered when any reproductive technology is developed.

Endocrine methodologies

Like most other discipline progress in endocrinology is largely depends on the analytical techniques. The most important step in the endocrinological study is the characterization of endocrine system, and understand how different organs function. This knowledge can be used to improve the reproductive performance of the animals. The information needed to characterize the hormone are: chemical structure of the hormone, biosynthesis and storage, and pathway of metabolisms. In most cases the fist assay when dealing with an unknown hormone is a bioassay. It is based on the measurement of physiological response caused by a hormone. In a bioassay, the hormone is injected directly into the animal to observe the phenotypic effect, or it is dissolved in a medium in which an appropriately responsive tissue can be cultured. An ideal bioassay is one that produces a graded response to the hormone, rather than an all or none response. The response of the assay is then transferred to numerical scale, by which the relative activity of the hormone can be quantified. Hormone concentrations are generally expressed in titers because they are usually determined indirectly by progressive dilution (titration) of the hormone sample being tested until its activity in the bioassay is no longer detectable. The goal of the bioassay is to detect the approximate concentration of hormone normally occurs in the animal. When excessive hormone is used in bioassay a non specific response may occur. This abnormal physiological response is called pharmacological response

Chemical structure, biosynthesis and pathways of hormones can be studied by various chemical analytical methods (Table 3). Today, assay of peptide hormones take advantages of powerful recombinant DNA and antibody technologies. If aminoacid sequence of a hormone is known, it is possible to synthesis nucleic acid probes that can recognize gene that codes for the hormone. The knowledge gene sequence also helps to understand the temporal and spatial pattern of expression of mRNA.

Table 3 Conserved hierarchy of endocrine methodologies.

Sl no	Study level	Techniques
1	Gene	mRNA expression pathways (RT PCR, qRT PCR, microarrayn, Northern Blot
2	Protein	PAGE, western blot, ELISA
3	Functional cell	Histology Histochemistry Immunohistochemistry Insitu hybridization

Future Directions of reproductive physiological research

Basic research on reproductive physiology/endocrinology inevitably leads to the advances in applied research and eventually the practices of aquaculture. Achievements in the fin fish reproductive physiology is an excellent example for how basic endocrinological findings can be effectively integrated into broodstock management (Zohar and Mylonas, 2001). On the contrary, we are still far from achieving adequate understanding on control of reproduction in penaeid shrimp. The growth of penaeid shrimp aquaculture has been spectacular and regarded as a remarkable success story of modern aquaculture. Twenty five years ago, Primavera (1985) identified the replacement of eyestalk ablation and development of low cost maturation diet as key goals to increase spawner yield and

reduce the production cost of shrimp culture industry. Unfortunately, even after twenty five years later, endocrine replacement for eyestalk ablation yet to be achieved. When we look into the global scenario of farmed shrimp production, there is an obvious shift at production level. While *P. monodon* production remains at constant level, there is a drastic hike in the production of *Litopenaeus vannamei* (Fig. 7). The poor performance of *P. monodon* is essentially due to the non availability of specific pathogen free (SPF) broodstock. The most significant hindrance for the development of SPF broodstock is the poor reproductive performance of captive *P. monodon*. Although findings on reproductive physiology of penaeid shrimps have been increased during the recent years, findings are still inadequate to resolve sufficient background for successful broodstock management. Information on the key hormones involved in reproduction is being just published.

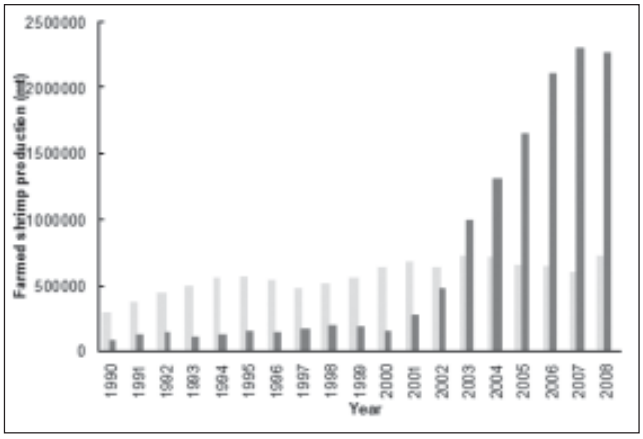


Figure 7. Global farmed shrimp production of Penaeus monodon (yellow bar)and Litopenaeus vannamei(green bar)

Many of the research work in crustacean physiology are criticized as semi scientific (Adiyodi and Subramoniam, 1985) or lack sound experimental design (Benzie, 1997). Further, most endocrinological studies in aquaculture are based on non model wild caught animals, and, therefore, the likelihood for the genetic and physiological heterogeneity is extremely high. The data generated by these studies does not have much value to develop a model for reproductive control.

The endocrine manipulation for induced maturation and spawning should be made at different physiological command levels. The choice at which level these interventions to be made is largely determined by at which level reproductive cycle disruption occurs due to the impact of captivity. These disruptions vary from simple inhibition of spawning to complete lack of gonad development. The success of endocrine manipulation largely depends upon the physiological stage of female. If female is not competent for oocyte maturation, it is less likely for having responded for hormonal therapy.

Modern technology has shifted endocrine research from traditional reductionist approach (part by part study) to an integrative approach. Although traditional approach progressed the science of endocrinology, it has limitations. The integrative approach investigates the complex system at a time. The modern molecular techniques such as microarray techniques would assist to unravel the many complexities of endocrinology

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Nutrition and Feed Technology

15

Nutritional Biotechnology in Aquatic Nutrition

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Besides the application of classical nutrition principles and practices in aquatic nutrition, nutritional biotechnology in general is also gaining momentum. The areas where a beginning has been made are, functional foods or feeds, application of phytases, bio-floc technology and nutrigenomics.

Functional food or medicinal food is any healthy food claimed to have a health-promoting or disease-preventing property beyond the basic function of supplying nutrients. The general category of functional foods includes processed food or foods fortified with health-promoting additives, like "vitamin-enriched" products. Fermented foods with live cultures are considered as functional foods with probiotic benefits. Functional foods are an emerging field in food science due to their increasing popularity with health-conscious consumers. The term was first used in Japan in the 1980s where there is a government approval process for functional foods called Foods for Specified Health Use (FOSHU) (Wikipedia).

The term 'functional foods' covers a broad range of products including, for example, DHA- and selenium-enriched eggs, selenium-enriched pork, stanol- and sterol-enriched margarine, etc. Also included under this category are dietary 'probiotics' and 'prebiotics'.

A probiotic is defined, in the strict sense, as "a viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract" (Roberfroid, 2000). It should be noted that the term has also been widely and incorrectly applied in aquaculture, and especially shrimp culture, to include the use of live microbes to beneficially alter the microbial balance in the culture system itself (Fegan, 2010).

Prebiotics, on the other hand, have been described as "non-digestible substances that provide a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria" (Gibson and Roberfroid, 1995).

A 'functional nutrient' can be further defined as a dietary ingredient that exerts possible positive effects on health in addition to its direct role as a nutrient.

Having defined these, let us have a look at the application of some of them in aquaculture. Some functional foods developed for application in aquaculture are meant to replace fishmeal. Among them are products derived from single cell proteins (SCPs) like yeast. The search for alternatives to fish meal as a source of protein in aquaculture diets has been an important area of research in recent years. Much of this research has focused on increasing the proportion of plant

proteins, such as soybean, in feeds for fish and shrimp. However, many sources of vegetable protein have disadvantages, including low nutrient densities, anti-nutritional factors, high carbohydrate content, imbalanced amino acid and fatty acid profiles, low palatability, seasonal variability and potential mycotoxin contamination (Ceulemans et al., 2003; Spring and Fegan, 2005). In this scenario SCP from yeast when tested showed that their cell walls are indigestible, low protein content, and poor amino acid profile. Nevertheless, many aquaculture feeds contain some SCP, usually yeast, at levels from 1-5% of the diet. Products have been developed after complete removal of the wall enriching the protein content which is called yeast extract. This has been used to replace fish meal completely for the feeds. As a protein source yeast extract (with the brand name NuPro from Alltech) has been investigated for a number of fish and shrimp species including tilapia, cobia, black tiger prawn and Pacific white shrimp. In a series of trials to develop organically certifiable feeds for fish and shrimp, it has been shown that complete replacement of fish and soybean meal with NuPro is possible, although cobia, a marine carnivore, showed a reduced growth rate at levels of 50% replacement and higher (Craig and McLean, 2005)

Nucleotides

In the form of nucleic acids, nucleotides are of fundamental importance as the basis of the genetic code. Genetic information is stored in DNA (except in the case of RNA viruses) providing the basic information coding for all the proteins produced in the body whereas RNA acts as a chemical messenger relaying the information stored in DNA from the nucleus to other parts of the cell. Other than their role in genetics and protein production, nucleotides also play major roles in almost all biological processes including:

- Storage of energy, mainly through adenosine tri-phosphate (ATP).
- As components of several important coenzymes such as nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD) and coenzyme A, all of which are involved in carbohydrate, protein and fat metabolism (Mateo, 2005).
- Mediation of important cellular processes through messengers such as cyclicadenosine monophosphate (cAMP) and cyclic guanine monophosphate (cGMP).
- Control of several enzymatic reactions.
- Serving as intermediates in biosynthetic reactions, especially in glycogen and glycoprotein synthesis and synthesis of polyunsaturated fatty acids (Gill *et al.,* 1985).

Nucleotides have been recognized as important elements in mammalian nutrition especially during periods of rapid growth or physiological stress as well as appearing to play a key role in efficient immune system function (Uauy, 1989; Barness, 1994; Van Buren, 1994). They can be synthesized directly or scavenged by salvage pathways in the body although it appears that exogenous dietary sources are preferentially used (Uauy, 1994). However, immune cells and intestinal cells cannot synthesize nucleotides and depend on nucleotides from other sources (Quan, 1992). Synthesis and salvage of nucleotides are thought to be energy intensive in metabolic terms and dietary nucleotides may reduce the metabolic cost of *de novo* nucleotide synthesis. Dietary sources of nucleotides may also benefit rapidly dividing tissues, such as those of the immune system, especially

under a challenge, and the term 'conditionally essential' has been used to describe their role in nutrition (Carver and Walker, 1995). In many biochemical processes, primary nucleotides such as 5'AMP, 5'CMP, 5'GMP, 5'IMP and 5'UMP are used to produce a number of intermediate metabolites through a series of enzymatic reactions. Supplementation of primary nucleotides in the diet provides a ready source of nucleotides for use in the synthesis of intermediate nucleotides when required.

Sources of nucleotides : Any ingredients of animal and plant origin containing cellular material are potential sources of nucleotides, usually in the form of nucleoproteins. The nucleotide content is particularly high in ingredients such as fish solubles, animal protein solubles, fish meal, legumes (adenine content is particularly high in black-eyed peas), yeast extracts and unicellular organisms such as yeasts and bacteria that are rich in RNA or DNA. The content, proportion and availability of nucleotides differs among ingredients. Muscle protein is a poor source of nucleotides as they are mainly in the form of actin-myosin protein. Oilseeds, such as soybeans, grains, fruits, vegetables and processed milk products are also poor sources of nucleotides (Barness, 1994; Devresse, 2000; Mateo, 2005). Among marine protein sources, anchovies and sardines, for example, have much higher guanine levels than squid, clams or mackerel. Availability and digestibility are also important issues. Whole yeast is much less digestible than yeast extract, possibly due to the need to digest the yeast cell wall and yeast extract having much higher levels of soluble protein. Fish and animal protein solubles are highly digestible but they leach easily, affecting overall availability (Devresse, 2000).

Phytases

Phytase is an enzyme that can break down the undigestible phytic acid (phytate) part found in grains and oil seeds and thus release digestible phosphorus, calcium and other nutrients.

Basically, phytase is a phosphate enzyme that hydrolyzes the ester phosphoric acid and inositol existing in the plants resources. The enzyme phytase is normally produced (endogenous phytase) in ruminants. Non-ruminants (monogastric animals) like human beings, dogs, birds, etc. do not produce this enzyme. Research in the field of animal nutrition has put forth the idea of supplementing phytase enzyme, exogenously, so as to make available bound nutrients like calcium, phosphorus, other minerals, carbohydrates and proteins.

Phytase releases the artophosphate as well as proteins in the intestine to be absorbed by the body, thus largely mitigates the need of phosphate like mono and di-calcium phosphate in the feeds and improve animal growth, reduce the phosphate expulsion to make the environment less polluted from excessive amounts of phosphate and finally decrease the cost for poultry feeds and nutrition.

Phytase is used as an animal feed supplement - often in poultry and swine - to enhance the nutritive value of plant material by liberation of inorganic phosphate from phytic acid (myo-inositol hexakisphosphate) and, thereby, to reduce environmental phosphorus pollution.

Phytase can be purified from transgenic microbes. Phytase has been produced recently in transgenic canola, alfalfa and rice plants. Phytase can also be massively produced through cellulosic biomass fermentation using genetically modified (GM) yeast. Phytase can also be isolated from basidiomycetes fungi. A strain of transgenic pig can produce phytase, thus reducing their environmental impact.

Fish utilize phytate P poorly because they lack phytase, the enzyme that hydrolyses phytic acid (Riche and Brown, 1996). But there might be differences between fish species, because in tilapia an intestinal phytase of physiological significance was shown. However, this failed in hybrid striped bass and in koi carp (Ellestad et al., 2002). Phytases (myo-inositol hexakisphophate 3-phosphorylase, EC 3.1.3.8 and myo-inositol hexakisphosphate 6-phosphorylase, EC 3.1.3.26) are acid phosphatase enzymes of the histidine acid phosphatase family, which liberate inorganic phosphate from phytate (Mitchell et al., 1997). Increased bioavailability of phytic P with the use of phytase has been mainly reported in carnivorous fish species such as channel catfish (Jackson et al., 1996), Atlantic salmon (Storebakken et al., 1998), striped bass (Hughes and Soares, 1998), Japanese flounder (Masumoto et al., 2001) as well as in the omnivore Nile tilapia species (Liebert and Portz, 2005). Common carp is an agastric species with a peculiar gastro intestinal tract and has an intestinal pH above 6.0 (Nwanna and Schwarz, 2006). But phytase activity is highly dependent upon the gut pH (Baruah et al., 2004). Furthermore, plant feedstuffs like cereals, legumes or by-products such as soybean meal, which have P mainly as phytate, play an important role in feeding carp. In these feedstuffs phytate will also affect the availability of some other minerals like Ca and Mg or Zn and Cu (Masumoto et al., 2001).

Table 1. Phytate content of cereals and roots (Ravindran et al. 1995)

	Phytate P[g/100 g dry matter]	**Phytate P[% of total P]**
Cereals		
Corn	0.24	72
Wheat	0.27	69
Barley	0.27	64
Oats	0.29	67
Sorghum	0.24	66
Rice, unpolished	0.27	77
Roots and tubers		
Cassava	0.04	28
Sweet potato	0.05	24

The first commercial phytase products derived from *Aspergillus niger* with the capacity to release phytate-bound P and reduce P excretion, was introduced into market in 1991. After mid-1990s, more and more studies about the effects of supplemental phytase on nutrient utilization or growth of fish have been started in common aquaculture species such as rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio* L.), channel catfish (*Ictalurus punctatus*), salmon (*Salmo salar*), striped bass (*Morone saxatilis*), Nile tilapia (*Oreochromis niloticus*). Phytase has been utilized by spraying onto pellets, pre-treating or dephytinizing feedstuffs before pelleted. Various parameters to evaluate phytase effects have been used including nutrients digestibility, nutrients retention and fish growth performance. Currently, research focus is mainly on phytase effects on digestive systems in different fish growth phases, the dose–response study, specific kinds of phytase for distinct fish species and the most efficient ways of supplement. Besides, the addition of organic acid along with phytase, especially in agastric fishes, is of special interest, and gains serious attention. It is well

documented that the use of microbial phytase in fish feed can enhance the bio-availability of phytate-bound P and nitrogen and thus less P discharged into the aquatic environment. Therefore, phytase is increasingly considered as an additive for cost-effective and environmentally friendly fish feeds. Table 2 summarizes commercially available products with costs fluctuating from ‘ 150-500 per kg

Table 2. Commercial production information of microbial phytases (Stefan et al. 2005)

Company	Country	Phytase source	Production strain	Trademark
AB Enzymes	Germany	*Aspergillus awamori*	*Trichoderma reesei*	Finase
Alko Biotechnology	Finland	*A. oryzae*	*A. oryzae*	SP, TP, SF
Alltech	USA	*A. niger*	*A. niger*	Allzyme phytase
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*P. lycii*	*A. oryzae*	Bio-Feed
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	Finland	*A. awamori*	*T. reesei*	Avizyme
Genencor International	USA	*P. simplicissimum*	*Penicillium funiculosum*	ROVABIO
Roal	Finland	*Aspergillus awamori*	*T. reesei*	Finase
Novozymes	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme, Roxazyme

Effects of phytase application in fish feeds can be summarized as (1) increase in bioavailability of phytate-P. Since supplementation of phytase can improve the apparent digestibility of P in soybean meal or canola meal-based diets, it is possible to improve the P retention of diets and reduce the P discharge into water that was considered as one of the main pollution elements in water environment. (2) Generally, growth improvements were observed in the studies that used diets entirely or almost entirely based on plant protein sources. Many studies reported that the addition of phytase to P inadequate diets has been shown to enhance growth performance. (3) In pigs, phytase was reported to improve protein and amino acid utilization through breakdown of phytin–protein complexes. In fish, however, the results are somewhat controversial. Variations in the outcome of different authors may be attributed to variation in phytic acid content in different feedstuffs, species used and various other inherent characteristics of feed ingredients, or probably due to the presence or absence of the stomach in different fish species, as phytase activity is pH specific. (4) Phytate also can chelate with other minerals to decrease their bioavailability to fish. Phytase supplementation can hydrolyze phytate and increase the concentration of minerals like magnesium, calcium, manganese, and zinc in plasma, bone and the whole body . (5) In dose–response studies, phytase addition of 250–1500 U/kg is usually considered feasible in many fish species. The optimum dose changes along with many factors such as fish species, different phytase sources, diet formulation (amount of substrate for phytase) and selected response parameters. Conclusive studies dealing with the mechanism of phytate degradation of different fish species depending on different diet formulation, specific characteristics of digestive tract and varying activity from different supplemental phytase sources are needed.

Biofloc technology (BFT)

AMR, ZEAH, bacterial floc, heterotrophs, autotrophs – these terms maybe new to the ears of fish and shrimp farmers but not for scientists. AMR stands for Aerated Microbial Reuse while ZEAH stands for Zero-Exchange, Aerobic, Heterotrophic. Both refer to the same thing: a system of intensive aquaculture that has been around for at least ten years and is becoming more popular– starting in the Western hemisphere but now spreading in Southeast Asia. The basic technology was developed at the Waddell Mariculture Center in the USA in the early 1990s. AMR or ZEAH, whichever term you prefer, has been found to reduce feeding cost, makes possible operation of a farm with very little or even zero water exchange, while still producing 10 to 30 tons of shrimps per hectare and from 10 to 100 kg of tilapia per square meter per crop.

Autotrophic vs Heterotrophic

AMR is basically a "heterotrophic" system as against the conventional culture system that fish and shrimp farmers in Asia which is considered "autotrophic." To understand the difference between the two systems, one has to go into the respective roots of the two terms. Heterotrophic comes from "heterotroph" – an organism which rely on carbon in organic form (i.e. other organisms) for food. Animals, fungi, parasitic plants and most bacteria are heterotrophs. In contrast autotrophic comes from the term "autotroph" an organism capable of sustaining itself due to its ability to produce their own food (or organic carbon) from inorganic materials which are basically water, carbon dioxide and nitrogen. The food is synthesized using energy from light or photosynthesis or inorganic chemical reaction or chemosynthesis. Autotrophs include all (except parasitic) plants and some bacteria.

A pond where in where food is produced by autotrophs, mainly plant organisms, whether microscopic and in the water column such as phytoplankton or resting on the bottom such as benthic algae is considered an autotrophic system. In contrast a pond where food has to be introduced is considered a heterotrophic system. It of course does not mean the system is completely free from any phytoplankton. In fact the presence of phytoplankton is believed by some to be essential as oxygen source and reduce aeration need in the daytime.

Extensive vs Intensive

In conventional pond culture farmers are familiar with great pains are taken to prepare the pond so that plankton, particularly diatoms flourish before stocking the shrimps or fish. Presumably the plant plankton becomes food to tiny animal plankton and the two types of plankton together becomes natural food for the newly stocked shrimp or fish fry. This is thought to be the ideal condition for the shrimps or fish because it simulates their natural habitat. Because food is produced within the pond itself the system is considered "autotrophic".

When the stocking density is low, the food that is generated within the pond is sufficient to support the shrimp or fish stock and no feeding is required. This is what is known as extensive aquaculture. Such a system is capable of producing at most a few hundred kilograms per hectare. In an effort to increase production it is inevitable for farmers to try stocking more – to the extent that the natural food that is produced becomes insufficient to support the stock. In such case feed has to be introduced in order to supplement the nutrition coming from naturally occurring food in the pond. Such system which relies on a combination of naturally occurring food and introduced food or feeds is often referred to as "semi-intensive" aquaculture.

At high stocking density, and as the animals grow, more feed is required to the extent that the role of natural food becomes insignificant and the culture becomes "intensive" in nature. Since no more than 30% of the carbon, nitrogen and phosphorus in feeds is assimilated or converted into flesh by the fish or shrimps, more of it serves only to pollute the water in the form of uneaten feeds and excretory wastes which is high in ammonia – a substance that stresses fish and shrimps, reduce growth rate and at high levels even cause mass mortality. At low stocking density, this poses no problem since they can still be fully utilized by phytoplankton and bacteria. At high densities however, the amount of such wastes overwhelms the system and if left unchecked accumulates in the system. The conventional approach is to change the water in order to reduce the level. Considerable skill and experience is required to maintain the phytoplankton population at the right level but which due to weather variation may collapse and create havoc. Furthermore, the water discharged is high in organic load – one aspect of intensive aquaculture that is at the forefront of environmentalist's list of negative effects of aquaculture to the environment.

Shifting from Phytoplankton to Heterotrophic Bacteria

As now practiced in Belize by the Belize Aquaculture Ltd (BAL) and many other farms the world over, applying AMR technology requires deliberately converting the pond ecosystem from one that is autotrophic or phytoplankton-based to a heterotrophic system dominated by bacteria after the fry has been stocked and has established itself. This requires providing a low-protein, high carbohydrate diet with nitrogen to carbon or C:N ratio of 16:1 (18 protein) so that the carbon-hungry bacterial population has adequate food to multiply even as the shrimps are also being fed with regular starter feed The culture is deliberately overfed at 200 to 250 of the shrimp biomass. The addition of wheat flour or even molasses, both of which are carbon rich, has been found also to hasten the growth of heterotrophic bacteria. Details of this technology can be had from Schryver et al. 2008. The only institution in India working on this technology is the Department of Industrial Fisheries, Cochin University of Science and Technology, Lakeside Capmus, Fine Arts Avenue, Ernakulam, Cochin. Contact madhukurup@hotmail.com

Nutrigenomics

Term	Defenition
Nutrigenomics	The study of genome wide effects of diet or components therof on the transcriptome, proteome and mebolome of cells, tissues or organisms at a specific moment in time
Genome	The entire complement of genetic material of an organism
Transcriptomics	The monitoring of the complete set of RNA transcripts produced by the genome at any given time
Proteomics	The examination of proteomes – the complete set of proteins in a cell or tissue – at a specific moment in time. Proteomics attempts to determine the role of specific proteins and how they interact with the molecules
Metabolomics	The identification and quantification of large sets of metabolites from cells or biological fluids and how these may change following physiological disturbance
Epigenomics	The detection and examination of DNA methylation patterns both spatially and temporally

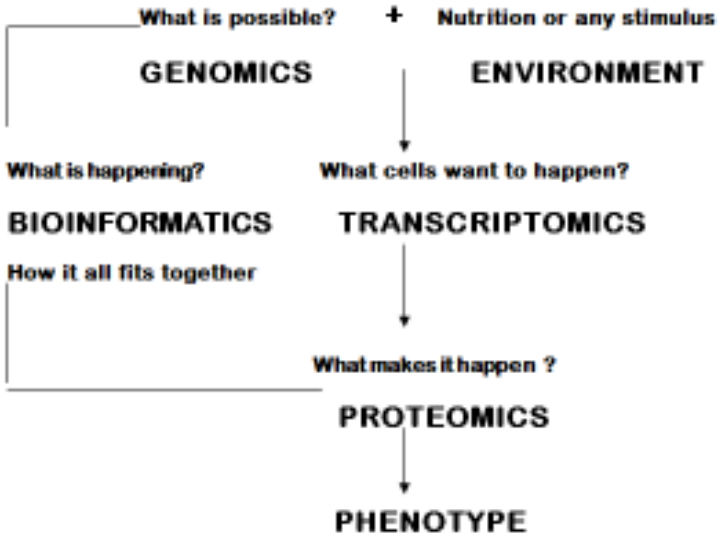
Nutrigenomics - possibilities in aquatic nutrition

Functional genomics refers to how the genome (in biology the **genome** of an organism is the whole hereditary information of an organism that is encoded in the DNA or, for some viruses, RNA. This includes both the genes and the non-coding sequences. The term was coined in 1920 by Hans Winkler, Professor of Botany at the University of Hamburg, Germany, as a portmanteau of the words **gene** and chromosome) of an organism regulates homeostasis (the property of and open system especially living organisms to regulate their internal environment to maintain a stable constant condition by means of multiple dynamic equilibrium adjustments controlled by interrelated regulation mechanisms) and responds to stimuli. In here we shall examine the application of functional genomics in nutrition research now known as ***nutrigenomics*** a term coined in 2002.

Influence of feed (nutrients) on the organism at a molecular level is the simplest explanation of nutrigenomics. Traditionally the response of a feed or nutrient is measured in terms of a standard set of parameters with strengths and weaknesses.

A simple example is measurement of growth as the change in body weight. It can be growth of muscle or growth of bones (in vertebrates). The role the genes have in these processes even though understood and appreciated gets sidelined and remains unaddressed. Thus research in food producing animals got limited to arriving at nutrient requirements, diet formulation, and the monitoring of performance. This approach has often involved the assessment of targeted metabolic pathways such as carbohydrate or lipid metabolism where biochemical and enzyme assays could also be used to monitor the effects of diet. With the development of cutting edge tools in molecular biology monitoring the affect of a nutrient at the molecular level is reality now. In reality, the scope of this field is significantly larger. The interaction of an organism with its diet (or nutrition source) is an intimate and complex physiologic affair that is typically based on multiple organ systems and, in higher eukaryotes, the endogenous microflora working in concert. The regulatory control mechanisms of these processes can be based at all levels from genetics and gene expression to the feedback of specific metabolites. Modern technology is providing a new opportunity to monitor the regulation of these processes on a systems-wide basis. Nutrition researchers are just beginning to utilize these tools to ask key scientific questions about diet and its effects on the organism using functional genomics. It has been known for some time that diet and specific nutrients can affect the function (expression) of genes. It has been well documented by many clinical studies that two humans fed an identical diet respond individually. The mechanism by which nutrients specifically regulate the expression of genes in vertebrates in general is poorly understood. The fundamental understanding of regulation gene expression in response to nutritional changes came from bacteria. However due to the complexity of the mechanisms in eukaryotic cells research in this area progressed slowly. One of the earliest reports of a micronutrient influencing gene expression in eukaryotes comes from zinc deficiency influencing gene expression of the hormones cholecystokinin, uroguanylin and the enzyme ubiquinone oxidoreductase. Nutrigenomics research aims at development of consensus responses to dietary stimuli so that anomalies can be studied further. This knowledge will provide information on how genes and nutrients interact and the effect of individual genetic differences on diet and nutrition. This research will be directly applicable to other species whose genome sequencing projects are underway including several agricultural and animal species including chicken, cattle, swine and fish. Developing within this genome era were technologies that were increasingly broad

in scope and were automated, high throughput, and data intensive. Many of these technologies also involved miniaturization of standard techniques to suit the new high throughput experimental designs. These technologies have significant implications for nutrition research and include aspects of genomics (polymorphism), functional genomics (gene expression), proteomics (protein expression), and bioinformatics (data storage and integrated data analysis). The organization of these data sources is shown in Figure 1 (below).



Functional genomics

Functional genomics aims at measuring the level of expression of all or a selected subset of genes based on the amount of mRNA (the trascriptome) present in the sample. The most powerful tool available today for this is the DNA array technology.

In a microarray comparison of gene expression from two experimental groups (A and B), RNA is extracted and reverse transcribed into cDNA. Either during this step by direct incorporation of fluorescently labeled nucleotides or indirectly by reaction of a modified nucleotide with the fluorescent label, cyanine 3 (Cy3) and cyanine 5 (Cy5) are used to differentiate each sample. Equimolar amounts of labeled cDNA are then mixed and hybridized to a single array of gene specific probes. These arrays are constructed by 'printing' as many as 80,000 'spots' onto a coated glass slide, each spot corresponding to a unique gene sequence that will hybridize or bind to the labeled cDNA. Post-hybridization the labeled array is scanned using a microscope and the amount of fluorescent signal from each of the dyes (Cy3/Cy5) measured at each spot. The relative signal present is representative of the ratio in gene expression between the two starting mRNA samples. Where gene expression is equivalent, equal signals in the Cy3 (green) and Cy5 (red) channels are observed producing a yellow spot. When one sample has significantly higher expression of a specific gene, the signal from that sample predominates, producing a more green or red spot.

The limitations of this technology are many. The main one being sensitivity in the data analysis. Statistically significant measurements are obtained only for most abundantly expressed genes. For smaller changes repeated measurements required are costly but attainable. Analytical precision

and standardization has been the main hindrance in microarray based functional genomics. Micro array gene expression database (MGED) founded in 1999 facilitates adoption of standards for DNA array experiment annotation and data representation. Standard experimental controls and data normalization methods can be found at www.mged.org. Therefore, high per analysis cost reduces the number of measurements per study and different platforms used; cDNA Vs oligonucleotide array, printed micro arrays Vs on chip synthesis etc., regular modifications to incorporate new genes and improved probe sequences complicates the scenario further. Off the shelf arrays or oligonucleotide collections available for custom spotting provides the first step in standardization of this technology. Because of these problems studies meeting rigid statistical requirements are relatively scarce.

Primarily, this technology should be thought of only a screening. In relationship between nutrition and health (unlike nutrition and disease) it is necessary to develop a biomarker which should reflect subtle changes in homeostasis; and efforts of the body to maintain homeostasis thorough cellular systems, organs and inter-organ interactions.

Integrated approach

By helping to understand the interaction between nutrients and molecules in an organism, the implementation of molecular biology and biochemistry in 'classical nutrition' research, followed by the technological revolution of the '-omics' technologies, will greatly affect nutritional sciences. Although the complexity of this proposed integration is exceeding the current bioinformatics tools and capacities, its implications for nutritional research can be enormous. Unlike biomedical interventions (drug therapy), nutrition is chronic, constantly varying, and composed of a very large amount of known and unknown bioactive compounds. Furthermore, nutrition touches the core of metabolism by supplying the vast majority of ingredients (both macro- and micronutrients) for maintaining metabolic homeostasis. This homeostasis stretches from gene expression to lipid metabolism and from signaling molecules to enzyme cofactors. Thus, nutrition by its nature needs to be studied in an integrated way (systems biology).

So far, most of the tools for this integration have been lacking, thus maintaining an unbridgeable gap between classical nutrition (studying physiology with a focus on biochemical pathways) and biomedical sciences (determination of disease-related molecular mechanisms). In applying systems biology to nutritional sciences, these paradoxical extremes are bridged and the complexity of the relationship between nutrition and health can be met by the complexity of the integrated approach. Many hurdles need to be taken, most of them in the field of bioinformatics, before this research area reaches maturity.

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16

Chemical and Biological Evaluation of Feeds

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Chemical evaluation of feeds and feed material

Due to the rapidity and ease the scheme of proximate analysis of feeds has remained unchanged. Gravimetric methods have been automated and expensive equipments are available today. The underlying principles remain the same.

Moisture and Dry matter (DM)

Proximate composition of feeds are generally expressed on a dry matter basis. For this pre-weighed samples are dried at constant temperature in a hot air oven at 100±5 ^{o}C and the loss of weight is the moisture content. Moisture in prepared feed must be monitored because levels over 8% favour the presence of insects, and over 14% there is the risk of contamination by fungi and bacteria. Since the moisture content is variable generally proximate composition tables are presented on a dry matter basis. If not they have to be converted to dry matter basis as shown below.

As fed nutrient content = % nutrient on DM basis x (100 – moisture content)
Suppose a feedstuff contains 35 % protein on DM basis and its moisture content is 9%
Then, as fed protein content = 35 x (100-9)/ 100 = 38.46
Similarly if a feed stuff contains 32% protein as fed with a moisture content of 20%
Then, % protein on DM basis = 32 x 100/ (100-20) = 40%

Organic matter (OM)

Dry matter fraction of the feed contains all nutrients which can be broadly divided into organic matter and ash. Organic matter contains all the macronutrients and vitamins and ash contains all minerals. Therefore Dry matter – total Ash = Organic matter (OM)

Crude protein

Protein nitrogen in the feed sample is converted into ammonium (NH_4^+) ions by digestion with Con. H_2SO_4 in the presence of a catalytic mixture. Ammonia is determined after steam distillation by capturing it in excess of boric acid (4%) and titrating it with standard acid (HCL or H_2SO_4)**.**

Digestion

$(C_6H_{10}O_{5)+}\ n\ H_2SO_4 \rightarrow 6nC + 5n\ H_2O$

$C + 2\ H_2SO_{4+}\ 4H_2 \rightarrow CO_{2+}\ 2H_2O + 2SO_2$

$4RCOONH_{2+2}\ H_2SO_4 + 4H_2 \rightarrow 2(NH_4)_2SO_4 + RCOOH$

Distillation

$2(NH_4)_2SO_4+4NaOH \rightarrow 2Na_2SO_4+4NH_3+4H_2O$

$4NH_3+4H_2O \rightarrow 4NH_4OH$

$NH_4OH+H_3BO_3 \rightarrow NH_4[B(OH)_4]$ (Ammonium borate)

Titration

$2NH_4[B(OH)_4] + H_2SO_4 \rightarrow (NH_4)_2SO_4+2H_3BO_3+2H_2O$

Reagents: Con. H_2SO_4, 40% solution of boric acid, Indicator 0.1% methyl red +o.2% Bromocresol green in 95% ethanol. Prepare 4% solution of boric acid and mix it with 10 ml indicator. 40 g boric acid in 1 L water and add 5-10 ml indicator solution. Catalyst mixture. $CuSO_4$ and KSO_4 in the ratio 1:9 or 1:10, Standard N/10 H_2SO_4 or HCL.

Procedure

Weight 1-2 grams of feed sample, oven dried and transfer into kjheldhal flask (800 ml). Add to it 2–10 grams of catalyst mixture, followed by 25ml of concentrated H_2SO_4 (increased the amount of concentrated H_2SO_4 by 10 ml for each 2 gram of sample for samples low in N). Kept it on the heater for digestion maintained at 100°C. Continue heating till frothing clears. If frothing comes up to the neck stop heating for some time. During digestion turn the flask every 30 minutes till the solution clears and all the carbon has been oxidized. Change in color indicates completion of digestion. Digestion is completed when the content gives transparent fluish white color. Then cool it at room temperature, transfer the content to 100-250ml volumetric flask as desired and dilute it to the volume required. Then take 5 – 10ml of the above digested samples in the micro kjeldhal distillation flask. Then take 10 ml of boric acid with mixed indicator in a beaker and place it under the condenser tube of the distillation flask, making sure that the condenser tube extends beneath the solution. Add about 20-25 ml of NaOH (40%) solution to the samples in the distillation flask. Start steam distillation, continue distillation until the volume in the receiver beaker is about 100-150 ml then stop distillation while stopping just remove the receiver beaker to avoid the possibility of back suction. Then titrate this excess boric acid us the N/10 H_2SO_4 and find out the CP by % by calculation. %CP=Nx6.25.

$$\%N = \frac{\text{Vol. of N/10 } H_2SO_4 \text{ used x dilution factor x 0.0014 x 100}}{\text{Vol. of aliqout taken x weight of sample digested}}$$

Ether Extract:

This represents the fraction consisting of substances such as glycerides of fatty free fatty acids, sterols, phospholipids, chlorophyll, alkaloid substances, volatile oils, waxes, resins etc. The fraction is extracted from moisture free samples with petroleum ether. Ether is continually volatilized condensed and allowed to pass through the samples, extracting ether soluble materials. After completion of the process ether is recovered, remaining crude fibre is dried and weighed.

Apparatus:

Soxhlet extraction apparatus, Extraction thimbles and hot plate.

Reagents:

Petroleum ether (BP 40°C - 60°C) if anhydrous ether is not available proceeds as follows. Wash commercial ether with 2 – 3 portions of water, add solid NaOH or KOH and let it stand still till most of the water is absorbed from the ether, decant into a dry bottle, add small pieces of carefully cleared metallic sodium and let it stand until hydrogen evolution causes. Keep the ether thus dehydrated over metallic sodium in loosely stoppered bottles (AOAC 1970 11th edition).

Procedure:

Weigh 2 grams of oven dried sample and transfer it into the ether extraction thimble. If the sample is not dried dry it by weight thimble overnight at 105°C. Place the thimble in the soxhlet extractor filled with a condenser and a pre-weighed flask containing anhydrous ether approximately 2 siphons, heat the flask on a hot plate with temperature regulated for about 8 hours. The extraction is complete when a drop of ether taken from the dripping of the extractor has no greasy stain on a filter paper.

After the extraction is complete, lower the heater and allows the thimble to drain empty. Remove the thimble weigh the sample from extraction apparatus and pour it back into the container. Dry the flask completely evaporates, dry the flask at 105°C in explosion proof oven for about 30 minutes. Cool in a desiccator to room temperature and weigh. Increase in weight of the flask is the estimate of ether extract. Alternately the ether extract can also be estimated by recording the loss in weight of moisture free sample following its extraction with anhydrous ether and drying it completely in the drying oven

Calculation:

$$\% \text{ EE} = \frac{\text{Weight of EE}}{\text{Weight of sample}} \times 100$$

Crude fibre:

Crude fibre is the loss on ignition of dry residue remaining after digestion of sample with 1.25% H_2SO_4 and 1.25% NaOH, under specific conditions crude fibre represents a fraction which is composed of substances making up the frame work of plants and include cellulose, hemi cellulose and lignin of the cell wall. This portion of the feed is supposed to be in digestible and hence the estimations based on treating the moisture and fat free sample with dilute acid and dilute alkali thus imitating the gastric and intestinal action in the process of digestion.

Apparatus:

Digestion apparatus consisting of individual controlled heaters and water condensers designed to maintain constant volume of the solution throughout digestion. Digestion containers 600 ml or 1 litre beaker, muslin cloth, aluminium dish or sintered crucible.

Procedure:

Take 2 grams of moisture free fat free sample in a beaker fitted with a round bottomed condenser. Add 200 ml of 1.25 % H_2SO_4 and boil half an hour on a hot plate, with a temperature regulator fitted

through a muslin cloth and make the residue acid free using a suction pump. If needed, make the residue acid free with hot water washings and suction if needed. Transfer the residue to the same beaker and add 200 ml of 1.25 % NaOH, again boil for half an hour, again filter through a muslin cloth and make the residue free of alkali… using hot water and then acetone. Transfer the residue to the weighed crucible filter the excess water and dry it at constant temperature at 100°C. Ignite the recidue in a muffle furnace at 600°C and cool the crucible in a dessicator. Crude fibre can be determined by loss of weight due to ignition.

Calculation:

$$\frac{\text{Lose of weight on ignition}}{\text{Weight of sample}} \times 100 = \text{CF\%....}$$

Ash:

Ash is the inorganic residue in the forage sample left after igniting the sample at about 60°C in a muffle furnace. Though in the feeds crude ash do not seem to have any direct nutritional use but it is to be determined in proximate analysis when NFE is determined by difference or when the figure of total organic matter is required.

Ash from plant materials is a poor index of inorganic constituents/nutrients the reason for this may be that the ash component of these materials is highly variable, not only in the total amount but in its component parts also. One of the specific examples is of silica which is of no nutritional value but never the less may be a factor in the total crude ash reported in the sample. Especially in forages, this figure may be much magnified because of adhering sand or soil etc.

Apparatus:

Muffle furnace, dessicator, porcelain crucibles and metal tongs.

Procedure:

Place new or clean crucible in a dessicator and cool to room temperature, weigh them immediately to prevent moisture absorption.

Take 5-10ml of oven dried finely powdered sample in a weighed crucible. Char the sample on a burner till the smoke is removed and then keep it in the muffle furnace at about 600°C for about 3 hours. Remove the crucibles, cool in a dessicator and weigh. Calculate the percentage of ash from the increase in weight of the crucible.

Calculation:

$$\text{Ash \%} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

Nitrogen free Extract (NFE)

Nitrogen free extract is determined by substracting the sum of percentage of moisture, EE, CF, CP and contents of a sample from 100. The value obtained is again in percent. However, it will be affected by the chemical errors in the analysis of all the five separate fractions.

This fraction comprises of starch and sugars of the sample plus some hemicellulose and much of lignin through originally considered as representing the highly indigestible carbohydrates of the feed, this assumption is incorrect as in many cases it has been shown that this fraction has lower digestibility in ruminanat animals than even the CF fraction of the same feed if results partially from extraction of indigestible hemi cellulose in fibre determination. Nevertheless it is still practically a useful index of non cellulose portion of the feed carbohydrates.

Calculation:

NFE % = 100 - (CP %+CF %+EE %+Ash %)

PROTEIN – Folin – Ciocalteu phenol method, Lowry *et al*. (1951)

Tissue preparation (ovary, liver & muscle)

1. 25 mg tissue + 1 ml 10% TCA
2. Homogenized & centrifuged at 3000 rpm for 15 mts.
3. The resultant supernatant (clear solution) for carbohydrate estimation & precipitate for protein estimation

Standard

4. 25 mg BSA standard crystals + 5ml 1N NaOH = Standard
5. 0.3 ml standard + 0.7ml 1N NaOH = ie. 1 ml total
6. This 1 ml + 5ml alkaline mixture (freshly prepared) + 0.5ml Folin-ciocalteu phenol reagent (2N stock solu.diluted1:1), (Conc. of protein in this dilution = 1.5 mg/1ml NaOH)
7. Adjust OD blank to 'zero'
8. OD = approximately 0.7 (660nm)

Estimation (ovary, liver & muscle)

9. Precipitate + 5ml of 1N NaOH (digestion)
10. 1ml from the above + 5ml alkaline mixture* (freshly prepared)
11. Mix well and keep it for 10minutes
12. Add 0.5 ml 1N Folin-phenol reagent
13. OD measured after 30 mts. at 660nm (blue colour)

*Alkaline mixture = 50ml of 2% Na_2CO_3 in 0.1N NaOH + 1ml of 0.5% $CuSO_4$. 5H2O in 1% sodium potassium tartarate

$$\text{Mg protein / 100 mg tissue (mg\%)} = \frac{\text{OD sample}}{\text{OD standard (0.7)}} \times 5 \times 1.5 \times 100 \times \frac{}{25}$$

Estimation (serum)

0.1 ml serum + 1ml 10% TCA (precipitate - on sample preparation, steps 2,3,9 -13 are the same)

$$\text{mg protein /100ml serum} = \frac{\frac{\text{OD sample}}{\text{OD standard (0.7)}} \times 5 \times 1.5 \times 100}{0.1}$$

CARBOHYDRATE ESTIMATION – DUBOIS *et al.* (1956) (phenol-sulphuric acid method)

Standard

1. 20 mg D.Glucose/100 ml saturated Benzoic acid = Standard
2. 0.2ml Standard + 0.8ml Satu. Ben. Acid + 1ml 5% phenol +5ml conc. H_2SO_4
3. Read at 490 nm after 30 mts.(OD approximately 0.47)

Estimation (ovary, liver & muscle)

0.2 ml supernatant (tissue sample) + 0.8 ml Ben. Acid + 1ml 5% phenol+5ml conc. H_2SO_4

Read at 490 nm after 30 mts. (orange- yellow colour)

$$\text{Mg carbohydrate/100mg tissue} = \frac{\frac{\text{OD sample}}{\text{OD Standard}} \times 0.04^* \times 100}{25}$$

* conc. of standard

Estimation (serum)

0.1 ml serum + 1ml 10% TCA (on sample preparation)

1ml clear solution (supernatant) + 1ml 5% phenol + 5 ml conc. H_2SO_4

$$\text{mg protein /100ml serum} = \frac{\frac{\text{OD sample}}{\text{OD standard}} \times 0.04 \times 100}{0.1}$$

CHOLESTEROL (Henly's method)

Standard

Stock = 1mg pure cholesterol in 1ml Glacial Acetic Acid (Std.)
Working solution = 1ml Stock + 24ml $FeCl_3$: CH_3COOH mixture (0.05% $FeCl_3.6H_2O$ in GAA, ie. 0.2 mg/ 5ml $FeCl_3$)
This 25 ml + 3ml conc. H_2SO_4 (reddish brown colour)
Read at 560 nm after 20 mts. (OD approximately 0.55)

Blank

5ml $FeCl_3$: CH_3COOH mixture + 3 ml conc. H_2SO_4

Estimation (ovary,liver & muscle)

25mg tissue + 10 ml $FeCl_3$: CH_3COOH mixture
Mix well – overnight in refrigerator
Centrifuge at 3000 rpm – 15 mts. – proteins precipitate
5 ml supernatant transferred to a glass stoppered centrifuge tube (keep it on ice)
Add 3 ml conc. H_2SO_4
read after 20 mts. at 560 nm

$$\text{Mg cholesterol / 100mg tissue} = \frac{\text{OD sample X 100 X 0.2}}{\text{OD standard X 12 .5}}$$

Estimation (serum)

0.1 ml + 10 ml 10 ml $FeCl_3$: CH_3COOH mixture

$$\text{mg cholesterol/100ml serum} = \frac{\text{OD sample X 100 X 0.2}}{\text{OD standard X 0.05}}$$

TOTAL LIPIDS – Sulphophosphovanillin method of Barnes & Blackstock (1973)

Standard

1. Stock solu. = 8mg cholesterol in 10ml 2:1 V/V $CHCl_3$:CH_3OH (equivalent to 10mg total lipid/10ml 2:1 $CHCl_3$:CH_3OH)
2. Working standard solu. = 0.2 ml stock + 0.3 ml, 2:1 V/V $CHCl_3$:CH_3OH (conc. of liquid = 0.2 mg/0.5ml)
3. This 0.5 ml working standard is mixed well
4. Dry in vacuum over silica gel in a desiccator
5. Add 0.5ml conc. H_2SO_4 (content 0.2mg/0.5 ml H_2SO_4)
6. Shake well and plug with non-absorbent cotton wool
7. Heat exactly for 10mts. at 100^0C in boiling water bath
8. Cool rapidly in running water to room temp.
9. Take 0.1 ml of acid digest in pipette &pour to clean test tube (0.4mg/0.1ml)
10. Add 2.5ml of vannilin reagent and mix well in a cyclo mixer (pink colour)
11. Read after 30-60mts. at 520nm (OD approximately 0.6)

Blank

0.5 ml, 2:1 $CHCl_3$:CH_3OH solution.
Read at 520 nm after 30-60 minutes.

Estimation (ovary, liver & muscle)

1. 10mg wet tissue in 1ml, V/V 2:1 $CHCl_3$:CH_3OH
2. Mix well using a glass rod in cold
3. Kept overnight in tightly stoppard test tubes in refrigerator for complete extraction
4. Mix well once again &centrifuge at 3000 rpm for 15 mts

5. Take 0.5 ml of extract into clean dry test tubes (supernatant)
6. Dry in vacuum over silica gel in a dessicator
7. Add 0.5 ml conc. H_2SO_4 & shake well (content 5.0 mg/0.5 ml H_2SO_4)
8. Plug the tubes with non-absorbent cotton wool
9. Heat for 10mts at 100^0C in boiling water bath
10. Cool rapidly to room temperature under running tap water
11. Take 0.1 ml of this acid digest into a clean dry test tube (1mg/0.1ml)
12. Add 2.5 ml phosphovanillin reagent & mix well in a cyclomixer (pinkish red colour)
13. Read after 30 mts. at 520 nm

Total lipid mg% (mg/100mg tissue) $= \frac{\text{OD sample}}{\text{OD Std.}} \times \frac{0.04}{0.1} \times \frac{0.1}{1} \times 100$ or $\frac{\text{OD sample}}{\text{OD Std.}} \times 4$

Estimation (serum)

0.1mlserum + 1ml 2:1 V/V $CHCl_3$:CH_3OH (steps 2-13 is the same)

Total lipid in mg/100ml serum $= \frac{\text{OD sample}}{\text{OD Std.}} \times \frac{0.04}{0.1} \times \frac{0.1}{0.01} \times 100$ or $\frac{\text{OD sample}}{\text{OD Std.}} \times 400$

TOTAL LIPIDS (Bligh & Dyer with Folch extraction)

1. 100mg tissue ground in a mortar & pestle with 10ml distilled water (in case of serum 0.5 or 1ml)
2. pulp transferred to 250ml conical flask or test tube
3. add 3ml $CHCl_3$:CH_3OH (2:1, V/V)
4. mix well & keep overnight in refrigerator in Stoppard tube in dark
5. add 0.5ml of 0.9 % NaCl, allow to separate into two phases in a separating funnel
6. lower $CHCl_3$ phase with lipids collected carefully
7. make upto 3ml with $CHCl_3$
8. pour into a clean dry preweighed beaker
9. carefully evaporate in a vacuum desiccator (covered with a dark paper)
10. weight difference of beaker = calculated as mg total lipids/100mg tissue and mg total lipids/ 100 ml serum

TOTAL CAROTENOIDS, OLSON (1979)

Standard

Crystalline â – carotene (E-Merck)

Estimation

1. Quickly remove 1g tissue from animals to screw capped vial + 2.5g anhydrous sodium sulphate

2. Gently mash the sample with a glass rod against the side of the vial until it is reasonably well-mixed (never grind it to a fine powder)
3. Add 5 ml CHCl3 over the caked residue
4. Seal the vial & place it at 0^0C overnight (8-12 hrs.)
5. $CHCl_3$ should form a clear 1-2 cm layer above the caked residue
6. Take 0.3 ml (extract) + 2.7 ml ethanol
7. read at 290, 350, 380, 450, 475, & 500 nm
8. plot the readings on a graph

Result

$$\text{mg carotenoids/gm tissue} = \frac{\text{absorption at 450 nm X dilution factor}}{0.25 \text{ X sample wt. (g)}}$$

0.25 = extinction coefficient

Biological Evaluation of feeds

Evaluation of feeds is a very important component in feed development. After the analysis of feed material, feed formulation and feed making or production are the steps which follow logically. After the feed production certain physical parameters like water stability (hydrostability) of the feeds can be assessed using standard procedures which are available in Goldbaltt et al., 1980, Jayaram and Shetty, 1981, Ruscoe et al. 2005. Bulk density is another physical parameter which can be looked into because the final product property and packaging designs are based on this physical property as shown in the Table below

Nutritional evaluation of aquatic feeds can be done in different ways depending upon the objectives of the study. First let us look at the indoor wet laboratory situation where the culture system is either static, recirculating or flow through. In all these systems water quality should not interfere with the response to nutritional actors. Therefore the basic water quality parameters specified for normal aquatic life has to be maintained. There is a wide choice of filteration, oxygenation and disinfection systems available. Water with abnormally high levels of hardness (in the case of freshwater), iron and alkalinity should not be used. Pesticides, heavy metals, ammonia and nitrite depress growth.

Final Product Bulk Density Correlation with Buoyancy Properties

Pellet buoyancy	**Sea water @ 20ºC (3% salinity)**
Fast sinking	> 640 g/l
Slow sinking	580-600 g/l
Neutral buoyancy	520-540 g/l
Floating	<480 g/l

Experimental organisms

1. Feeding history should be known otherwise probability of obtaining highly inconsistent data increases
2. Nutritional status should be standardized by a conditioning period which is longer for adult animals and short for juveniles
3. Preferably select organisms from a single brood if hatchery bred stock is used
4. Age specific requirements for a particular nutrient exists

Experimental diets

1. Since the role of nutrition has been overlooked for too long standard diets and standard experimental protocols are necessary
2. Standard reference diets (SRD) recommended by NRC for penaeid shrimp has been successfully used in many nutrient requirement studies
3. Experimental feeds should normally contain small number of ingredients
4. For requirement studies purified ingredients should be used
5. Ingredient nomenclature should conform with published guidelines of International Union of Nutritional Sciences

Experimental diets

1. Factors influencing mixing of dietary ingredients are particle size, density, static charge, hygroscopicity and adhesiveness
2. Manufacturing process should be directed towards minimizing loss of heat labile nutrients
3. Cold extrusion is preferable, cold air drying is preferred and freeze drying is the most conservative and safe method

Interactions

1. Alginates and phytic acid reduced bioavailability of divalent metal ions such as Ca
2. Some carbohydrates are found to reduce the uptake of proteins and amino acids
3. Alginates and guar gum reduces digestibility of protein in rainbow trout

Digestibility

1. Markers are used in aquatic nutrition studies
2. Procedure dependant errors should be minimized
3. Markers do not pass though the gut homogenously due to sequestering
4. If concentration of digestibility indicator in the faecal material is increasing, total collection of faeces would be the accurate procedure
5. Each ingredient under investigation should be added at levels of both 15 and 30%
6. Remaining 70-80% consists of the base formulation
7. All experimental animals should be fed a conditioning diet for at least one week
8. Faeces from experimental feeding must be collected from two independent feedings

9. Approximately one hour after feeding all uneaten food should be removed and faeces collected 3-5 h later
10. Entire faecal production for each day is then pooled for three consecutive days to compensate variability in faecal production (quantity generally) among days

Energy content

1. Digestible energy value (DE) of feedstuffs used are not determined
2. Gross energy (GE) values are determined by calorimetry
3. Apparent DE can be determined from the difference between GE and faecal energy
4. Apparent DE should be based upon calorific equivalents

Evaluation of nutrient requirements

1. The responses should minimally consist of weight gain, survival, tissue levels and related biochemical indices whenever possible
2. A minimum of 4 dietary levels or more should be used in any experiment devoted to determining the quantitative requirement
3. If less than four levels are tested then fitting the data to a descriptive response curve cannot be conducted to a high degree of confidence
4. Desired and actual levels of nutrients should be equivalent
5. Actual availability of the nutrient to the organism should be attempted

Experimental period

1. Tissue levels of nutrient under investigation need to be determined prior to the experiment
2. High tissue level of a specific nutrient at the initiation of an experiment can influence a response elicited
3. Deficiency signs may escape detection simply because duration of the experiment was insufficient to allow depletion
4. 300% increase in weigh has to be recorded in shrimp
5. Duration will thus depend on the nutrient under investigation

The next scenario is outdoor systems in which photoperiod cannot be controlled. These evaluations are more expensive than indoor evaluations because all the requirements more. Finally, on-farm evaluations are the ultimate where level of controls decrease but the results obtained would be realistic. On-farm research involves evaluations at multiple locations also.

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Anti-nutritional Factors and Toxins in Feeds

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To minimize of the cost of feed for livestock, poultry and aquaculture, nutritionist attempt to reduce the use of costlier ingredients and replace them with locally available cheaper feed ingredients. The continuous rise in the cost of fish meal and decline in the availability of quality fish meal, necessitate the search for alternative protein sources. The use of plant-derived materials such as legume seeds, different types of oilseed cake, leaf meals, leaf protein concentrates, and root tuber meals as fish feed ingredients is limited by the presence of a wide variety of anti-nutritional substances (ANF). Important among these are protease inhibitors, phytates, glucosinolates, saponins, tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters. Evidently, little unanimity exists between the results of different studies as to the specific effects of anti-nutrients, since most studies have been conducted using an ingredient rich in one particular factor and the observed effects have been attributed to this factor without considering other anti-nutrients present in the ingredient, or interactions between them. Tentatively, protease inhibitors, phytates, antigenic compounds, and alkaloids, at levels usually present in fish diets containing commercially available plant-derived protein sources, are unlikely to affect fish growth performance. In contrast, glucosinolates, saponins, tannins, soluble non-starch polysaccharides, gossypol, and phorbol esters, are more important from a practical point of view. The common processing techniques such as dry and wet heating, solvent extraction and enzyme treatment are effective in removing the deleterious effects of antinutrients from feed materials.

ANF in oilseed (Tacon, 1992)

OILSEEDS	
Groundnut *Arachís hypogaea*	1,2,5,6,8
Rapeseed *Brassíca campestris napus*	1,3,5,7
Indian mustard *Brassica juncea*	1,3,12
Sunflower Helíanthus annuus	1,6,7,17
Cottonseed *Gossypiurn* spp.	5,8,9,11,18
Linseed *Línum usítatissimum*	4,5,8,12,14
Sesame *Sesamum indicum*	5
Crambe *Crambe abyssíníca*	3
Soybean *Glycine max*	1,2,3,5,6,8,10,11,13,14,16,19

1. Protease inhibitors (T-trypsin, C-chymotrypsin, Pl.- plasmin,)
2. Phytohaemagglutinins,
3. Glucosinolates,
4. Cyanogens
5. Phytic acid,
6. Saponins,
7. Tannins,
8. Estrogenic factors,
9. Gossypoll
10. Flatulence factor,
11. Anti-vitamin E factor,
12. Anti-thiamine factor,
13. Anti-vitamin A factor,
14. Antipyridoxine factor,
15. Anti-vitamin D factor,
16. Anti-vitamin BI 2 factor,
17. Arginase inhibitor,
18. Cyclopropenoic acid,
19. Allergens

Despite the presence of anti-nutrients within raw pulses and oilseeds the prospects for the increased use of 'processed' oilseed meals and pulses as 'fishmeal' replacers for livestock, poultry and aqua feeds is very encouraging by using different feed processing techniques, including mechanical extraction, solvent extraction, aqueous extraction, heat processing, enzymatic processing. Although plant protein sources may be a cheaper and more sustainable source of dietary protein for use by the livestock / poultry /aquaculture industry, their success or not depends on the skills of the feed ingredient processor, the formulator, the manufacturer, and last but not least, the farmer.

Table 1 Common Anti-nutritional factors found in feed ingredients.

ANF GROUPS	ANF CLASSES	FEED INGREDIENT	HARMFUL EFFECTS	CORRECTIVE MEASURES
PROTEIN INHIBITORS	Protease inhibitors	Most legumes	1. Inhibits proteolytic enzymes 2. pancreatic hypertrophy 3. Increases dietary sulphur containing amino acids	Heat treatment
	Haemagglutinins (lectins)	legumes seed	Agglutination of vertebrate RBC	Moisture heat treatment
	Toxic amino acids	Mimosine in Soobabul	Enlargement of thyroid gland, low serum thyroxine and triiodothyronine	Drying and ensiling

	Food allergens	Soybean (globularproteins)	Gastrointestinal hypersensitivity reactions and digestive disturbances, including mucosalinflammation and luminal osmolality	
GLYCOSIDES	Goitrogens	cruciferous plants, rapeseed and mustard seed	Binds with iodine moietythyroid hyperplasia and reducedgrowth	Addition supplementation of iodineextraction with hotwater, dilute alkali, or organic solvent mixtures
	Cyanogens	cassava, chickpea, kidney bean, lima bean, hyacinth bean,field pea, pigeon pea, jack bean, and the oilseed linseed	Reduces oxygen carrying capacity,inactivate the cytochrome oxidase	Drying reduces the enzymes responsible for converting to the toxic compound (HCN)
	Saponins	kidney bean, lentil, pea, chickpea, alfalfa, soybean, groundnut, lupin and sunflower	Interference with the absorption of lipids, cholesterol, bile acids, andvitamins A and E. Lowers surface tensions and haemolyze redblood cells	extraction with hot water or ethanol
	Estrogens	wheat, rice, chick-pea, alfalfa,lupin, groundnut, linseed and soybean	produced elevated zinc in the liverand bones and increased the deposition of calcium, phosphorus and manganese inthe bones	
PHENOLS	Gossypol	Cottonseed	liver and kidney abnormalities (including thickening of glomerularbasement membrane, liver necrosis, and ceroid deposition in the liver, spleen and kidney) reduction in haematocrit, haemoglobin, and plasma protein levels	Supplementation of iron
	Tannins	Most widely distributed (Present in high amount in Salseed meal, mangoseed kernel, Mustard oil cake, red sorghum)	Bind with protein Depress cellulose activity	Soaking and cooking Anaerobic storage Addition of PEG or PVPAkalines, formaldehyde, acetone or hydrogen peroxide treatment
MISCELL-ANEOUS	Phytic acid	All vegetable foods / feeds	Phytate bound mineral-P, Zn, Fe, Mg, Cu are not available	Supplementation of phytase enzymes
	Oxalate	Leaves – level are high in matured leaves	Binds with calcium and make it insoluble	Addition supplementation of calcium
	Anti-vitamin A	Raw soybean	Destroys vitamin A	Heat treatment
	Anti-vitamin E	Kidney bean	Destroys vitamin E	Autoclaving treatment
	Anti-vitamin K	Sweet clover	Reduce prothrombin	

			in blood and affects blood clotting	
	Anti-vitamin D	Raw soybean	Rachitogenic activity	Autoclaving
	Anti-pyridoxine	Linseed	Destroys pyridoxine	Autoclaving
	Anti-niacin	Maize, Wheat bran		
	Anti-enzymes	1. cholinesteraseinhibitor solanine present in green potatoes, 2) amylase inhibitors present in wheat, oats and rye, and 3) an arginase inhibitorpresent in sunflower seeds, which is a derivative of chlorogenic acid .		
	Toxic fatty acids (Cyclopropene fatty acids)	Cottonseed	inhibition of fatty acid desaturase systems and consequent altered lipid metabolism, and histological abnormalities, including hepatocycte necrosis, unusual liver glycogen deposition, appearance of fibres in the hepatocytecytoplasm, proliferation of bile duct, and fibrosis	removed from oilseeds by lipidextraction
	Erucic acid	Rapeseed	Cardiac toxic and necrosis of muscle fibres.	

Mycotoxins:

Fungal contamination of foods and feeds is not a new phenomenon; it has been well established since biblical times. Mycotoxin is derived from the word myco meaning fungi. Mycotoxins are secondary metabolites produced by a wide range of fungi, mainly mold. There are more than one hundred species of molds that produce mycotoxins; the three most important species are *Aspergillus*, *Penicillium* and *Fusarium.* Nearly three hundred types of mycotoxins have been recorded so far. Livestock and birds consuming feeds contaminated with mycotoxins suffer from mycotoxicosis, though the problem has been known for several centuries, until the early sixties it was mainly thought in terms of toxicity to animals and birds and the consequent economic loss (Cheeke and Shull, 1985).

Classification of mycotoxins based on their biological effects:

Ingestion mycotoxin contaminated feed by livestock and poultry cause variable effects which ranges from drop in production, depression in growth rate to mortality, depending on the intensity of the contamination. The toxic effects of these mycotoxins vary according to the type of toxin. Based on the biological effects to the livestock and poultry the toxins have been classified below (adapted from Cheeke and Shull, 1985).

Hepatotoxins:-	Aflatoxin, Sporidesmin, RubratoxinB, Sterigmatocystin, Tricothecenes, Ochratoxin A, PhomposinA, Cyclopiazonic Acid
Nephrotoxins:-	OchratoxinA, Citrinin, Aflatoxin, Oosporin, Cyclopiazonic Acid, Sterigmatocystin
Neurotoxins:-	Trichothecenes (vomitoxin, satratoxin), Salframine, PenitremA, Ergot alkaloids, Ochratoxin A
Genitoxins:-	Zearalenone, Ergot alkaloids, Aflatoxins (mostly ruminants)
Dermitoxins:-	12,13-Epoxytrichothecenes (T2 toxin, nivalenol)
Carcinogens: -	Aflatoxin, Sterigmatocystin, Luteoskyrin, Patulin, and Penicilic acid, T2, OchratoxinA and Citrinin
Teratogenes:-	Aflatoxin. Ochratoxin
Immunosuppressents:-	Aflatoxin, T2, Ochratoxin, Citrinin, Oosporin
Hematological agents:-	Aflatoxin, Trichothecenes.

Mycotoxins Recorded in our laboratory:

In Namakkal with the layer population reaching nearly two crores the daily requirement for feed is nearly 2500tonnes and an equal quantity of feed is needed for the broilers in the adjacent Coimbatore district. Nearly 95% of the raw materials are procured from throughout India. Further, we receive samples for testing from almost all the states in the country, hence the results obtained in this laboratory can be taken as an indication of the toxin contamination of feed and ingredients in India.

Aflatoxin: Aflatoxin is the most prevalent of all the mycotoxins. A total of 31307 samples were screened in this laboratory from May 1994 to December 2001 for the contamination of aflatoxins, of which 58% were oilcakes, 27% were feed and 15% were cereals. The bulk of the positive samples were de-oiled groundnut cake (DOGNC) and maize.

Oil Cakes:

Out of the 18290 samples of oil cakes analyzed De-oiled groundnut cake DOGNC was the major component (7882). Invariably all the DOGNC samples (99.1%, Table 1) were found to be contaminated, in 48% of the samples the level was below 100 ppb, but in 46% samples it ranged between 100 and 500 ppb and 5% of the samples the level was above 500ppb. Of the 5717 samples of sunflower cake (SFOC) samples screened 17% were negative for AFTB1 in 63% of the samples the level of toxin ranged between 1 and 20 ppb , while in 17% of the samples the levels of the toxin ranged from 21 to 100ppb and only in 2% of the samples the levels were above 100ppb. Soybean meal (SBM) was the third predominant oil cake subjected for aflatoxin analysis, 84.3% of the meal screened were negative for aflatoxins. Further, 15.2% recorded below 20ppb of AFTB1, and in only 0.5% of the samples the level exceeded 20ppb. Reviewing the influence of season on the toxin contamination of the samples, it was found that even during non rainy season nearly 99% of DOGNC, 70% of SFOC and 36% of SBM were positive for the presence of AFTB1.

Cereals: Maize was the predominant cereal screened (85%), nearly 56% (Table 3) of the maize samples were negative and in 22% the level of AFTB1 was below 20ppb. In 12% of the maize samples the level ranged between 21 and 100ppb, 4% of the samples the level was between 101 and 500 ppb, in 1% of the samples the level was above 500ppb, studying the influence of the season it was found that nearly 35% of the maize samples tested in the non-rainy season were found to be contaminated with AFTB1. Among the other cereals tested 65% of jowar and 39% of bajra were positive for the presence of AFTB1. In Jowar the majority (55%) contained below 20ppb and only 10% contained levels above 20ppb of AFTB1, 8.2% contained between 21 and 100ppb and only 1.8% contained above 100ppb of AFTB1. In bajra only 1.5% contained levels above 20ppb of AFTB1.

Mycotoxins other than aflatoxins

A 2-Dimensional thin layer chromatographic method was modified and standardized in this laboratory from an earlier procedure (Tapia, 1985, Chandrasekaran,et al. 2001). Quantification of ochratoxin A (OA), citrinin, T2-toxin, zearalenone, sterigmatocystin, aflatoxins was done. A total of 4131 samples were received for analysis between April 1997 and December 2001. While 2407 samples were oil cakes, 1405 samples were feed and 319 were cereals. OA, citrinin and aflatoxin were the mycotoxins predominantly observed in the feed ingredients and feeds.

Ochratoxin: Seventy eight per cent of SFOC samples were found to be contaminated with OA. While 55.3% samples contained up to 100 ppb, 22.8% contained levels higher than 100ppb and 63.6% samples of SFOC were co-contaminated with AFTB1. Of the 1405 feed samples, 138 samples were positive for OA. In 63 samples, both OA and AFTB1 were found to co-occur up to 20ppb level and in 48 samples they co-occur at higher levels (21 to 200 ppb). Very little contamination was observed in SBM and rapeseed meal.

Citrinin: Among the cereals, maize was predominantly contaminated both with citrinin and AFTB1. While 45 maize samples out of 229 were positive for citrinin, 22 contained AFTB1 also. Citrinin was present in 171 feed samples and all of them were contaminated with AFTB1. Among these, 53 samples contained above 50ppb of citrinin and 21-50ppb of AFTB1, 5 bajra samples were contaminated with citrinin (100 – 200ppb) out of 11 samples received for analysis. Only 6 SBM samples were found to have citrinin and all the samples were negative for AFTB1.

Zearalenone: Out of 79 jowar samples received, 37 jowar samples contained zearalenone ranging from 1 to 2 ppm level. Out of 11 bajra samples, 8 were positive for zearalenone (0.5 to 1ppm).

Emerging new toxins: Apart from the above mentioned toxins the presence of cyclo piazonic acid, oosporin, patulin, deoxynivalenol (DON), diactoxyscirpernol (DAS), fumonisins and sterigmatocystin have also been recorded occasionally. Further, presence of toxins different from those mentioned above have been suspected due to the fact that mortality has been recorded in birds showing hepatitis and nephritis even though the feed was free from the above mentioned mycotoxins. Several new fluorescent spots are encountered in the analysis of multimycotoxins and some are consistently seen in the feeds obtained from farms with a history of feed related problems, indicating the possibility of new toxins.

The results of the various screening process indicates that the mycotoxin contamination of the feed ingredients has reached an alarming level, where even in dry seasons the feed/ingredients are not free from mycotoxins. The seriousness of the situation has not been well understood either by the agricultural farmers or the raw material suppliers or the poultry farmers.

Conclusions:

1. The use of vegetable protein supplements in livestock feeding is on the increase.
2. The ANFs in the ingredients are being inactivated before use.
3. Quality checks which are available to assay the inactivation are available have to be employed to ascertain that the ANFs are properly inactivated
4. Mycotoxins have become the major problem as the ANFs are natural constituents and the effects can be anticipated. The problem of mycotoxicosis is increasing day by day for which a coordinated effort is needed

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Formulation of compounded feeds

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Feed formulation is a technique to be learnt and refined by experience. To date numerous descriptions have appeared in nutrition textbooks. The exercise, taught over and over again is difficult to comprehend especially by the beginners. The purpose of this article is to describe and share some of this authors experience in teaching and learning feed formulation; mainly for aquatic animal research. Previous descriptions on the subject can be had from New, (1987) and Houser and Akiyama (1997) among which, New (1987) describes the subject with a lay man in mind. The emphasis here is to describe the technique in the simplest way by which it is presumed that, beginners in the subject of aquatic animal nutrition shall be benefited.

Let us start by organising the data or information required for the exercise. The checklist can be as follows.

1. List of feed ingredients available for the formulation and their proximate chemical composition and cost.
2. Specifications of the feed to be formulated in terms of protein, lipid, fibre, soluble carbohydrates (NFE or nitrogen free extract) vitamins and minerals etc.
3. Safe levels and suitability of the levels of incorporation of certain materials for the animals to be fed (Table 4)

The primary step in formulating a feed is to organise this information in a manner in which it is convenient to the person doing the formulation. Readymade Tables of the aforementioned information are available. These Tables may be too huge to be referred frequently (e.g. Appendices IV, IV Part A and IV Part B in New 1987). Hence, it is advisable to construct a Table to suit ones own convenience as follows. The Table1, shown below, includes a list of feed ingredients commonly used in and around Cochin, India and their retail cost. Tables 2 and 3 presented are also relevant in the Indian context, which may or may not be representative; however they are useful in the absence of analytical data.

The best bank of this information would be ones own analysis of the material if he/she has access to analytical facility. If not, samples can be got analysed from approved laboratories in the government sector or private sector. Government laboratories providing this service are (1) Nutrition laboratory of PNP Divison at CMFRI, Cochin – Write to The Director, CMFRI, P.O.Box 1603, Tatapuram (P.O.), Ernakulam, Cochin-682 014, Kerala (2) Animal feed quality control laboratory (AFQCL), TANUVAS, Namkkal, Tamilnadu. – Write to: Dr.D.Chandrasekaran, Prof. and Head, AFQCL, Veterinary College and Research Institute, Namakkal, Tamilnadu.

Table 1. List of feed ingredients commonly used in and around Cochin, India and their retail cost

Ingredient	DM[1]	CP[2]	EE[3]	CF[4]	NFE[5]	Ash	AIA[6]	Cost [Rs./kg]
Fish meal	95.16	68.50	8.49		0.61	17.56	2.71	25.00*
Shrimp waste meal	92.51	67.45	3.29		5.27	16.50	4.39	20.00*
Clam meal	94.37	59.79	13.01		15.10	6.47	1.94	20.00
GNOC	94.55	43.75	8.13	5.49	30.10	7.08	2.36	9.20
Tapioca flour	87.18	2.82	0.29	1.79	80.26	2.02	0.10	7.00

[1]Dry matter, [2]Crude Protein, [3]Ether Extract, [4]Crude Fibre, [5]Nitrogen-Free Extractives and
[6]Acid Insoluble Ash in %
*Costs indicated are for dried unsalted anchovies and *acetes* spp. respectively. It may go up to Rs.70 and Rs.120 depending upon availability.

Table 2. **Proximate composition of selected feed ingredients of plant origin in India***

Ingredient	DM(%)	CP(%)	EE (%)	CF(%)	NFE(%)	Ash (%)
Rice polish	87.4	14.5	17.3	7.5	n.a.	n.a.
Rice polish	90	12.2	16.0	9.0	46.8	6.0
Rice polish	91.6	11.4	15.3	11.0	41.0	12.9
Rice, broken	90	12.0	4.2	5.3	65.4	3.1
Rice bran	89.9	12.6	11.3	19.3	36.5	10.2
Rice bran	92.2	7.8	6.1	14.4	43.4	20.5
Rice bran	91.6	2.9	5.0	18.0	38.4	27.3
Rice bran	91.3	9.4	4.7	13.5	32.3	31.4
Defatted rice bran	92.8	12.1	1.3	15.2	40.4	23.8
Wheat bran	87.7	15.8	4.3	8.7	n.a.	n.a.
Wheat bran	90	13.5	2.6	12.2	58.7	3.0
Wheat bran	87	8.2	6.6	33.5	34.5	4.2
Wheat bran	90.7	12.6	7.5	11.9	54.5	4.2
Wheat, broken	91	11.5	1.9	4.0	73.4	0.2
Wheat flour	87.4	14.5	3.7	2.7	64.2	2.3
Groundnut cake	92.2	28.6	13.8	7.5	28.9	13.4
Groundnut cake	94	37.7	11.5	13.2	24.3	7.3
Groundnut cake	90	42.0	7.3	13.0	25.2	2.5
Groundnut cake	91.7	46.6	7.7	6.5	23.2	7.7
Groundnut cake	92.9	35.8	8.5	8.2	29.9	10.5
Groundnut extr.	93	48.0	2.0	11.2	29.1	2.7
Sunflower extr.	92	31.0	2.1	18.4	39.0	1.5
Sunflower extr.	89.8	30.1	2.9	24.7	25.6	6.5
Palm kernel cake	91.1	12.2	4.9	25.6	45.8	2.6
Soybean meal	88.2	46.3	1.3	5.0	n.a.	n.a.
Soybean meal	97	58.6	1.4	0.4	31.3	5.3

Soybean meal	90	46.0	0.9	7.3	35.2	0.6
Soy sauce waste	88	13.5	8.2	5.8	55.2	5.3
Rapeseed cake	89	35.9	0.9	13.2	32.1	6.9
Salseed cake	91.4	8.2	2.9	1.7	68.4	10.2
Sesame cake	91.7	41.9	9.2	6.2	19.6	14.8
Sesame cake	90	29.0	12.9	18.3	19.8	10.0
Sesame cake	90	42.7	6.9	5.7	21.8	12.9
Mustard cake	91.5	30.8	9.3	6.2	34.9	10.3
Mustard cake	90.8	23.6	9.6	6.3	40.9	10.4
Cotton seed cake	93	37.0	6.7	13.0	35.3	1.0
Cotton seed cake	91.8	42.7	1.0	12.6	27.3	8.2
Gingely cake	91	34.0	7.8	7.9	38.2	3.1
Gingely extr.	93	40.0	2.0	9.7	38.4	2.9
Niger extr.	93	35.0	2.0	19.0	33.5	3.5
Copra cake	88	22.0	6.5	12.2	42.1	5.2
Copra cake	91.6	20.3	11.4	16.2	37.5	6.2
Copra cake	n.a.	22.0	6.0	12.0	n.a.	2.1
Tobacco seed extr.	92.3	30.6	0.3	-	47.7	13.7
Maize meal	86.5	9.5	4.0	4.0	67.5	1.5
Maize	89.6	4.6	7.8	3.5	72.7	1.0
Sorghum	90	9.0	2.8	3.0	75.1	0.1
Spirulina	91.3	50.5	1.0	2.1	26.7	11.0
Tapioca flour	88.5	3.1	2.3	2.0	78.8	2.3
Tapioca flour	92	1.8	1.3	1.8	86.9	0.2
Coffee pulp	87.7	14.0	1.2	20.8	43.5	8.2
Colocasia meal	94.2	24.6	4.5	8.2	47.0	9.9
Eichornia meal	96.7	19.5	2.3	18.3	47.3	9.3
Pistia meal	95.1	19.5	1.3	11.7	37.0	25.6
Leucaena meal	88.2	33.1	4.7	9.0	34.2	7.2
Mulberry leaf, dry	91.1	27.7	2.4	11.5	41.4	8.1
Salvinia meal	97.4	16.2	1.1	18.5	39.6	22.0

Table 3. Proximate composition of selected feed ingredients of animal origin in India*

Ingredient	DM(%)	CP(%)	EE(%)	CF(%)	NFE (%)	Ash (%)
Fish meal	91.4	64.4	7.5	0.3	-	19.2
Fish meal	90.5	53.6	5.4	3.1	7.5	20.9
Fish meal	90.8	56.1	2.5	17.8	11.9	2.5
Fish meal	85.4	37.2	2.7	22.7	16.1	6.7
Fish meal	89.4	14.4	8.7	30.0	19.8	16.5

Fish meal	86	47.8	10.3	2.6	7.0	18.3
Fish meal	90	72.0	10.0	0.5	n.a.	n.a.
Fish meal	91	50.0	7.0	1.0	29.0	4.0
Fish meal	90	45.0	8.0	1.2	29.8	6.0
Shrimp waste	90	28.0	2.7	12.5	n.a.	n.a.
Shrimp waste	91	22.5	3.6	35.3	11.0	18.6
Shrimp waste	96.4	34.2	6.7	12.2	15.4	27.9
Shrimp waste	84.4	28.3	1.1	7.1	16.3	31.6
Squilla meal	85.9	46.0	2.6	13.5	5.8	18.0
Squid meal	92	75.0	6.5	4.0	n.a.	n.a.
Clam meal	93	52.0	11.6	5.5	n.a.	n.a.
Clam meal	91.9	50.7	8.9	3.9	22.0	6.4
Silkworm pupae	92.9	43.9	25.7	4.2	3.3	15.8
Defatted silkworm pupae	91.9	68.0	2.6	1.3	12.8	7.2
Blood meal	90	65.3	0.5	n.a.	n.a.	n.a.
Blood meal	87.1	76.6	1.1	1.0	4.6	3.8
Meat meal	92	50.0	4.4	6.8	25.8	5.0
Meat meal	90	71.2	13.3	0.7	n.a.	n.a.
Liver meal	93	65.0	3.4	1.2	21.0	2.4
Earthworm meal	95	51.7	3.4	12.8	14.6	12.5

*Source: Modified from Nandeesha (1993)

A fair understanding of the Table containing the compositional information is a definite prerequisite before proceeding any further. The heads in which the nutrient composition is quantified can be further described as,

1. DM Dry matter-indicates the total nutrient content excluding water.
2. CP Crude protein-indicates the total protein content inclusive of non-protein nitrogenous substances also.
3. EE Ether extract or crude fat indicates the total fat content, which may include other fat-soluble substances.
4. CF Crude fibre-indicates the cellulose content or the indigestible (for simple stomached) carbohydrate content.
5. NFE Nitrogen free extract or the total soluble/digestible carbohydrate content is contained in this fraction.
6. Ash Indicates the total content of mineral salts
7. AIA Acid insoluble ash indicates the indigestible impurities/adulterants in feed material which are mainly sand and silica

With the extensive use of computers it is desirable that if this information is fed into a computer in a Microsoft Excel spread sheet available in the MS Office; it would be helpful in avoiding repetitive calculations which my be required if a simple calculator is used.

Now, let us get ahead with the computation *per se*. Pearson square method used for blending two ingredients or products to attain a specific nutrient concentration until the advent of computers is not explained here. This is because of the basic drawback of this method to blend only two ingredients at a time. When more than two ingredients need to be blended multiple Pearson squares have to be used or grouping of ingredients based on criteria like 'protein rich' and 'energy rich' has to be adopted. Simple ways of doing it is described by Ali (1987), New (1987) and a detailed review is available in Church and Nipper (1984).

The method intended to be described here in detail is commonly known as the hit and trial method which is not only convenient but also clears the concept of blending of feed ingredients/ products in definite proportions and the resultant changes it brings about in the blend.

After organising the data bank to be used for the formulation the next step is to define the requirement or composition of the feed to be made.

Taking marine shrimp as an example, let us assume the requirement in terms of nutrients for animals grown in culture ponds at a stocking density of less than 5 animals per square meter as protein ≥35%, fat not more than 7%, fibre < 3%, soluble carbohydrates (NFE) 20-40% ash ≤ 15%, AIA ≤ 3% containing gross energy (GE) of >400 kcal/100g and digestible energy (DE) of <300 kilocalories/100g. GE and DE are explained later. Let us also fix that our feed mix should not cost more than Rs.25/-.

Table 4. **Proximate composition of the feed ingredients, approximate cost (Rs/kg)**

Ingredients	**Cost Rs./kg**	**DM**	**CP**	**EE**	**CF**	**NFE**	**Ash**	**AIA**
Fish meal	25.000	95.160	68.50	8.49	0.00	0.61	17.56	2.71
Shrimp waste meal	20.000	92.510	67.45	3.29	0.00	5.27	16.50	4.39
Clam meal	20.000	94.370	59.79	13.01	0.00	15.10	6.47	1.94
GNOC	9.200	94.550	43.75	8.13	5.49	30.10	7.08	2.36
Tapioca flr	7.000	87.180	2.82	0.29	1.79	80.26	2.02	0.10
Oil	50.000							
Cholesterol	100.000							
Lecithin	100.000							
Vitamin mix.	75.000							
Mineral mix.	30.000							
	% inclusion	**Cost of Incl. [Rs./kg]**	**CP**	**EE**	**CF**	**NFE**	**Ash**	**AIA**
Fish meal	0.200	5.000	13.70	1.70	0.00	0.12	3.51	0.54
Shrimp meal	0.200	4.000	13.49	0.66	0.00	1.05	3.30	0.88
Clam meal	0.200	4.000	11.96	2.60	0.00	3.02	1.29	0.39
GNOC	0.200	1.840	8.75	1.63	1.10	6.02	1.42	0.47
Tapioca flr.	0.090	0.630	0.25	0.03	0.16	7.22	0.18	0.01
Oil	0.050	2.500		5.00				

Cholesterol	0.005	0.500						
Lecithin	0.005	0.500						
Vitamin mix.	0.030	2.250						
Mineral mix.	0.020	0.600						
Total cost/nutrients	1.000	21.820	48.15	11.61	1.26	17.44	9.70	2.29
GE kcal/100g			264.83	105.65		71.50		**441.99**
DE kcal/100g			200.59	41.76		33.05		**275.41**
	% inclusion	**Cost of Incl. [Rs./kg]**	**CP**	**EE**	**CF**	**NFE**	**Ash**	**AIA**
Fish meal	0.150	3.750	10.28	1.27	0.00	0.09	2.63	0.41
Shrimp meal	0.150	3.000	10.12	0.49	0.00	0.79	2.48	0.66
Clam meal	0.150	3.000	8.97	1.95	0.00	2.27	0.97	0.29
GNOC	0.200	1.840	8.75	1.63	1.10	6.02	1.42	0.47
Tapioca flr.	0.240	1.680	0.68	0.07	0.43	19.26	0.48	0.02
Oil	0.050	2.500		5.00				
Cholesterol	0.005	0.500						
Lecithin	0.005	0.500						
Vitamin mix.	0.030	2.250						
Mineral mix.	0.020	0.600						
Total cost/nutrients	1.000	19.620	38.79	10.41	1.53	28.43	7.98	1.85
GE kcal/100g			213.33	94.77		116.56		**424.66**
DE kcal/100g			160.61	83.31		53.99		**297.90**
	% inclusion	**Cost of Incl. [Rs./kg]**	**CP**	**EE**	**CF**	**NFE**	**Ash**	**AIA**
Fish meal	0.100	2.500	6.85	0.85	0.00	0.06	1.76	0.27
Shrimp meal	0.100	2.000	6.75	0.33	0.00	0.53	1.65	0.44
Clam meal	0.100	2.000	5.98	1.30	0.00	1.51	0.65	0.19
GNOC	0.300	2.760	13.13	2.44	1.65	9.03	2.12	0.71
Tapioca flr.	0.290	2.030	0.82	0.08	3.63	23.28	0.59	0.03
Oil	0.050	2.500						
Cholesterol	0.005	0.500						
Lecithin	0.005	0.500						
Vitamin mix.	0.030	2.250						
Mineral mix.	0.020	0.600						
Total - cost/nutrients	1.000	17.640	33.52	10.00	5.28	34.40	6.76	1.64
GE kcal/100g			184.34	91.02		141.05		**416.42**
DE kcal/100g			136.17	80.02		61.87		**278.06**
Cholesterol	100.000							

In the spread sheet where the information regarding composition and cost if fed another portion of the spread sheet involving the required number of rows and columns can be used for computation as shown in Table 4 above.

The trial to blend the ingredients available can be started by assigning the number of parts of a particular ingredient or it can be expressed as percentage inclusion. Knowledge of the safe levels of incorporation of a particular ingredient, cost, gelling properties, pelletability, bulk density etc., aids in assigning the number of parts of a particular ingredient. Let us start with assigning 20 parts of fishmeal, 20 parts of shrimp meal, 20 parts of clam meal, 20 parts of GNOC and 5 parts of oil which totals to 85 parts per 100. These values are entered in the Excel spread sheet by taking 100% as 1. Hence, part per100 is entered as a decimal value. Thus, 20 part per 100 = 0.2, 5 parts per 100=0.05, 3 parts per 100 = 0.03 and 2 parts per 100 = 0.02 and 0.5 parts per 100 = 0.005. Out of the remaining 15 parts 3 parts of mineral mixture and 2 parts of vitamin mixture may be assigned. Since the dietary essentiality of cholesterol and phospholipids especially lecithin is proven in marine shrimps they may be provided at levels of 0.5% each making the total mix 91 parts per 100. The remaining nine parts can be either filled up with inert filler, filler with binder or an energy source like tapioca flour.

Now let us examine the nutrient profile (in %) of the mix by finding out the contribution of 20% fishmeal to the formulated feed as follows.

Crude protein (CP) available from 20% fish meal (FM) = CP in FM x 20/100
= 68.5 x 20/100
= 13.70

Ether extract (EE) or crude fat available from FM = EE in FM x 20/100
= 8.49 x 20/100
= 1.70

Crude fiber (CF) available from FM = 0

Nitrogen free extract (NFE) or soluble carbohydrates available from FM = NFE in FM x 20/100
= 0.61 x 20/100
= 0.12

Ash (total minerals) available from FM = Ash in FM x 20/100
= 17.56 x 20/100
= 3.51

Acid insoluble ash (AIA) or inert material contributed by FM = AIA in FM x 20/100
= 2.71 x 20/100
= 0.54

Similarly gross energy (GE) is the quality of the feed, which indicates the amount of heat, which means energy, liberated when the material is burnt completely and digestible energy (DE) is that portion of the energy available to the animal to which it is fed. Technically it is the GE – energy lost

as faeces or faecal energy. Standard values are in use for converting protein, fat and carbohydrate content. The values recommended by aquaculture coordination and development programme (ADCP, 1983) are used here which are as follows.

Nutrient	**Gross energy (GE)kcal/g**	**Digestible energy (DE) kcal/g**	
Protein	5.5	Animal protein	4.25
		Vegetable protein	3.8
Fat	9.1		8.0
Carbohydrate	4.1	Animal carbohydrate	3.0
		Vegetable carbohydrate	2.0

In the Excel spread sheet the formulae may be entered in the cell right below CP as = A1*0.2, where A1 is the cell number which contains the % CP of fishmeal. If we are working with a calculator and a work sheet these values have to be calculated as shown above and entered in the respective cells. However, in and Excel worksheet this formula if copied using the **+** sign appearing on the right hand bottom corner of the cell from which we intend to copy the formula, these calculations can be completed in no time. Similarly, for all ingredients the respective formulae may be copied and the work sheet may be filled.

The total GE and DE content of the blend are calculated by multiplying the total CP, EE and NFE contents with these values. That is, total GE form protein in the first formulation will be 48.15x5.5+11.61x9.1+71.50x4.5 = 441.99. Similar calculations with appropriate energy values for fat and carbohydrate (NFE) given above, gives us the total GE and DE values of the blend. Care should be taken to enter multiplication formulae which begin with an = sign followed by the cell to be multiplied followed by the * and then the energy value.

The last step is to total the individual nutrient components in the bottom row for which the formula is = sum (cell No.: cell No.) where the first cell number is the cell from which the totalling should start and the second cell number is the cell from which the totalling should end. Thus, we get the final composition and cost of the feed mix, which may or may not comply with the requirements we had defined.

After completion of this we have at hand a base form, which we work, through the innumerable permutations and combinations to achieve the required nutrient specifications and cost. If a calculator and work sheet are the tools available we have to use several worksheets to manipulate the computations. In the Excel worksheet, once a formulation is made, just by altering i.e., increasing or decreasing the part/100 figures all the corresponding figures change automatically. If several blends have to be made by modifying the inclusion rates, Tables containing nutritional and cost information of the first blend can be retained as it is and a copy of the same can be used right below as shown in the Table 4. To copy the information in an Excel spread sheet containing formulae 'paste link' button has to be clicked after selecting the area to be copied from the drop-down 'edit menu'. Simple copy and paste menu if used, a copy of the data will show erroneous calculated values because destination cells change while copying.

In conclusion, a brief description of using Excel, which is used in feed formulation here, is appended here for beginners. Once Excel is open, we can see a grid of rows and columns. A

number labels each row, and a letter of the alphabet labels each column. The limit within a worksheet is 256 columns and 16,384 rows. Note that there are tabs at the bottom of the screen that indicate the sheet tabs (Sheet 1, Sheet 2 and so on); the programme will open with 16 worksheets available within the overall workbook.

The programme opens with the highlight on cell A1 (Column A, Row 1) in the upper right-hand corner of the sheet. As a means of learning let us use some basic functions of the programme.

Enter number 10 into cell A1 and the number 20 into cell B1. Move the cursor back to cell A1, hold down the shift key, and use the arrow keys to move the cursor over to cell B1. Both the cells will now be highlighted. From the drop-down edit menu, click on Copy. Use arrow keys or the mouse to move to cell A2. Click on the Edit menu again, then click Paste. The contents of cells A1 and B1 have now been copied to cells A2 and B2.

An alternative method of copying is to use the mouse and a built-in copying feature of Excel. First, use the Delete key to clear the contents of cells A2 and B2. Then highlight cells A1 and B1 as we did above. Now, note the black square in the lower right-hand corner of the highlighted area. When we place the mouse cursor directly over this black square, it will change to a thick white cross to narrow back cross, hold down the mouse button and drag the narrow black cross down to cell B2. When we release the mouse button, the contents of cells A! and B! will be copied to cells A2 and B2. Another convenient feature of a spreadsheet is that it allows the user to input formulae into cells. Formulae can use data in various cells to perform calculations, and formulae can be easily copied to other cells using copying methods described above. To illustrate this point let us add the contents of cells A1 and B2. Make sure these cells still contain 10 and 20, respectively. Use arrow keys or the mouse to move to cell C1. In Excel, formulae always start with '=A1+B1' into cell C1. Once we input this formula and, over the cursor (or hit the Enter key), Excel will calculate the formula. If desired, the formula can be copied down the column as described in the previous example on copying.

In addition to copying formulae down a column, they can be copied across a row. Note, however, when formulae are copied across a row that the cell references change. To illustrate this, copy the formula in cell C1 to cell D1. Now look at the cell pointer and the formula bar, which are located just above the column headings. The formula in cell D1 shown on the formula bar is '=B1+C1'; hence, the cell reference shifted one column to the right. In situations where you desire that a formula refers to a specific cell or a range of cells, the formula can be written with the '$' modifier, which points to a specific cell, row, or column depending on how it is used. For example, if we wanted to use the value in cell A1 as the divisor for a series of numbers in column B, you would input the equation into cell C1 as '=B1/A1'. Then when we copy this equation down column C every value in column B would be divided by the value in cell A1. With this brief introduction, and with the use of Help menu in the programme or with the assistance of someone well versed in Excel spread sheet operations a construction of a diet formulation spread sheet should be easy.

Linear programming software is inbuilt in Excel and can be accessed though Solver in the Tools menu is very popular among by professional animal nutritionists.

Suggested further reading:

Ali, S. A. (1987) Feed formulation methods. In CMFRI Special Publication No.8. Manual of Research methods for Fish and Shellfish Nutrition pp. 95-98

Church, D.C. and Nipper, W. A. (1984) Ration formulation. Pages 195-227 In D.C.Church editor, Livestock fedds and feeding, 2 edition O.B.Brooks Inc., Conrnvalis, Oregon, USA

Houser, R.H. and Akiyama, D.M. (1997) Feed formulation principles. In Crustacean Nutrition, Advances in World Aquaculture, Vol.6 Eds. D'Abramo, L.R., Conklin, D. E. and Akiyama, D. M. World Aquaculture Society, pp.493-519

NANDEESHA, M.C. 1993. Aquafeeds and feeding strategies in India, p. 213-254. *In* M.B. New, A.G.J. Tacon and I. Csavas (eds.) Farm-made aquafeeds. Proceedings of the FAO/ AADCP Regional Expert Consultation on Farm-Made Aquafeeds, 14-18 December 1992, Bangkok, Thailand. FAO-RAPA/AADCP, Bangkok, Thailand, 434 p.

New, M.B. (1987) Feed and feeding of fish and Shrimp – Manual on the preparation and presentation of compounded feeds for shrimp and fish in aquaculture. FAO/ADCP/87/26.

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Application of Linear Programming in feed formulation

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In fish feed formulation we determine the types and proportion of ingredients to be mixed to produce a complete feed at possible low cost. The fish species in culture pond should be fed with a feed that imitates as far as possible the nutrient levels in their natural food. This is achieved to some extent by mixing different feed ingredients in a suitable way. Here, several factors need to be taken into consideration, as the feed should be nutritionally viable and economically feasible. The problem then is to determine exact optimum proportions of different feed ingredients, which meets the necessary nutritional requirements and have the minimum possible unit cost for the formulated feed. This is achieved through Linear Programming.

The word “Programming” is used here in the sense of “planning”; the necessary relationship to computer programming was incidental to the choice of name. The importance of linear programming derives in part from its many applications and in part from the existence of good general-purpose techniques for finding optimal solutions. Linear programming is about making the most of limited resources. It deals with maximizing / minimizing a linear function of variables subject to a set of linear constraints. Applications range from economic planning and environmental management to diet problems.

What is Linear Programming?

It is a Mathematical technique and not computer programming. It allocates scarce resources to achieve an objective. Linear Programming was pioneered by George B. Dantzig in World War II while being assigned to the U.S. military. He developed a workable solution called Simplex Method in 1947. A program can be thought of as a set of blocks, or activities, of different shapes that can be fitted together according to certain rules. Before linear programming and the simplex method were invented, it was not possible to computationally determine the best combination.

Requirements of a Linear Programming Problem

- Must seek to ***maximize*** or ***minimize*** (put together knows as ***Optimize***) some quantity (the objective function)
- Presence of restrictions or constraints – limits ability to achieve objective
- Must be alternative courses of action from which to choose
- Objectives and constraints must be expressible as linear equations or inequalities

An example

A company produces drugs I and II using machines M1 and M2. One ton of drug I requires 1 hour of processing on machine M1 and 2 hours of processing on machine M2. One ton of drug II requires 3 hours of processing on machine M1 and 1 hour of processing on machine M2. Available times are 9 hours of processing on M1 and 8 hours on M2. Each ton of drug produced (of either type) yields a profit of 1 million rupees. To maximize its profit, how much of each drug should the company produce?

Linear Programming set up:

Let x_1 be the number of tons of drug I produced, and x_2 be the number of tons of drug II produced. The problem is therefore:

Maximize	$x_1 + x_2$	(Profit)	-	Objective Function
Subject to	$x_1 + 3x_2 \leq 9$	(Processing by machine M1)	-	Constraint
	$2x_1 + x_2 \leq 8$	(Processing by machine M2)	-	Constraint
	$x_1, x_2 \geq 0$	(Non negative values)	-	Constraint

Here x_1 and x_2 are the decision variables and to get maximum profit from the available resources it is desirable to obtain the maximum value for the objective function $x_1 + x_2$ retaining the conditions (constraints).

A general Feed Formulation problem:

b_i - required number of units of nutrient i, $i = 1, \ldots, m$.

a_{ij} - number of units of nutrient i in one unit of food j, $i = 1, \ldots, m$, $j = 1, \ldots, n$.

c_j - cost per unit of food j.

x_j - number of units of food j in the diet.

$$\begin{array}{lrcl} \text{We seek to} & \text{minimize} & c_1x_1 + \cdots + c_nx_n & \\ & \text{subject to} & a_{i1}x_1 + \cdots + a_{in}x_n & \geq \quad b_i \text{ for all } i, \\ & & x_1, \ldots, x_n & \geq \quad 0. \end{array}$$

In matrix notation, we can write this as

$$\min_{\mathbf{x}} \mathbf{c}^T\mathbf{x} \quad \text{subject to} \quad \begin{array}{rcl} A\mathbf{x} & \geq & \mathbf{b} \\ \mathbf{x} & \geq & 0 \end{array}$$

Define the following:

For a Linear Programming solution for feed formulation, information necessary are (i) a list of ingredients that are available for use in the feed and their cost. (ii) The nutrient contents for each of the ingredients. (iii) The nutritional requirements of the species in terms of minimum, maximum or exact quantities needed, and (iv) any physical or non-nutritive limitation which might be imposed because of ingredient characteristics such as limitation of supply, effects on feed mixture, toxic factors, ability of feed to be pelleted etc. There are certain attributes such as palatability or acceptability on which it is difficult to place a numerical value.

Linear programming is one of the optimisation techniques in mathematics wherein an objective function, in terms of *n* variables; say $x_1, x_2, ..., x_n$ of the linear form $c_1x_1 + c_2x_2 + ... + c_nx_n$ is optimised (minimized or maximized) subject to a set of linear constraints formulated as

$$\begin{array}{c} a_{11}x_1 + a_{12}x_2 + ... + a_{1n}x_n \geq b_1 \\ a_{21}x_1 + a_{22}x_2 + ... + a_{2n}x_n \geq b_2 \\ \vdots \quad \vdots \quad \ddots \quad \vdots \quad \vdots \\ a_{k1}x_1 + a_{k2}x_2 + ... + a_{kn}x_n \geq b_k \end{array}$$

Using matrix algebra this is denoted as

$$\min_x c'x$$

Such that

$$Ax = b$$

and

$$1 \leq x \leq u$$

Where,

$$c' = (c_1,...,c_n), x' = (x_1,...,x_n), l' = (l_1,...,l_n), u' = (u_1,...,u_n), A = (a_{ij})_{k \times n}$$

Also some of the equations may hold ""instead of "≤" sign and upper bound and lower bound are set for the values of x_1's. In the context of finding an optimum feed formulation, the Linear programming set up is the following. Let, $b_1, b_2, ...b_k$ are the requirements for a species with regards to *k* essential/ nonessential nutrients (some may be minimum/maximum levels). Suppose there are *n* numbers of feed ingredients available for preparation of the feed composition that are the sources for the *k* nutrients. Let the proportion of the i^{th} nutrient available in the j^{th} ingredient is denoted by a_{ij}, then we get the *k* equations of constraints as $a_{i1}x_1 + a_{i2}x_2 + ... + a_{in}x_n = b_i$ and for the *k* nutrients we get *k* such equations of constraints, where $x_1, x_2, ...x_n$ are the proportions of *n* feed ingredients. Now if $c_1, c_2, ...c_n$ are the unit costs of the ingredients, the objective is to minimize $c_1x_1 + c_2x_2 + ... + c_nx_n$ which is the total cost subject to the above mentioned constraints.

Examples

Market prices of Ingredients at Tuticorin.				
No.	Ingredients	(X_i)	Rs. / kg	(c_i)
1	Fish meal	X_1	55.00	c_1
2	Shrimp meal	X_2	45.00	c_2
3	Clam meal	X_3	75.00	c_3
4	Blood meal	X_4	50.00	c_4
5	Poultry byproduct meal	X_5	7.50	c_5
6	Hydrolyzed feather meal	X_6	9.65	c_6

Nutritional composition of different feed ingredients (% of dry matter, DE in kcal/kg of dry weight)								
No.	Ingredients	Protein	(a_{ij})	Lipid	NFE	Fibre	DE	Met
1	Fish meal	55.60	a_{11}	12.00	8.10	3.00	3566	1.67
2	Shrimp meal	35.50	a_{12}	7.00	16.00	12.70	2549	0.71
3	Clam meal	55.20	a_{13}	9.70	23.90	4.20	3839	1.96
4	Blood meal	88.50	a_{14}	1.20	3.90	0.40	3974	0.95
5	Poultry byproduct meal	59.90	a_{15}	17.10	5.40	2.10	4076	1.13
6	Hydrolysed feather meal	91.40	a_{16}	3.90	0.50	0.40	4212	0.59

Nutritional Requirements of Grouper fry			
No	Nutrients / Ingredient	Requirements	(b_i)
1	Protein	≥ 47.8 %	b_1
2	Lipid	≤ 14 %	b_2
3	NFE	≤ 20%	b_3
4	Fibre	≤ 6%	b_4
5	Digestible Energy	≥ 3400 Kcal/Kg	b_5
6	Met	≥ 0.97%	b_6

Optimal Solution: Corner Point Method - Graphical method of solving LP (for 2 variables)

The company producing two types of hats

	Hours per unit		
	Types of hat		
Machine	**Type-1**	**Type-2**	**Capacity**
	(X1)	(X2)	(Hrs/Week)
Machine-1	1 hr	2 hr	40 hr
Machine-2	4 hr	3 hr	120 hr
Profit per hat	Rs.4	Rs. 5	

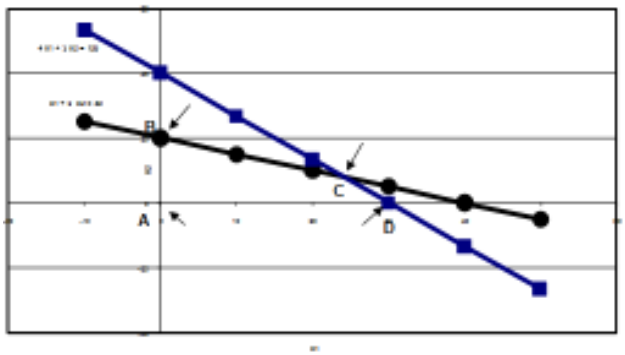
TProblem setup is

Maximize Z = $4X_1 + 5 X 2$

Subject to: $1 X_1 + 2 X 2 \leq 40$

$4 X_1 + 3 X 2 \leq 120$

$X_1, X_2 \leq 0$



The constraints represent 4 planes defined by their respective linear equations

$1 X_1 + 2 X_2 = 40; 4 X_1 + 3 X_2 = 120; \quad X_1 = 0; \quad X_2 = 0$

These planes intersect and the common area (plane of intersection) will have four vertices shown by the arrows in the above graph. These four points, denoted by A, B, C and D, co-ordinates of the points and value of the objective (profit) function are given in the following table.

Profit: $Z = 4X_1 + 5X_2$

Point	Co-ordinates	Z = 4X1 + 5X2	Profit
A	(0, 0)	(4)(0) + (5)(0)	0
B	(0, 20)	(4)(0) + (5)(20)	100
C	(24, 8)	(4)(24) + (5)(8)	136
D	(30, 0)	(4)(30) + (5)(0)	120

The vertex that corresponds to maximum value of the objective function is the solution of the linear programming problem. Hence, the solution of the LP is X1 = 24 and X2 = 8. So the company will have maximum profit when they produce 24 units of Type-1 hats and 8 units of Type-II hats and the maximum profit is 136 Rupees.

When there are more than two variables in the LP problem, as in most of the cases, the graphical procedure will not be useful. Iterative mathematical procedures (optimization algorithms) are then used to solve the LP problem. The most popular method, which is widely used, is the Simplex method. A recent development in solving general optimization problems is using genetic algorithms which is a method developed based on the theory of evolution in biology. This is a principle borrowed into mathematics from biological science and it involves principles of selection, crossing over, mutation etc. There are different computer sofware availiable in market specifically for LP. The famous mathematical sofware MATLAB has a **linpro** module for solution of LP. The SOLVER add-in of Excel in MS Office is also one software for solving LP.

Linear Programming Example - How to use SOLVER in Excel

Machine	Hours per unit Types of hat Type-1	Type-2	Capacity
	(X1)	(X2)	(Hrs/Week)
Machine-1	1 hr	2 hr	40 hr
Machine-2	4 hr	3 hr	120 hr
Profit per hat	Rs.4	Rs. 5	

The company producing two types of hats

Problem setup is

Maximize $Z = 4X_1 + 5X_2$

Subject to:

$1 X_1 + 2 X_2$ d" 40

$4 X_1 + 3 X_2$ d" 120

X_1, X_2 e" 0

Developing necessary equations for solving the LP in Excel's Solver:

Objective Function: $Z = 4X_1 + 5X_2$

Data Preperation:

Variables:	**X1**	**X2**	
Coefficients:	4	5	array1 is C21:D21
Solution:	1	1	array2 is C22:D22 (Initially some arbitrary value)
Value	9		Determined using the sumproduct function as =SUMPRODUCT(C21:D21,C22:D22) This is the target cell (C23 - to be maximized)

Left hand side of the constraints

Coefficients	**X1**	**X2**	**Value = Sumproduct(array1,array2)**	
Equation-1	1	2	3	=SUMPRODUCT(C32:D32,C22:D22)
Equation-2	4	3	7	=SUMPRODUCT(C33:D33,C22:D22)

Right hand side of the constraints

	RHS
Equation-1	40
Equation-2	120

Solver execution:

- Now select solver from the Tools menu of excel after preparation of data.
- Then the window for feeding solver parameters will appear. In the set target cell box click and select the target cell where the value of the objective function for a given solution of variables is given (cell address: C23).
- Choose the maximization option in the radio button options
- In the by changing cells box enter the range where an arbitrary solution for the variables is entered (cell array: C22..C23)
- Press the Add button and you will get a window box to enter the constraints equations.
- For each of the constraints the value calculated using the *sumproduct* function is to be given as the left hand side of the constraint equation is the cell reference.
- Choose the correct sign (<= or >= or =) at the middle and the RHS value reference in the constraint cell and then press add button.
- This is to be repeated for each constraint equation.

- After finishing this close the window box to come back to the window for solver parameters.
- Click the options button to get another window, solver options window.
- Tick the assume non-negative checkbox and press OK button to go back to solver parameters window.
- Now press the solve button to carry out the calculations.
- Once it is finished, the Solver Results window appears where in the Reports portion there is an Answer option. Double click on this to add the results to your workbook as a separate worksheet.
- Final values of the variables as well as the objective function will be availiable in this worksheet.

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Nutritional Requirements of Fish and Shellfish

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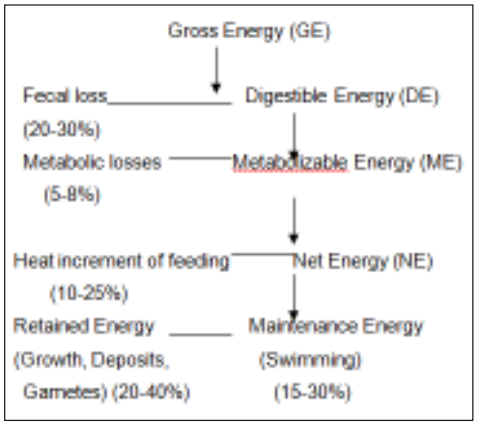
Introduction

Finfish and shellfish (shrimp) farming have shown phenomenal growth in the last decade in India producing protein rich health food and earning valuable foreign exchange. Feed is a major input in fish and shrimp farming. The development of nutritionally balanced feed involves understanding the dietary requirements of candidate species, selection of feed ingredients, formulation of feeds and appropriate processing technology for producing water stable pellet feeds. Depending upon the type of farming, a wide range of feeds is used for feeding stocked shrimp and fish. While no feed is used in traditional farming systems, supplementary and balanced feeds are used in extensive and semi intensive aquaculture. Data base on nutritional requirements of candidate species help to develop high efficiency practical feeds, which in turn led to the development and propagation of semi-intensive and intensive aquaculture leading to increased fish and shellfish production and productivity per unit area.

Nutritiional Requirements of Finfish

Feeding fish with excess nutrients than needed leads to wastage and economic loss. Similarly under feeding nutrients leads to poor performance. Hence balanced feeding is essential for optimum performance with economic viability. To evolve balanced feed, study of nutrition of candidate species is imperative, which helps to understand what fish requires in its diet for optimum and healthy growth and cost-effective conversion of feed into biomass. Dietary requirements are influenced by digestive physiology and environmental conditions.

Gross Energy Metabolism in Fish



Fish need energy for cellular function of maintenance and production. Energy in diet is stored as chemical energy and it is liberated by oxidation of organic compounds, protein, fat and carbohydrate.

Fish require 10 – 30% lower maintenance energy than land animals, because they are poikilotherms and hence there is no regulation of body temperature. Their aquatic mode of life also does not require much energy. Secondly fish are ammoniotelic and excrete nitrogen as ammonia

expending less energy than the other animals that excrete nitrogen as urea and uric acid. The maintenance energy needs of different animals are compared with that of fish are as given below

Animal	**Basal metabolic energy ($MJ/kg^{0.75}$)**
Cow	0.32
Pig	0.31
Sheep	0.29
Foul	0.36
Rat	0.30
Chick	0.36
Man	0.29
Average	**0.27**
Fish	0.01 – 0.07

The estimated metabolic rates in fish are maintenance energy requirement 85-110 J/kg/day and heat increment 16-24KJ/kg/d. The digestible energy levels for maximum growth of fish are 14-17 MJ/kg dry diet, while the gross energy of the diet is 17-20 MJ/kg dry diet. A 300-400 g fish needs 270-320 KJ/kg/d for maximum growth. Young fish need less energy (protein deposition) than old fish (fat deposition). Gonadal maturation depletes 60% body energy reserves. The energy content of egg in fish is estimated to be 27 KJ/g dry and the total energy stored in eggs is 8-15% of gross body energy. This is found equal to gonado-somatic **index.**

Protein Nutrition

Protein requirement of all teleosts is higher than that of land animals because fish utilizes significant portion of protein for energy needs. But fish appears to be efficient in converting protein into growth – Protein Efficiency Ratio (PER) is around 2.2 to 2.7, which is higher than that of land in animals. Therefore production of fish by aquaculture is far more efficient than any other animal production. It is estimated that protein required for maintenance is 1.5-2.0 g/kg/day and for maximum growth it is 7 – 11 g/kg/day. Fish retains about 40% of the protein fed. The protein requirement of some selected fishes is given below

Freshwater fishes	**% Protein in diet**
Atlantic salmon	45.0
Rainbow trout	40.0
Common carp	31-38
Grass carp	41-43
Tilapia Nile	30.0
T. zilli	35.0
T. mossambica	40.0
Eels	45.0
Siberian sturgeon	40.0
Snake head	52.0

Marine fishes

Gilthead sea bream	40.0
Red sea bream	55.0
European sea bass	50.0
Asian sea bass	40-45
Sole	55-60
Turbot	65.0
Plaice	57.0
Yellow tail	55.0
Estuarine grouper	40-50
Puffer fish	50.0
Red drum	40-50
Milkfish	40.0
Mullet	35-40

Amino acids

The same ten Essential Amino Acids (EEAs), which are essential for other animals(arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine threonine, tyrosine, tryptophan and valine) are also found essential for fish. EAAs are required for protein synthesis (growth), maintenance-protein lost from body surface, gastro-intestinal tract, and oxidation of AAs, synthesis of other N compounds such as purines (glycin, glutamic acid), polyamines (methionin), catechol amines (phenyalanine), thyroxine (tyrosin), carnitine (lysin), creatine (arginine, glycin) histamine (histidine), taurine (cystine) and serotonin (tryosin). EAAs constitute 1/3 of total protein and 2/3 are non EAAs. Estimates show that 30-40% of protein N is retained in body. EAA requirement determined by dose response studies of some of the fishes is compared with that of other animals as given below

EAA requirement of animals (as % of P)

EAA	Rat	Chick	Pig	Salmon	Cat fish	Carp
Arginine	5.0	6.3	1.3	5.0	4 .3	4.3
Histidine	2.5	1.5	1.2	1.8	-	-
Ileucine	4.2	3.5	3.2	2.3	-	-
Leucine	6.3	6.8	3.8	4.0	-	-
Lysine	5.8	5.2	4.8	5.0	5.1	5.7
Methionine + Cystine	5.0	4.0	2.8	4.0	2.3	3.1
Phenyalanine + Tyrosine	6.7	6.7	4.4	5.3	5.0	6.5
Threonine	4.2	3.3	2.8	2.3	2.0	3.9
Tryptophan	1.3	1.0	0.8	0.5	0.5	0.8
Valine	5.0	3.6	3.2	3.3	-	-
Protein in Diet%	12.0	23.0	20.0	>35.0	24.0	38.5

Lipid and Fatty Acid Nutrition

Fat levels of 6 – 8% are adequate in most of the fish diets. However, higher fat levels can help sparing protein in the diet. The quality of fat in terms of fatty acid composition is more important from nutrition point of view. Polyunsaturated fatty acids (PUFA) are essential in fish diets. The following fatty acids are found to be essential for fish

Oleic acid	-	18 : 1n-9
Linoleic acid	-	18 : 2n-6
Arachidonic acid	-	20 : 4n-6
Linolenic acid	-	18 : 3n-3
Eicosapentaenoic acid	-	20 : 5n3
Docosahexaenoic acid	-	22 : 6n-3

PUFA are involved in synthesis of important physiological compounds, such as steroid hormones and prostaglandins. Deficiency of EFA affects maturation and spawning. Rainbow trout fed EFA deficient diets produced eggs with poor hatching rate. Similar observations were also made in red sea bream – defective oil globule formation in the eggs. Higher levels of EFA seem to result in depressed growth and FCR and also alter the fatty acid composition of phospholipid. The essential fatty acid requirement of some of the fish species is given below

Fish	**EFA**	**% required in diet**
Rain bow trout	18:3n-3	0.8 - 1.0
	n-3 HUFA	10% of lipid
Carp	18:2n-6	1.0
	18:3n-3	1.0
Eel	18:2n-6	0.5
	18:3n-3	0.5
Salmon	18:3n-3	1.0
	18:2n-6	1.0
	n-3 HUFA	0.5
Tilapia zilli	18:2n-6	1.0
	Or 20:4n-6	1.0
T.nilotica	18:2n-6	0.5
Red sea bream	n-3 HUFA	0.5
	Or 20:5n-3	0.5
Turbot	n-3 HUFA	0.8
Yellow tail	n-3 HUFA	2.0
Coho salmon	18:3n-3	1.0 – 2.5

Fresh water fish show requirement for n-6 & n-3 EFA, where as marine fish show requirement for n-3 and also HUFA. Phospholipid, lecithin is essential in lipid transport, cell membrane structure

and brain function. Tocopherol plays an important role in fish. It is essential to prevent oxidation of PUFA in the body of fish. Carotenoids are required for pigmentation of fish. Vitamin K is involved in prothrombin factor of blood, which helps in quick clotting of blood.

It is established that fishes cultured in the temperate (low temperature) regions show higher requirement of polyunsaturated fatty acids of the n-3 series. This is mainly because that in these fishes it is necessary to maintain membrane fluidity in the cells as the fatty acids of n-3 series have melting points in the range of –45 to –55^{o} C. Whereas the melting points of fatty aids of n-6 series are in the range of –10 to 15^{o}C. Hence the fishes of tropical region such tilapia and snakehead show requirement for the n-6 fatty acids.

Carbohydrate Nutrition

Omnivorous fishes have enzymes to digest carbohydrates (CHO) while carnivorous fishes have poor digestibility of CHO. Polysaccharides are better utilized than monosaccharides. Monosaccharides are rapidly absorbed and poorly utilized. Polysaccharides are slowly absorbed and better utilized. Insulin secretion seems to be poor in fishes. Generally carbohydrate utilization by fish is found to be lower than that of higher animals. Carps can utilize dietary carbohydrate as energy source up to 30 – 40%. For carnivorous fishes the CHO levels in the diet are generally in the range of 10 – 20%. Adequate levels of CHO and lipid in diet as energy source can spare dietary protein. Cooked starch is better digested and utilized by fish á – starch is the best source of CHO for salmon. On the other hand sea bream, yellow tail, carp and other warm water fishes better utilize â-starch. Cellulose (roughage) in diet up to 10% helped to improve FCR and PER.

Vitamin Requirements

Vitamins are complex organic substances which are essential nutrients. They are needed for healthy growth. Their deficiency may cause disease. There are 11water soluble vitamins and 4 fat soluble vitamins. Water soluble vitamins act as co-enzymes. The functions of fat soluble vitamins are Vitamin A – visual pigment, muco-polysaccharide metabolism, Vitamin D in Ca absoption (Ca homeostasis), Vitamin E a natural lipid anti-oxidant and vitamin K in electron transfer oxidative Phosphorilization and blood coagulation

Vitamin requirements of fish for growth

Vitamin	**requirement in diet mg/kg**		
	Trout	**Salmon**	**Carp**
Thiamine	10-12	10-15	2-3
Riboflavin	20-30	20-25	7-10
Pyridoxine	10-15	5-20	5-10
Niacin	120-150	150-200	30-50
Pant-acid	40-50	40-50	30-40
Folic acid	6-10	6-10	-
Cyanoco-amin	-	0.015	-
Inositol	200-300	300-400	200-300
Biotin	1-1.5	1-1.5	1-1.5

Ascorbic acid	100-150	100-150	30-50
Vit. A I.U	2000-2500	-	1000-2000
Vit. E	-	-	80-100

Vitamin Deficiency Diseases in Fish

Thiamine	poor appetite, oedema, poor growth
Pyridoxine	nervous disorders, oedema
Riboflavin	corneal vascularization, poor growth
PABenzoic acid	Mortality
Pantothenic acid	clubbed gills, necrosis, poor growth
Inositol	distended stomach, poor growth
Biotin	loss of appetite, convulsions, poor growth
Folic acid	Lethargy, anemia, poor growth
Choline	kidney haemorrhage, poor growth
Nicotinic acid	loss of appetite, jerky Motion, poor growth
Cyanocobalamin	poor appetite, anemia
Ascorbic acid	scoliosis, lardosis, poor collagen formation

Mineral Requirements

Minerals are needed for fish both for nutritional functions and osmoregulation. Fish absorb some minerals from water, because of this requirement studies are difficult. There are major minerals that are required in bulk and minor minerals or micro mineral nutrients which are required in small quantities. Deficiency of minerals in the diet may lead to deficiency disease.

Bulk minerals required for fish: Ca, P, Cl, Mg, K, Na

Micro mineral nutrients needed for fish: Co, Cu, I, Fe, Mn, Se, Zn

Ca and P are required for bone mineralization

Mineral requirements in fish diets

Mineral	**Requirement in kg diet**
Calcium	5.0 g
Phosphorus	7.0 g
Magnesium	0.5 g
Sodium	1.0-3.0 g
Potassium	1.0-3.0 g
Sulphur	3.0-5.0 g
Cholrine	1.0-5.0 g
Iron	0.05-0.1 g
Copper	1.0-4.0 g
Manganese	20-50 mg

Cobalt	5-10 mg
Zinc	30-100 mg
Iodine	100-300 mg
Molybdenum	trace
Chromium	trace
Fluorine	trace

Mineral Deficiency Diseases in Fish

Ca & P Deficiency : poor growth & FCR, low bone ash, low haematocrit levels
Magnesium- found in bones and metallo-enzymes
Deficiency: poor pg & FCR, renal calcinosis, Muscle flaccidity
Zn -found in enzymes – superoxide desmutase Carboxypepsidase
Deficiency : Cataract, dwarfism
Fe: For haemoglobin
Deficiency: Anemia
Cu: found in enzymes
Deficiency: symptoms not known
Manganese: found in enzymes
Deficiency: malformation of bones, poor growth
Selenium: found in enzymes- glutathione peroxidase
Deficiency: muscular dystrophy, exudative diathesis, higher levels of toxic- spiral swimming, mortality
Iodine: Thyroxin formation

Nutritional Requirements For Indian Major Carps

The dietary nutritional requirements of Indian major carps (Catla, Rohu and Mrigal) and catfish are summarized in the table below

Nutrient in the diet	**Requirement %**	
	Indian major Carps	**Catfish (*Clarias batrachus*)**
Crude Protein	30-45	30-35
Crude fat	7-9	6-7
Carbohydrate	30	30
Gross energy, kcal per kg	3500	3500

Fatty acids, vitamins and minerals are required in the diets of Indian major carps and catfish as per the general discussions presented above.

Nutritional Requirements of Freshwater Prawns

Aquaculture of fresh water prawns has made very impressive progress in the world. The giant fresh water prawn *Macrobrachium rosenbergii* is the most popular species having high commercial potential. It has also emerged as a good alternative species for penaeids. The other important fresh prawns suitable for aquaculture are the river prawn *Macrobrachium malcomsoni* and *Macrobrachium idella*.

Dietary Nutrition

Nutritional studies recommended a protein requirement of 25-30% in the diet of *Macrobrachium rosenbergii*, although other studies suggested up to 35% protein for this prawn. Protein digesting enzymes such as carboxypepsidase and aminopepsidases, arylamidases and dipeptidases have been found in the prawn. There is no pepsin like enzyme in the prawn. However, trypsin like activity was not found in *M*. *rosenbergii* unlike in penaeids. Qualitatively the same ten amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine threonine, tyrosine, tryptophan and valine) were found essential for *M.rosenbergii* as in the case of penaeid shrimp. Supplementation of amino acids has been shown to improve the performance of diet. The amino acid taurine is reported to be involved in cardiac and neural processes and alanine is having a role in osmoregulation.

The lipid levels suggested in prawn feeds are in the range of 6-9%. Fresh water prawns seem to require linoleic fatty acids (18:2n-6) more than linolenic fatty acids (18:n-3). They are capable of elongating the chain of fatty acids from 18:2n-6 to 20:2n-6 and desaturate 20:2n-6 to 20:3n-6. The ratio of n-6 and n-3 fatty acids in the diet of prawns was found to affect the growth. Addition of lecithin and cholesterol to diet did not increase weight gain and survival of juvenile giant prawn.

Freshwater prawns are able to utilize complex carbohydrates. Dietary carbohydrate can be used for sparing protein. Gelatinized starch is better utilized by prawns. In *M. rosenbergii* high á–amylase and chinase activities were found. Dietary cellulose level up to 30% does not seem to affect the growth of prawns under experimental conditions.

Water soluble vitamins are important for prawns. Deficiency of vitamin C in diet led to mortality in *M. rosenbergii* . Srivastava et al. (2000) studied the effect of dietary vitamin Feed efficiency improved with dietary vitamin E up to 300 mg/kg diet and declined at higher levels.

Advances in Brackishwater Fin Fish Nutrition

The brackishwater aquaculture although largely remained synonymous to penaeid shrimp culture, finfish culture in this sector has involved mainly three species namely, the Asian sea bass (*Lates calcarifer*), milkfish (*Chanos chanos*) and grey mullets. The progress made in the nutritional research of these species is also scanty.

Asian Sea bass (Barramundi)

Investigations on Asian sea bass (also known as Bhetki) have been mainly concentrated on energy nutrient requirement in the diet. The fish, being carnivorous showed a dietary requirement of 45 – 55% protein, 6 – 18% of lipid and 10 –16 % of carbohydrate as determined by different workers. Subsequently Catacutan and Coloso (1995) suggested 42.5% and 10% lipid with a protein – energy ratio of 128mg protein/kcal as optimum for the juveniles *L. calcarifer* for growth and good FCR and PER. When the lipid level was raised to 15% in the diet the protein sparing effect was not observed in this fish.

The highly unsaturated fatty acids (HUFA) of n-3 series are reported to essential for sea bass suggesting its marine species characterisitics. The requirement in the diet is suggested to be 1.72% for this fish. Deficiency of HUFA in the diet caused red colouration of the fins in the fish fed such diets.

Studies on carbohydrate and lipid nutrition for juveniles of Asian sea bass, reported that best growth and FCR were observed in fish fed with 20% carbohydrate (bread flour as source) and 12% or 18% lipid (cod liver oil and soybean oil in 1:1ratio).

Diets for weaning the larvae of *L. calcarifer* were successful and research is progress for the development of suitable indigenous feeds for the culture of sea bass. In India the culture of *L. calcarifer* is progressing steadily as an alternative species for penaeid shrimp. However, Asian sea bass is cultured in Australia and Thailand using formulated feeds.

Milkfish

The dietary requirement of protein, lipid and energy requirements for the milkfish, *Chanos chanos* were determined. It was reported that milkfish requires 44% protein in its diet for growth. The essential amino acid requirement of milkfish is reported as given below following levels of the EEAs.

EEA	Requirement as % of dietary protein
Arginine	5.25
Histidine	2.00
Isoleucine	4.00
Leucine	5.11
Lysine	4.00
Methionine	2.50 (cystine 0.75%)
Phenylalanine	4.22 (tyrosine 1.00%)
	or 2.80 (tyrosine 2.67%)
Threonine	4.50
Tryptophan	0.60
Valine	3.55

The lipid requirement is 7 – 10%. As for the quality of lipid the fish seems to have a requirement of n-3 fatty acids at 1.0 – 1.5%. Other studies have reported that a high protein/15-24% fiber diet gave better feed conversion than low protein/high fiber diet.

Grey Mullets

Very little information has come out on the dietary nutrition of grey mullets except a few studies on lesser mullet species. The dietary protein requirement for *Liza macrolepis* fry was reported as 40%. In addition to protein a lipid requirement of 5% and a digestible energy of 372kcal/100g diet were also reported for this fish. For *L. subviridis* the protein requirement was found to be 43.98%. However, for *Mugil auratus* and *M. capito* a high dietary protein requirement of 70% is reported.

Nutritional Requirements of Shrimp

Shrimp diet should have adequate energy, not only to meet the needs of body maintenance called basal metabolism, but also for growth. In nature shrimp feeds on a variety of food items and derive their balanced nutrition for healthy growth. When shrimps are cultured in confined systems (ponds), they should be provided with a balanced diet as close to natural food as possible. It is for this reason understanding the nutritional requirements of candidate species is essential.

Proten Requirement

Protein is the most important and essential nutrient in the diet of shrimp. It also contributes a major share to the cost of feed. The requirement of protein varies with size of shrimp and also with the source of protein used in diet. The dietary requirement of protein for tiger shrimp *Penaeus monodon* ranges from 35 to 45% and for *P. indicus* it ranges from 30 to 43%, which are the most sought after species for culture. It has been demonstrated that postlarvae and juveniles require higher protein in diet and the requirement decreases, as the shrimp grows larger in size.

Amino acids

The growth of shrimp is directly related to the quality of protein in terms of amino acids. Out of the twenty-five odd amino acids that are generally found in proteins, ten are essential amino acids (EAA). These are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine threonine, tyrosine, tryptophan and valine. Shrimps are not capable of synthesizing these amino acids and should be provided through diet and hence they are termed as essential. It is found that if the amino acid composition of the protein in the feed matches with the amino acid composition of shrimp body tissue, such feed promotes good growth. The quantitative requirement of EAA in the diet is related to protein level in diet and their recommended levels in shrimp feeds are given below:

Essential Amino Acid requirement in shrimp feed

Amino acid	As % of protein	% of feed at protein level in feed			
		36.0	38.0	40.0	45.0
Arginine	5.8	2.09	2.20	2.32	2.61
Histidine	2.1	0.76	0.80	0.84	0.95
Isoleucine	3.5	1.26	1.33	1.40	1.58
Leucine	5.4	1.94	2.05	2.16	2.43
Lysine	5.3	1.91	2.01	2.12	2.39
Methionine	2.4	0.86	0.91	0.96	1.08
Methionine+ Cystine	3.6	1.30	1.37	1.44	1.62
Phenylalanine	4.0	1.44	1.52	1.60	1.80
Threonine	3.6	1.30	1.37	1.44	1.62
Tryptophan	0.8	0.29	0.30	0.32	0.36
Valine	4.0	1.44	1.52	1.60	1.80

Source: Modified from Akiyama and Dominy, 1989

Lipid Requirement

Lipid is a complex mixture of simple fat, phospholipids, steroids, fatty acids and other fat soluble substances such as pigments, vitamins, A, D, E and K. The quantitative requirement of fat in the diet of shrimp is in the range of 5 to 10%. However, the quality of fat in terms of fatty acids is more important.

Fatty acids

Fats are tri-esters of glycerol. Among the long chain fatty acids polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2n6), linolenic acid (18:3n3), eicosapentaenoic acid (20:5n3) (EPA) and docosahexaenoic acid (22:6n3) (DHA) are essential for growth, survival and good feed conversion ratio (FCR) for *P. monodon* and other penaeid shrimps. The n3 fatty acids are more essential than the n6 acids (also known as ù fatty acids). The fatty acids, EPA and DHA, which are known as highly unsaturated fatty acids (HUFA) of n3 series, are particularly important. Quantitatively EPA and DHA are needed at 0.5% to 1.0% in the diet of larvae and juvenile shrimp. Studies in *P.indicus* have shown that oils rich in PUFA such as fish (sardine) oil, squid oil and prawn head oil produce superior growth when incorporated in its diet. These oils are rich in HUFA.

Phospholipids

The phospholipid, phosphatidylcholine (lecithin), is essentially required in the diet of shrimp for fast growth and good survival. Soya lecithin is a good source of phospholipid for shrimps. It is required at 2% level in the diet. The development and survival of larvae is significantly improved when the diet contained lecithin. It was established that phospholipids lipid having choline and ethanolamine are only effective. Those phopholipids having other groups such as serine are not as effective as these derivatives. Phospholipids are found to be involved in the transport of lipid, especially steroids in the haemolymph.

Steroids

Shrimps grow through the process called moulting in which they periodically shed body skin (shell). Steroid hormones called, ecdysones, are responsible for moulting. To synthesize these hormones, the steroid cholesterol is required in the diet. Shrimps are not capable of synthesizing cholesterol in their body and hence must be supplied through diet. The requirement of cholesterol in shrimp diet was shown to vary from 0.5% to 1.0%. For *P. monodon* and *P. indicus* the dietary requirement of cholesterol is 0.5%. Plant sterols, such as phytosterol, ergosterol and â sitosterol were also tested for shrimp *P. japonicus*. Though these sterols support growth and survival, the performance of cholesterol is superior to these sterols. 24 methylcholesta 5, 22 dienol was also found to be as effective as cholesterol. Phytosterols are converted to cholesterol and utilized by the pathway suggested by. Many natural feed ingredients, such as prawn head waste and squid are good sources of cholesterol which can be included in the feed formulations.

Energy Requirements

The major components of shrimp diet are protein, fat and carbohydrate, which are the main sources of energy to animals. One gram of protein is approximately equal to 5.5 kcal of energy for shrimp, while fat is the highest energy source equal to 9.5 kcal/g. The energy equivalent of carbohydrate is 4.5 kcal/g. The total digestible energy content of a diet varies with the proportion of protein, fat and carbohydrate. While keeping minimum essential levels of these nutrients, the energy requirement in the diet of penaeid shrimp was found to be 2800 kcal to 4300 kcal/kg for tiger shrimp, *Penaeus monodon* and 3500 to 4000 kcal/kg for *P. indicus*.

Carbohydrate Requirement

Carbohydrate is an inexpensive source of energy in shrimp diet. Among the different types of carbohydrates available, shrimp are found to utilize disaccharides and polysaccharides better than monosaccharides. The Indian white shrimp *P. indicus* showed superior growth on diets containing maltose and starch than those containing glucose, fructose, galactose and glycogen. Tiger shrimp *P. monodon* showed preference for trehalose, sucrose and glucose for growth. However, diets containing maltose and molasses gave inferior results.

The quantitative requirement of carbohydrate in the diet of shrimp is related to dietary protein and lipid levels. Depending upon the total energy content required in the diet, carbohydrate can be used from 10 to 40% level. Carbohydrate has protein sparing effect in. Using starch as source of carbohydrate in diet has dual advantage. Besides being energy source, it can act as binder if gelatinized by cooking with moisture and improves water stability of diet. Corn flour, wheat flour, tapioca flour and other grain flours are good sources of starch in shrimp feeds.

Another polysaccharide, cellulose (also known as crude fiber) is also found to be required in shrimp diet. The enzyme cellulase is detected in digestive tract of penaeid shrimp. But the digestibility of cellulose in shrimp is negligible. However, it is needed in the diet as roughage for improving the feed efficiency. Cellulose levels in shrimp diet should be in the range of 1 to 3% for best results and should not exceed 6%. However, in good quality feeds the crude fiber levels are maintained below 3% level.

The dietary requirements of major nutrients for tiger shrimp and Indian white shrimp are summarized in Table 2.

Table 2: Requirement of major nutrients of shrimps cultured in India

Nutrient		Dietary requirement		
		P. monodon	***P. indicus***	***Leptopenaeus vannamei***
1.	Energy (Kcal/kg)	2800 4300	3500 – 4000	2800 3500
2.	Protein %	35.0 45.0	30 – 43	30.0
3.	Lipid ,,	5 15	6 – 10	6-8
4.	Carbohydrate ,,	20 25	25 30	25-30
5.	Phospholipids ,,	0.1 2.0	0.1 2.0	1-2
6.	Cholesterol ,,	0.5	0.5	0.2-0.5%

Vitamin Requirement

The dietary requirement of vitamin C received utmost attention of the research investigations in the recent times. The requirement of vitamin C has been demonstrated in many shrimp species. The interesting aspect is that the requirement of vitamin C for the survival of *P. vannamei* decreased with size of the shrimp and was found to be 120 mg/kg diet for the shrimp with an initial weight of 0.1 g and for those with 0.5 g weight the dietary requirement was found to be only 41 mg/kg diet.

Plain Vitamin C being very sensitive to heat and in the presence of moisture, it is easily destroyed during the processing of feed. Protected Vitamin C, which is a derivative of either Phosphate (L ascorbyl 2 phosphate) or sulphate (L ascorbyl 2 sulphate), is found to be stable during feed processing

and storage. Experiments have shown that the requirement of protected Vitamin C is far less (0.04%) than that of the plain Vitamin C (0.26%). The phosphate derivative was found to be more effective than the sulphate derivative. Shiau and Hsu (1994) reported a dietary requirement of 40.25 mg/kg of ascorbyl-2-monophsophate (equivalent to 18 mg/kg of plain ascorbic acid) for *P. monodon*. Hsu and Shiau (1998) have studied the use of stable vitamin C derivatives in the diet of *P. monodon* and recommended 48.4mg/kg diet (equivalent to 22.5 mg of plain Vitamin C).

Ascorbic acid is involved in the formation of amino acid hydroxyproline, which is needed in collagen synthesis (Lightener et al., 1977 and 1979). It also plays an important role as an antioxidant and free radical trap along with vitamin E. Vitamin C is required in the formation of folic acid from folinic acid, chondroitin sulphate and intercellular ground tissue. Recent investigations have shown that vitamin C plays an important role in increasing disease resistance capacity in shrimps. Feeding shrimps with vitamin C rich feed showed complete resistance to bacterial disease caused by *Vibrio* sp. when injected to them.

The requirement of other vitamins in the diet of shrimp for their healthy growth is also investigated (Deshimaru and Kuroki, 1979). Choline and Inositol are needed in the diet of shrimp at 60 to 600 mg and 200 mg in 100 g respectively (Kanazawa et al., 1976). Thiamine requirement for penaeid shrimp is found to be 4 to 6 mg%. Riboflavin and niacin (nicotinic acid) are found as tissue coenzymes associated with FMN, FAD, NAD and NADP. These enzymes are needed for the degradation of sugars, fatty acids and amino acids. The dietary requirement of riboflavin and niacin for shrimp is 8 mg% and 40% respectively. Pyridoxine and biotin requirements are reported to be 6 12 mg% and 0.4mg% respectively. Pyridoxine levels of 0.01-0.02% were found to be required in the diet of this shrimp. Similarly shrimp showed a dietary requirement of 0.075% of pantothenic acid.. The deficiency of water-soluble vitamins in the diet resulted in poor growth and the epithelial cells of the midgut gland were destroyed in *P. monodon* (Catacutan and Cruz, 1989). Shiau and Lung (1993) determined vitamin B_{12} requirement in the diet of *P. monodon* and recommended 0.2 mg /kg of the vitamin B_{12} for optimum growth of this shrimp.

Kanazawa (1985) demonstrated the requirement of calciferol (vitamin D), â carotene (pro-vitamin A), folic acid, cyanocobalamine (vitamin B12) and menadione (vitamin K) for *P. japonicus*. It is reported that pantothenic acid and para aminobenzoic acid are not needed for this shrimp. He and Lawrence, (1993) suggested that vitamin E (á tocopherol) is required for shrimp *Penaeus vannmei* at 99 mg per kg diet for optimum growth.. The requirement of vitamin K (menadione) in diet was determined as 160mg/kg diet for *Penaeus chinensis* by Shiau and Liu, (1994). The carotenoid, astaxanthin is in recommended in shrimp feed for proper pigmentation of cultured shrimp. Negre - Sadargues et al. (1993) studied the effect of a mixture of astaxanthin and canthaxantin in the diet at 100 and 200 mg/kg level on growth of the shrimp *P. japonicus* and found that there was no evidence to show the effect of the pigments on growth of shrimp. However the shrimp accumulated the pigments in the epidermis and exhibited highest survival rate over the control groups. The suggested level of carotenoids in shrimp diet is 50 ppm.

The recommended levels of vitamins in the practical feeds for *P. monodon* and *P.indicus* are shown in Table 3.

Table 3: Recommended vitamin levels in shrimp feeds

Vitamins (mg/kg feed)	*P .monodon*	*P.indicus*
Riboflavin	40	20
Thiamine	120	110
Pyridoxine	120	200
Pantothenic acid	100	75
Niacin	150	100
Folic acid	5	5
Biotin	1.0	1
Vitamin B_{12}	< 0.1	< 0.1
Choline chloride	600	750
Inositol	2000	3000
Vitamin C	10000	4000 8000
Vitamin E	200	200
Vitamin A	5000 I.U.	5000 I.U.
Vitamin D	1000 I.U.	15000I.U.
Vitamin K	40 I.U.	40 I.U.

Minerals Requirement

Among the mineral the requirement of calcium and phosphorus in diet shrimp was investigated the most. It was demonstrated in *P. japonicus* and *P.indicus* that shrimp can absorb calcium from seawater (Deshimaru et al., 1978; Rao et al. 1982). However, addition of calcium in the diet improves growth (Deshimaru et al., 1978; Ahamad Ali, 1999). Phosphorus is shown to be essential in the diet of shrimp. Its availability from seawater is negligible unlike calcium. The requirement of this element for penaeid shrimp is in the range of 1.0 to 1.5%. The ratio of calcium and phosphorus in diet seems to be important. Juveniles of *Penaeus indicus* (0.l5g) fed with diet having 0.53% of calcium and 1.05% of phosphorus gave significantly higher growth and low FCR, higher levels of dietary calcium suppressed growth and increased FCR (Ahamad Ali, 1999) though the survival of shrimp and their body calcium levels were unaffected. Shrimps fed with phosphorus deficient diet were sluggish and weak. Phosphorus levels above 1.05% showed no beneficial effect ; a dietary Ca-P ratio of 1:1.98 gave the best growth and FCR for *P.indicus*. Addition of 0.1% to 0.7% of magnesium in the diet suppressed growth and increased FCR (Ahamad Ali, 2000).

Despite the fact that shrimp were fed with diets having different magnesium levels, the body magnesium levels in the shrimp *P.indicus* remained constant. These results suggested that magnesium requirement for this shrimp might be satisfied through absorption from water. Davis and Lawrence (1992) evaluated the requirement of thirteen elements, Ca, P, Na, Cl, K, Mg, Mn, Fe, Zn, I, Se, Cu and Cr for the shrimp *Penaeus vannamei*. No significant difference in growth and survival of shrimp was found when the above minerals were deleted from the diet. However, deletion of Mg, Mn, Cu, Zn and Fe in diet resulted in reduced mineralization of these elements in carapace and hepatopancreas of the shrimp. Deletion of Ca and P did not affect the mineralization in the body. Subsequently Davis et al. (1992) supplemented iron (Fe) in the diet of *P .vannamei* at 0, 20, 40 and 80 mg per kg and found no difference in the performance of the treatments. Based on these results the authors suggested that Fe is not required to be supplemented in the diet of this shrimp.

Davis and Arnold (1994) determined the availability of phosphorus from various phosphorus salts for the shrimp *P .vannamei* and reported that the phosphorus availability (PA) from mono-

calcium phosphate (monobasic) is the highest at 46.3% and the lowest in the tri-calcium phosphate (tribasic) at 9.9%. The PA is 19.1% in di-calcium phosphate (dibasic). The PA from Potassium and Sodium phosphates (monobasic) is 68.1 and 68.2 respectively for the shrimp. Potassium is required at 0.9 to 1.0% for *P. japonicus*. The requirement of magnesium is reported to be 0.3%.

The juveniles of *P. indicus* were fed with purified diets supplemented that the diet with a total copper content of 22.7 mg% gave the best FCR and higher survival (Ahamad Ali, 2000a). The shrimp grew better on diet supplemented with 0% manganese (it had 0.21mg% manganese originally). Higher levels of dietary manganese suppressed growth. The growth of shrimp fed with zinc supplemented diets improved with dietary zinc up to 23.6 mg% and higher levels of zinc suppressed growth. The dietary needs of minerals for some penaeid shrimp are summarized in Table 4.

Table 4: Recommended mineral levels in shrimp feeds

Mineral	**Requirement in diet**		
	P.indicus	***P. monodon***	***P.japonicus***
Calcium %	0.5 0.6	2.0 2.5	1.24
Phosphorus %	1.05	1.2 1.4	1.04
Potassium %	1.26	0.7 0.9	0.9 1.0
Magnesium %	Trace	0.08 0.15	0.3
Iron (mg/kg)	—	60-80	Growth retarded at 0.07%
Copper (mg/kg)	13.6	8 10	60
Manganese (mg/kg)	Trace growth retarded	40 50	10
Zinc (mg/kg)	240	80 100	
Cobalt (mg/kg)		0.8 1.0	
Iodine (mg/kg)		4.0 5.0	
Chromium (mg/kg)		0.6 0.8	
Selenium (mg/kg)		0.17 0.21	

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Aquatic Feed Production Technology

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Introduction

Aquaculture feed technology evolved with the intensification of this food production system. There are wide varieties of methods that can be used to process aquatic feeds. For example, in early days the most common method to feed the fish was hand feeding of mixed, home- blended diets and trash fish. Then fish farmers started using cold forming of moist diets. Pelleting presses then became popular and a majority of the fish feed were processed using pellet mill technology. Shown below is the traditional method of dough ball making practiced in India.

Pelleting

In order to understand the pelleting presses the figure given below is self explanatory. The ingredient mixture falls on to a continuous spiral. A screw forces the feed against a knife and this knife rotates against a fixed die plate. Feed this forced through the die comes out as a compact pellet.

The components of a wet feed mincer/grinder/pelletizer are shown below.

Thus, pelleting is defined as an extrusion type thermoplastic moulding operation wherein finely divided particles of feed are packed into a compact pellet. It is thermoplastic because the protein and sugar of feed ingredients become plastic when heated and diluted with water (moisture). Extrusion is versatility inbuilt into the pelletizer which involves a cooking process where the major macronutrients viz., protein and starch gets cooked in such a way to from an inflatable gel.

Extrusion

Extruders are basically screw pumps through which feed mix forced is subjected to heat, pressure and shear forces. Extrusion is a process, which combines several unit operations including mixing; cooking, kneading, shearing, shaping and forming. The two factors that most influence the nature of the extruded product are the operating conditions of the extruder and the rheological properties of the food. The most important operating parameters are the temperature, pressure, diameter of the die apertures and shear rate.

The array of machines available is mind boggling due to their applications in food industry in general; and the array of value added food products produced are limitless.

Extrusion with steam preconditioning is known as wet extrusion and extrusion without steam preconditioning is called dry extrusion. Dry extruders were solely designed for processing soybeans, which inherently had sufficient oil to act as a lubricant during extrusion. Later, steam preconditioning

prior to extrusion was shown to improve the processing efficiency and product versatility. This lead to what is technically called 'retro-fitting' of dry extruders with steam conditioners. Thus, a clear distinction between dry and wet extrusion is absent today.

Extruders can also be classified according to the method of operation (cold extruders or extruder cookers) and the method of construction (single- or twin-screw extruders).

Twin screw extruders Twin screw extruder is a better design where one screw wipes out the cavity of the other screw thus ensuring positive displacement of feed materials though the barrel preventing burning out of products prevalent in single screw extruders. Moreover, a single screw extruder requires more moisture in the feed mix to make it move through the extruder barrel which results in residual moisture in the extruded product requiring elaborate drying utilizing higher energy. In a twin-screw extruder, lower moisture content in feed ensures less moisture in the extrudate requiring no or short drying.

Current aquatic feed manufacturing practices seem to fit into two simple categories; floating and sinking. Today 100 percent floating feed is extruded and nearly 60 percent sinking feed is extruded. Whereas, the rest of the 40 percent sinking feed is still pelleted. This is just simply because extrusion cooking offers several benefits to the aquatic feed manufactures. Following is the brief description of the advantages of the extruded feed.

Hygienic quality of extruded feed

Ingredients are cooked at high temperatures and pressures. Therefore, extrusion cooking provides hygienic processing of feed destroying the pathogens and most viruses and reducing the toxin levels in the feed ingredients. Growth inhibitors, allergens and other anti-nutritional factors largely inactivated during extrusion cooking.

Option of producing floating or sinking feed

There are many aquatic species that are cultured today. Some of them prefer to eat the pellet on the bottom of the pound where as some of species like to come on the surface to control of the density of the products and thus buoyancy properties are managed.

The use of wet material in the pellet

Extrusion allows raw aquaculture wastes and undercooked waste to be used in final feed without any problem. There are closed loop systems designed for recycling wet product with extruders. These systems allow us to use wet slurries at levels ranging from 22 to 42 percent (as a percentage of the dry recipe) depending upon the moisture contents of the slurries in single screw extruders. In a twin-screw extruder, addition of wet slurries can be as high as 60 percent depending upon the final moisture contents of the wet slurry. Extrusion allows raw aquaculture wastes and undercooked waste to be used in final feed without any problem. A reclamation system to recover wet, under-processed product that cannot be recycled through the dryer as dry rework are available to be used in extrusion processing. These are closed loop systems designed for recycling wet product with extruders. These systems allow us to use wet slurries at levels ranging from 22 to 42 percent (as a percentage of the dry recipe) depending upon the moisture contents of the slurries in single screw extruders. In a twin-screw extruder, addition of wet slurries can be as high as 60 percent depending upon the final moisture contents of the wet slurry.

High fat pellets

Extrusion processes allow feed manufacturers to produce high fat feed. With twin screw extruder a 20 percent fat can be added during extrusion of feed. Whereas, in a single screw extruder, it is limited to around 12 percent fat addition during extrusion. Additional fat is added to the pellet using different coating systems (vacuum or atmospheric). Extruded feed can absorb more fat externally in the coating steps as compared to pelleted feed. Nowadays aquatic feed is processed to contain almost 50 percent fat which is only possible by using extrusion technology. The main purpose of lipids (fats and oils) in feeds are as an energy source; to increase palatability; provide essential fatty acids; carrier for fat soluble vitamins; modified texture; density control and dust reduction. Fat level in fish diets can vary depending upon the species of the fish.

The buoyancy properties required in the feed for common aquatic species cultured is shown in the Table below. And their bulk density indices are also shown

Buoyancy Properties of Feed for Common Aquatic Species (Modified from Riaz 2009)

Floating	Slow-sinking	Sinking
Carp	Seabass	shrimp
Catfish	Grouper	crab
Koi	Cobia	
Tilapia		
Milkfish		

Final Product Bulk Density Correlation with Buoyancy

Properties

Pellet buoyancy Sea water @ 20ºC (3% salinity)
Fast sinking > 640 g/l
Slow sinking 580-600 g/l
Neutral buoyancy 520-540 g/l
Floating <480 g/l

Size of the pellets and water stability

Through extrusion pellets of sizes varying from less than 1 mm to 10 mm in diameter can be produced. Extrusion moisture content during processing can be controlled over a wide range, which helps to increase the water stability of the final feed. These pellets absorb more water, retains shapes for longer time and results in reduced losses of nutrients. Water Absorption and integrity after hydration is very important. It benefits pellet technical qualities and fish health.

Mechanical resistance of pellets

Feed made using extrusion technology is more resistant to mechanical durability and produces fewer fines in the finished feed during transportation. Extruded feed has an internal matrix system which tends to increase resistance to mechanical handling of feed. Extruded feed produces approx one-to-two percent fines in the finished products during bulk handling, where as pelleted feed normally generates five-to-eight percent fines during handling in bulk or bag form. Extrusion reduces 75

percent of the amount of fines which normally enters the water and ends up decaying on the bottom of the pond. In other words, fewer fines means: increased water stability; clearer ponds; lower fatality rates; less unde- sirable bacteria growth; increased is not going to eat 10mm pellet since the size of the pellet is bigger than the mouth.

Water stable pellet

Pellets that break down quickly in water will lose nutrients. Some farmers hydrate feed in water and nutrient solution prior to feeding and require rapid and excellent water stability for handling and feeding purposes. Pellets that breakdown quickly in the stomach of fish loses nutrients (during regurgitation) and may contribute to GDAS (Gastric dilation and air sacculitis) in certain species.

Manufacturing soft aquatic pellets

Some species like blue fin tuna prefer very soft pellet. Extrusion processing allows us to make a very soft pellet (moisture content up to 30 percent in finished feed), which is not possible by pelleting or other methods. This is accomplished using a preservation system in aquatic feeds (final product moisture of 16-28 percent) during extrusion processing. Lower Aw, (water activity) below 0.70 can be controlled with humectants at 10-12 percent levels and by reducing pH to 4.0-4.5 with acids at 1-2 percent levels or with fish silage/solubles. Mould inhibitors are also added at 0.2- 0.5 percent level in the formulation. Recent developments indicate that soft, gel-like aquatic pellets can be processed by coupling alkaline extrusion with a post- extrusion acid bath to adjust ph and set or 'firm up' the texture and pellet integrity.

According to Riaz (2009) the aquatic feed industry is further complicated by the presence of multi-national firms who will often know what extrusion process is required for their production strategies. Their requirements may not always fit the categories outlined above. Another group of aquatic feed producers that will have unique processes are those that manufacture starter feeds. These extrusion systems will be very specialized and often dedicated to the production of smalldiameter feeds. They also require several add-on's listed below.

- VFD (Variable Frequency Drive)
- DMS (Density Management System – vented head, stuffer, vacuum system)
- MBV (Mid-Barrel Valve)
- BPV (Back Pressure Valve)
- EDMS (External Density Management System)
- PDU (Product Densification Unit)
- SAS (Spherizing Agglomeration System)
- Some of these add-ons can help the feed industry make floating, sinking, and slow sinking feed on the same extruder. The following is detailed information for some of these add on equipments.

Back pressure valve

Final product characteristics such as density and texture can be controlled by extruder die restriction. The extrusion industry has developed the revolutionary back pressure valve (BPV) to adjust die restriction while the extrusion system is in operation. By changing the restriction at the discharge of the extruder during operation, the aqua feed density can be varied by up to 25% without changing the screw configuration or the final die. The variable opening BPV is mounted on

the end of the extruder prior to the final die. Specific Mechanical Energy (SME) and extrusion pressure are process parameters controlled by valve positioning. The BPV provides internal control of shear stress and SME for regulation of important product properties:

- Bulk density
- Size and uniformity of cell structure
- Starch gelatinization
- Shape definition
- Water and fat absorption

The BPV also reduces the need for altering the extruder configurations between different aquatic feed families. An integral part of the BPV is a by-pass feature to divert product from the die/knife assembly for service and start-up/shutdown procedures which also improves sanitation in this area.

Mid-barrel valve

Extrusion companies came up with the idea to install a valve in the middle of the extruder barrelto serve as an adjustable restriction device for controlling shear stress and SME during extrusion of aquatic feed. The name of this valve is mid-barrel valve (MBV). The MBV can be adjusted from a setting that adds little or no restriction to a setting that can almost completely restrict the passage of the extrudate, and has demonstrated SME increases of 100% or more. Insertion of this on-line valve can greatly enhance the flexibility of the extrusion process without the downtime associated with configuration changes. A mid-barrel valve can also be connected to the extruder control system to automatically adjusted and maintain the SME valve to its desired set-point in order to make pet food with a wide range of bulk densities.

SAS

In the sphereizer-agglomeration system (SAS); a uniformly mixed and pulverized formulation is passed through a low shear and low temperature extrusion process where it is conditioned with steam, water and other possible liquid additives and compressed through a special die to form extruded strands. These strands when transported into a sphereizer by cyclonic motion sizes and shape the strands into pellets with lengths about the size of the strand diameter. Through SAS™ feeds in the size range of 0.3 to 1.2 mm can be produced. Low processing temperature minimizes nutrient damage and favours production of medicated feeds and utilization of other temperature sensitive ingredients. However, the main disadvantages pointed out are, no pasteurization and capability to produce only sinking pellets.

According to experts, the global aquatic feed market is expected to grow by over six percent per year. This estimate is based on feed sales of 21,000,000 tonnes/year in 2005. The global aquatic feed market is expected to be 28,000,000 tonnes by 2010. (Form www.andritzsprout.com **andritzsprout@andritz.com)**

Suggested Reading

Riaz M.N. 2009 Advances in aquaculture feed extrusion. 17th Annual ASAIM SEA Feed Technology and Nutrition Workshop, June 15-19, 2009 f& Imperial Hotel f& Hue, Vietnam

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Larval Nutrition - a nutritional perspective

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Introduction

Fish naturally contain high levels of HUFA i.e., docosahexaenoic acid (22:6n-3), eicosapentaaenoic acid (20:5n-3) in their body tissues and juvenile marine fish require 0.5 to 1% (n-3) HUFA as dry weight. 5-21% substitution of triacylglycerol fish oil, present either as a natural constituent of fish meal, or as added fish oil meets this requirement. Problems in altricial fish are 1. Marine fish larvae grow more rapidly than juveniles. 2. Natural diets of marine fish larvae are rich in phospholipids rather than triacylgcerol and, 3. The ratio of 22:6(n-3): 20:5 (n-3) in phospholipids naturally consumed is ca. 2:1 whereas this ratio in triacylglycerols in fish oil is less than or equal to 1:1. Thus the marine larval fish feeds based on conventional fish oils with ratios of 22:6 (n-3): 20:5(n3) less than or equal to 1:1 are sub-optimal, either by not providing 22:6(n-3) or by providing an excess of 20:5(n-3). Over emphasis of (n-3) HUFA has resulted in the neglect of arachidonic acid (20:4n-6) as a dietary essential fatty acid for marine fish and the role of monounsaturated fatty acids as major energy yielding nutrients in fish.

Metabolic interrelationships, conversions and competitions.

18:3 (n-3) ⟶ 20:5 (n-3)
Linolenate EPA

Either non-conversion, or very low conversion due to Δ-5 fatty acid desaturase activity.

20:5 (n-3) ⟶ 22:6 (n-3)
EPA DHA

Conversion at low rates not likely to meet the high demands of larval fish growth fully Problems: Visual impairment due to impaired rod function leading to decreased efficiency in capturing prey at low light intensities.

22:5 (n-3) ⟶ 22:6 (n-3) is not by direct Δ-4 desaturation but by a complex pathway where,

20:5 (n-3) is chain elongated to 22:5 (n-3) and then converted to 24:5 (n-3). 24:5 (n-3) is converted to 24:6 (n-3) by Δ-6 desaturase and 24:6 (n-3) is chain shortened through peroxisomal β-oxidation to 22:6 (n-3) or DHA.

Or

18:2 (n-3) ⟶ 18:4 (n-3)

by Δ-6 desaturase

and interestingly, 18:4 (n-3) and 24:5 (n-3) are substrates for the same enzyme.

Thus, 18:3 (n-3) competitively depresses conversion of 20:5 (n-3) ⟶ 22:6 (n-3)

High concentration of 22:6(n-3) exists in the neural tissues. The acyslases and transacylases that esterify fatty acids into phospholipids do not have absolute specificities for particular fatty acids. Therefore, fatty acid compositions of tissues are partly determined by the levels of fatty acids available from the diet. This is true in the case of PUFA where an excess of one dietary PUFA e.g., 20:5n-3, can lead to and elevation of that PUFA in tissue phospholipids at the expense of another PUFA present in much lower concentrations in the diet e.g., 22:6n-3. This effect has been established for phospholipids of fish brain.

Artemia, rotifers and copepods contain substantial amounts of 18:3(n-3) linolenic acid and probably linolenic acid competitively inhibits 20:5(n-3) ⟶ 22:6(n-3) conversion, even if the fish has the capacity to carry out this conversion. *Artemia* nauplii supplemented with fish oils preferentially catabolize 22:6(n-3) relative to 22:5 (n-3). Thus final ratio of 22:6 (n-3): 20:5(n-3) is invariably substantially less than the starting feed. Oils with a high ratio of DHA: EPA should be used in live feed enrichment protocols. Relative excess of 20:5(n-3) over 22:6(n-3) can be harmful in larval feeds. 20:5(n-3) competitively inhibits production of eicosanoids from arachidonic acid 20:4(n-6). Arachidonic acid is the major precursor of eicosanoids in fish and higher vertebrates, despite the surfeit of 20:5(n-3) over 20:4(n-6) in fishes. Current emphasis is on a desirable ratio of 20:5(n-3):20:4(n-6) in larval fish feeds.

General understanding is that marine fish lack Δ-5 desaturase activity. Hence they cannot convert 18:2 (n-3) to 20:4(n-6). Therefore, 20:4(n-6) has been an essential function of producing eicosanoids making it an essential fatty acid (EFA) in marine fish, which has to be provided in larval feeds. Supplementation of marine fish larval feeds with (n-3) HUFA fish oils has obscured the potential importance of 20:4 (n-6) in larval nutrition.

Navas et. al. (1993) and Thrush (1993) fed a diet of fish meal + fish oil + vegetable oils rich in 20:5(n-3) and 22:6(n-3) to broodstock of sea bass (*Dicentrachus labrax*) and reported a production of lower quality eggs with lower survivability and hatchability. The diet contained 0.6%, 20:4(n-6) of the total fatty acids with a ratio of 20:5(n-3) : 20:4(n-3) in the range of 15:1. Animals receiving trash fish (*Boops boops*) produced better quality eggs in comparison. Trash fish diets contained 4.6%, 20:4(n-6) and the ratio of 20:5(n-3):20:4(n-6) was 1.5 : 1. Following up this work, Bruce (1997) found that phosphatidylcholine was the major egg lipid in sea bass and other marine fish eggs. And oil based diets had ratios of 20:5(n-3): 20:4(n-6) of 17.3:1. Trash fish diets had the ratio of 3.4:1. Phosphatidylinositol had the highest% of 20:4(n-6) and is the most likely source of 20:4 (n-6) for ecosanoid production. This is important in the context of broodstock diets because prostaglandins ($PGF2_{\alpha}$) is produced from 20:4(n-6) and has roles in natural shedding of eggs, synchronizing ovulation and spawning and avoids over ripening of eggs.

However, other than *Boops boops* no other natural feed source is found to contain 20:5(n-3):20:4(n-6) in the ration 1.5:1. Does this putatively high requirement of 20:4(n-6) in sea bass apply to other marine fishes?

Knowledge of the (n-3) PUFA requirements of different species of marine fish is still elementary. It is not sufficient to consider gross PUFA requirements, instead the ratio of 22:6 (n-3):20:5(n-3):20:4(n-6) has to be defined.

Requirements

Marine fish can neither biosynthesize 22:6(n-3) *de novo* nor from shorter chain precursors such as 18:3(n-3), therefore 22:6(n-3) and 20:5(n-3) are essential dietary constituents for marine fish. 22:6(n-3) is present in very high concentrations in neural and visual cell membranes and synaptosomal membranes, in fish as in mammals. An insufficiency of 22:6(n-3) in marine larval fish diet is likely to impair neural and visual development with significant if not serious consequences for a whole range of physiological and behavioural processes including those dependent on neuroendocrines. Abnormal pigmentation in cultured marine flatfishes is related to HUFA deficiencies.

Detailed studies examining the appropriate ratios of fatty acids, mainly 22:6(n-3), 20:5(n-3) and 20:4(n-6) have revealed that given a sufficiency of 22:6(n-3), excess of 20:5(n-3) is not deleterious, where as 20:4(n-6) is, because of a generalized biochemically-induced stress in the fish through excess eicosanoid production. In commercially available fish oils, 20:4(n-6) are found to be consistently at low levels (< 1% of the total fatty acids). Neither is an excess of dietary 22:5(n-6) a practical problem. But the major limiting fatty acid in commercial fish oils would be 22:6(n-3). The availability of the fatty acid through enriched *artemia* is also problematic because brine shrimp nauplii retro-converts 22:6(n-6) to 20:5(n-3). Thus, oils particularly rich in 22:6(n-3) are essential for the supplementation process and other than commercial (n-3) HUFA concentrates Tuna orbital oil (TOO) is the only identified natural oil, which has the levels and ratios of 22:6(n-3), 22:5(n-3) and 20:4(n-3) that lead to satisfactory, though not optimal survival growth and metamorphosis of turbot larvae which may not be applicable to all marine fishes. There is evidence that seabass larvae require more of 20:4(n-6) and TOO with ca. 2% proved to be satisfactory. Commercial fish oils with less than 1% arachidonic acid has to be blended with oils rich in arachidonate to achieve this objective.

Presentation

Nearly all mariculture production systems rely heavily on live feeds viz., rotifers, *atremia* nauplii and copepods of *Tisbe, Acartia, Eurytemora. Artemia* and *Brachionus plicatilis* are naturally deficient in 20:5(n-3) and no known strains of *artemia* contain significant levels of 22:6(n-3) making (n-3) HUFA enrichment necessary. Procedures for enrichment with emulsions of marine fish oils are well developed. Commercial products are readily available to achieve this objective. However, up gradation of current procedures in the light of recent knowledge of PUFA requirements is essential. Current problems in enrichment of live feed are – 1. 22:6(n-3) content is very small in triacylglycerol micelles generated in enrichment procedures and are prone to autooxidation, especially under vigorous aeration. 2. Natural antioxidants such as á-tocopheryl acetate and scorbyl palmitate are not effective especially until hydrolysed in the intestinal tract and absorbed. Ethoxyquin and Butylated hydroxy anisole minimizes peroxidation. However, the level of these in enrichment emulsions is an area where there is no information.

Lecithin can be used to considerable advantage in enriching the nauplii with 22:6(n-3) rich fish oils, because lecithin acts as a natural emulsifying agent and a natural protectant against autooxidation. Thus the ideal enrichment mixture tested is a combination of 90% 22:6(n-3) rich fish oil + 10% lecithin from fish eggs. Lecithin derived from fish eggs is superior to soy lecithin because

fish egg lecithin contains readily assimilable 22:6(n-3) and 20:5(n-3) in the ratio of 2:1. Soy lecithin contains only 18:2(n-6) linoleic acid. Commercial availability of (n-3) PUFA rich phospholipids is limited. This limitation has to be overcome by exploring fishery products other than fish roe and milt.

Alternatives to fish oil fractions rich in 22:6(n-3) are (1) a heterotrophic dinoflagellate *Crytothecodenium cohnii* which is mass produced commercially to produce triacyl glycerol rich in 22:6(n-3) - commercial product by MARTEK®; frozen thawed cells are used to supplement *artemia*. (3) Spray dried *Schizochytrium* spp. rich in PUFA is a single celled heterotrophic marine protist of the group labyrinthulomycota - commercial product KELCO®. (4) Copepods cultures have to be developed because they have a preponderance of phospholipids rather than triacylglycerols in their body. Levels and ratios of 22:6(n-3): 20:5(n-3): 20:4(n-6) more closely resemble larval natural diets and the probability of natural protection of PUFA by natural antioxidants and delivery to larvae is always advantageous. Copepods enriched with freeze thawed cells of *C. cohni* or *Schizotricodinium* spp. is another possibility ensuring the appropriate HUFA ratio delivery to larval marine fishes, which is not popular.

Sources

Traditional commercial fish oils especially byproducts of industrial pelagic fisheries are the richest sources of fats and fatty acids. Basically fish oils are rich in 20:5(n-3) and the ratio of 22:6(n-3):20:5(n-3) is found to be < 1:2. MAXEPA™ type oils available commercially contain 12% 22:6(n-3) and 18% 20:5(n-3) which are sourced from southern hemisphere low latitude fisheries, mainly, plichards, anchovies, sardines and menhaden. Northern hemisphere high altitude fisheries yield oils with decreased (n-3) PUFA mainly from capelin, sand eels, herring, sprat and mackerel. 20:5(n-3):22:6(n-3) ratios do not differ from the former with an increased % of 20:1 (n-9) and 22:1(n-11) serving as metabolic source of energy. Cod liver oil has a higher PUFA (n-3) content and a lower% of 20:1(n-9) and 22:1(n-11).

Commercially fish oils are enriched with 22:6(n-3) and 20:5(n-3) by fractional distillation, solvent extraction or by urea adduction or by a combination of all these methods. (n-3) PUFA's are available as ethyl ester, free fatty acids and rarely as triacylglycerols among which ethyl esters are already used to enrich *artemia*. Commercial fish oils can meet enrichment requirements because saturated and monounsaturated fatty acids in fish oils are as important as energy yielding molecules and (n-3) PUFA are useful for structural purposes. Eventhough (n-3) PUFA can be catabolized for energy, they are more difficult to catabolize than saturated or monounsaturated fatty acids. Thus over enrichment with PUFA could conceivably result in an insufficient energy content in the diet.

The only 22:6(n-3) rich natural fish oil known so far is tuna orbital oil (TOO), which contains 30% 22:6(n-3), 7% 20:5(n-3) and 2% 20:4(n-6). It has been proven that blending of 90% TOO with 10% lecithin from fish roe produces the most ideal enrichment emulsion known to date. However, maintenance of the levels of DHA: EPA: AA in *artemia* till the larval fish feeds on it has not been successful because all these fatty acids especially DHA is metabolized by *artemia* after bioencapsulation leading to lowering of its content in the enriched organism. Surprisingly, a strain of *artemia* from China designated as *Artemia sinica* is found to retain the levels of DHA up to 24 h post-enrichment.

The future direction of PUFA nutrition in mariculture is to blend the range of products available to us to achieve either economical larval survival or brood stock maturation and spawning. The clues have naturally come from the nutrient profiles of mature fish eggs.

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Microalgae - a reliable renewable feed stock for future fuel

Syamlal

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Microalgae are microscopic unicellular plants usually found in aqueous environments. The size ranges from a few microns to hundreds of microns. They exists individually or in colonies. Microalgae, capable of performing photosynthesis, are important for life on earth; they produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically

The biodiversity of microalgae is enormous and they represent an almost untapped resource. It has been estimated that about 200,000-800,000 species exist of which about 35,000 species are described. Over 15,000 novel compounds originating from algal biomass have been chemically determined. Most of these microalgae species produce unique products like carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols.

The chemical composition of microalgae is not an intrinsic constant factor but varies over a wide range, both depending on species and on cultivation conditions. It is possible to accumulate the desired products in microalgae to a large extent by changing environmental factors like temperature, illumination, pH, CO2 supply, salt and nutrients.

In addition, because the cells grow in aqueous suspension, they have more efficient access to water, CO2, and other nutrients.

While fish oil has become famous for its omega-3 fatty acid content, fish don't actually produce omega-3s, instead accumulating their omega-3 reserves by consuming microalgae.

Microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels, foods, feeds and high-value bioactives

Why microalgae

The idea of using microalgae as a source of fuel is not new, but it is now being taken seriously because of the escalating price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning fossil fuels. The fear of depleting oil reserves also contribute to the rush for renewable fuels.

There are several aspects of algal biofuel production that have combined to capture the interest of researchers and entrepreneurs around the world.

These include:

1) High per-acre productivity compared to typical terrestrial oil seed crops,

2) Non-food based feedstock resources,

3) Use of otherwise non-productive, non-arable land,

4) Utilization of a wide variety of water sources (fresh, brackish, saline, and wastewater),

5) Production of both biofuels and valuable co-products.

6) Nutrients from the wastewater and CO2 from the flue gas can be utilized.

Microalgae appear to be the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels.

Microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass biodiesel derived from microalgal oil; and photobiologically produced biohydrogen. Out of these biodiesel from algae oil is the major energy product. Biodiesel is produced by transestrification of algal oil (lipid).

Oil content of some microalgae

Microalga Oil content	**(% dry wt)**
Botryococcus braunii	25–75
Chlorella sp.	28–32
*Crypthecodinium cohn*ii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	20
Nannochloris sp.	0–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

Ideally, microalgal biodiesel would be carbon neutral, as all the power needed for producing and processing the algae would come from biodiesel itself and from methane produced by anaerobic digestion of biomass residue left behind after the oils has been extracted. Although microalgal biodiesel can be carbon neutral, it will not result in any net reduction in carbon dioxide that is accumulating as a consequence of burning of fossil fuels.

Farming algae for fuel

Culture requirements

Light
Temperature
Mixing
Gas exchange
Nutrients

Culture Systems

Open pond
Closed photobioreactors
Fermenters

Biomass harvest
Downstream processing
Economic analysis
Energy balance
Co-products

Critical issues of algae fuel industry

Production systems
Trained personnel
Algal strain selection
Input costs
Develop high lipid strains
Component separation
Light management
Extraction
Contamination
Nutrient delivery
Temperature management
Intellectual property
Monitoring systems
Mixing

Low cost microalgae biomass production system developed at M/s Energymicrolgae.

The system is able to produce the biomass @Rs. 4/ Kg DW in a commercial unit. The system powered by ACCaS technology allows the algae farm to be located any remote place where there is no CO2 source.

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World of Microalgae - Scope of Bioprospecting

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Microalgae and their Importance

Microalgae constitute a diverse group of prokaryotic (Blue green algae) and eukaryotic (Protists and unicellular algae) organisms which account for about 50% of global organic carbon fixation (Banares 2004). They are the chief producers and base of food chain in aquatic trophic structure. Recently these photosynthetic microbes have gained a great global consideration in various areas of R&D. Due to their high nutritive values and unique chemical composition, use of microalgae as food (e.g., Spirulina), feed and fertilizer is a years old practice. These autotrophs are important sources of proteins, PUFAs (poly unsaturated fatty acids), pigments (e.g., â Carotene) and vitamins. One of the latest tendencies in microalgal biotechnology is the production of bioactive compounds and their use in pharmaceutical, neutraceutical and cosmetic industries. Biofuels from microalgae are globally accepted as new generation biofuels. Microalgal phycoremediation has been proved to be the best technology of bioremediation in CO_2 sequestration and waste water treatments. Microalgal "green cell factories" are also succeeded in the field of transgenics. Present demand for microalgae for various applications has led to the large scale cultivation and thereby dramatic advancements in the microalgal industry. Their global distribution along with great adaptability to difficult agro-climatic conditions makes microalgae an appropriate commercially cultivable commodity.

Microalgae as food

Chemical composition: - Majority of microalgal applications are based on their chemical composition (Table1). The high protein content makes them an unconventional source of protein (Spolaore 2006). Microalgae can synthesize all amino acids hence provide all essential amino acids to the diets. The average lipid content of microalgae is about 1- 70% but can be 90% of dry weight under special conditions (Spolaore 2006). Microalgal fatty acids include all the essential fatty acids and are the rich sources of long chain poly unsaturated fatty acids (LC-PUFAs) like DHA (docosahexaenoic acid; 22:6(*n* "3)) and EPA (eicosapentaenoic acid; 20:5(*n* " 3)) (Mansour 2005). Microalgal carbohydrates include glucose, starch, sugars and other polysaccharides. Presence of highly digestible algal carbohydrates make them suitable for human consumption as a whole (Becker 2004). Microalgae are also found rich in all essential Vitamins including A, B_1, B_2, B_6, B_{12}, C, E, Nicotinate, Biotin, Folic acid and Pantothenic acid (Spolaore 2006). The pigments in microalgae include chlorophyll (0.5-1.5% of dry weight), carotenoids (0.1- 0.2%) and Phycobiliproteins.

As food: Today in many parts of the world microalgae are sold as health food or food supplement in the form of tablets, capsules and liquids and they act as antioxidants, probiotics etc., there by positively affecting the health. *Spirulina (Arthrospira platensis* and *A. maxima)* is one among the most popular microalga as an SCP (single cell proteins) and a health food for humans and animals. Other important species like *Aphanizomenon flos-aquae* and *Chlorella* are also consumed as SCPs where as *Dunaliella* and *Haematococcus* are used as source of natural colorants like *â* Carotene and Astaxanthin respectively. Moreover microalgae have various possible health promoting effects (Table B). Incorporation of *Spirulina* in poultry diets showed high growth rates and low mortality. Algal diet showed an improvement in weight gain for pigs and similar positive effects were noticed for ruminants as well (Becker,W, 2004). More over no adverse symptoms and unwanted side effects were reported so far in relation with microalgal consumption.

Table A. General composition of human food sources and different microalgae (% of Dry matter) (Becker, 2004)

Commodity	Protein	Carbohydrates	Lipids
Baker's yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
Anabaena cylindrical	43-56	25-30	4- 7
Chlamydomonas reinhardii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirulina maxima	60-71	13-16	6-7
Synechococcus sp.	63	15	11

Table B: Various health promoting effects of commonly used microalgae in nutrition (P. Spolaore, 2006)

Microalgal species	Health promoting effects
Arthrospira sp.	Alleviation of hyperlipidemia; Suppression of hyper tension;Protection against renal failure; Growth promotion of intestinal *Lactobacillus*; Suppression of elevated serum glucose
Chlorella vulgaris	Immune-stimulation; Free radical scavenging; Reduces blood lipids; Efficacy on gastric ulcers, wounds, and constipation; Prevention of Atherosclerosis and hypercholesterolemia; Antitumer action
Dunaliella salina	Ingredient of dietary supplements and functional foods; source of antioxidant and provitamin A
Aphanizomenon flos-aquae	Promotes good overall health

Microalgae for aquaculture

Microalgae are utilized in aquaculture as live feeds for all growth stages of bivalve molluscs (eg. oysters, scallops, clams and mussels), for the larval/early juvenile stages of abalone, crustaceans and some other fish species, and for zooplankton used in aquaculture food chains. Main application of microalgae in aquaculture is nutrition as they play a vital role in rearing of aquatic animals. PUFAs derived from microalgae, i.e. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA) are known to be essential for various larvae. For salmonids microalgae impart colour to the flesh along with other nutritional benefits. *Chaetoceros calcitrans, Skeletonema costatum, Tetraselmis sp., Isochrysis galbana, Pavlova lutheri, Chlorella sp*. etc. are some of the commonly used microalgae in aquaculture. In rearing aquatic animals the "Green water" technique not only improves water quality by oxygen production, pH stabilization etc., but also regulates bacterial population, probiotic effects and stimulates immunity of the reared animals. Microalgae could also be used in the fish feed formulations as growth promoter. The high production cost of microalgae remains a main constraint to the end users viz., fish and shellfish hatcheries. However the selection of appropriate species, use of mixed cultures and uptake of new technologies (like specialised mass-culture) may help to overcome the limitations to some extent (Brown 2002).

Biofuels from microalgae

Microalgae can be a suitable alternative feedstock for next generation biofuels because certain species contain high amounts of oil, which could be extracted, processed and refined into transportation fuels, using currently available technology (Gouveia and Oliveira 2009). Microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass; biodiesel derived from microalgal oil and photobiologically produced biohydrogen (Yusuf Chisti 2007). Microalgae have fast growth rate, permit the use of non-arable land and non-potable water, use far less water and do not displace food crops cultures. Rather their production is not seasonal and they can be harvested daily. Microalgae can couple CO_2-neutral fuel production by CO_2 sequestration and produce non-toxic and highly biodegradable biofuels (Schenk 2008). Furthermore the microalgal residue produced can be utilized either as manure or for the production of BTL (biomass to liquid), bio-ethanol and bio-methanol. (Benemann 2000). However microalgal biodiesel needs to be technically feasible and economically competitive, with reference to petrodiesel, microalgal production, harvesting and extraction and biotechnology(Gouveia and Oliveira 2009).

Table C. Comparison of crop-dependent biodiesel production efficiencies from plant oils (Modified from Schenk 2008)

Plant source	Biodiesel (L/ha/year)	Area to produce global oil demand (hectares × 106)	Area required as percent global land mass	Area as percent global arable land	
Cotton	325	15,002	100.7	756.9	
Rapeseed/canola	1,190	4,097	27.5	206.7	
Jatropha	1,892	2,577	17.3	130	
Oil palm	5,950	819	5.5	41.3	
Algae (10 g m"2 day"1 at 30% TAG)		12,000	406	2.7	20.5
Algae (50 g m"2 day"1 at 50% TAG)		98,500	49	0.3	2.5

Microalgae as biofertilizers

Cyanobacteria, the largest prokaryotic group of microalgae play a major role among the N2-fixing microorganisms in rice fields. They are ecologically fit and economically feasible than the chemical fertilizers (Mishra and Pabbi 2004). Free living forms have a modest potential of about 30kgNha^{-1} crop cycle^{-1}, which may translate to a yield increase of 300-450 kg ha^{-1} (Roger, P.A, 2004). The symbiotic association of *Anabaena azollae* with the fresh water fern Azolla is of great agronomic significance, as this has been used as green manure in rice fields for centuries, in Vietnam and China. Other important species are *Nostoc*, *Aulosira* and *Tolypothrix*.

Microalgal high value compounds and other bioactive products

a. Fatty acids

Fatty acids play important roles in membrane transport, energy storage and chemical precursors in many materials (Masaki ota, 2009) and poly unsaturated fatty acids (PUFAs) have many neutraceutical and pharmaceutical applications (V.Patil et. al., 2006). Eicosapentaenoic acid (EPA, C20:5 ù-3), docosahexaenoic acid (DHA, C22:6 ù-3) arachidonic acid (AA, C20:4 ù-6) and ã linolenic acid (GLA, C18:3 ù-6) are the most important among them. Recent studies indicate that DHA is essential for the development of nervous system and retina (A.Sukenik, 1999) and EPA to be effective in coronary disease and in certain types of cancer (Alonso and Castillo, 1999). Fish and microalgae are the primary sources of these PUFAs. Due to the negative aspects and drawbacks of fish oil (T.Tonon et al., 2002; V.Patil et. al., 2006), and as fishes obtain these PUFAs from microalgae through food chain, microalgae themselves are found to be an alternative source of good quality PUFAs (Table D). PUFA content in microalgae can also be modified by environmental factors like, light, nutrients, temperature etc. and by genetic approach (Alonso and Castillo, 1999) and this kind of strain improvement required to make PUFA production economically feasible for industrial production.

Table D. Particularly interested microalgal PUFAs, (modified from Pauline Spolaore, 2006)

PUFA	Potential Application	Microalga producer
GLA	Infant formulas for full term infants Nutritional suppliments	*Arthrospira*
AA	Infant formulas for full term/pre-term infants Nutritional suppliments	*Porphyridium*
EPA	Nutritional suppliments Aquaculture	*Nannocloropsis, Phaeodactylum, Nitzschia, Porphyridium, Monodus*
DHA	Infant formulas for full term/pre-term infants Nutritional suppliments Aquaculture	*Crypthecodinium, Schizochytrium, Isochrysis, Pavlova*

b. Pigments

Carotenoids: Microalgal carotenoids include â-carotene, astaxanthin, lutein, violaxanthin, lycopene, zeaxanthin etc. Among them â-carotene (from Dunaliella) and astaxanthin (from Haematococcus) are the most important commercially used ones. They are natural colourants with antioxidant property,

hence used as food colorants, and in cosmetics. â-carotene act as provitamin A and have anti-inflammatory properties. The green halophilic flagellate D. salina can produce â-carotene up to 14% of its dry weight (Spolaore, 2006). Dunliella products like powder or extract for human use and for feed use are available @ US$ 300-3000/kg. Astaxanthin is produced from *H. pluvialis* (up to 1.5 – 3% of dry wt.) and priced US$ 2500/kg. Salmon feed industry is the major consumer of astaxanthin, but human neutraceuticals and cosmetics industry have also show shown interest (Spolaore, 2006).

Phycobiliproteins: Phycobiliproteins (present in cyanobacteria, red algae and cryptomonads) are natural dyes with health promoting properties and pharmaceutical and cosmetic applications. They are highly sensitive fluorescent reagents, hence can serve as labels for antibodies, receptors etc., and are used in immunolabelling experiments and fluorescent microscopy or diagnostics. The cyanobacteium *Arthrospira* and the rhodophyte *Porphyridium* are the main commercial producers of phycobiliproteins, i.e., phycocyanin and phycoerythrin (Spolaore, 2006).

c. Others

Polysaccharides: Sulphated polysaccharides from red microalgae (Porphyridium, Rodella and Rhodosorus) are used as gelling agents, stabilizers, thickeners and emulsifiers in food stuffs, paints, photographic films, pharmaceuticals etc. These polysaccharides have also applications in human and animal health as dietary fibres and antiviral agents (S. Arad, 1999).

Polyhydroxyalkanoates (PHAs) (Bioplastics): PHAs are thermoplastic polymers produced by many organisms including microalgae. High molecular weight PHAs can be produced and accumulated by Cyanobacteria in the form of cytoplasmic amorphous granules with material properties similar to conventional synthetic plastics. Their biodegradability and high moisture resistance makes PHAs superior to other cheaper verities of materials used in place of synthetic plastics.

Antioxidants: Due to the phototrophic life and high oxidative stress, in microalgal cell there are antioxidative scavenger complexes producing antioxidative components. These natural antioxidants have applications especially in sun protecting cosmetics, neutraceuticals and in therapy of oxidation associated diseases (O. Pulz and W. Cross, 2004)

Antibiotics: Bioactive compounds from microalgae like fatty acids (GLA and EPA), pigments (carotenoids) and many others are proved to be antobiotically active as antibacterial, antifungal, antialgal, antiprotozoan substances (Michael A Borowitzka, 1999)

Antivirals: Cyanobacteria are the most significant sources of antivirals. Some of them are identified and reported for the activity against HIV and HSV-2. Calcium spirulan, a sulphated polysaccharide from *Spirulina platensis,* inhibits replication in HSV, HIV etc. Similar activity is also noted from the polysaccharides of red algae (Michael A Borowitzka, 1999).

Anticarcinogens: Cytotoxic and antitumor and antineoplastic substances are mainly isolated from Cyanobacteria, Dinoflagellates and Cryptophytes with inhibitory activity for proliferation of a variety of mammalian carcinomas and tumors.

Microalgae in Bioremediation - Phycoremediation

In Microalgal bioremediation, microalgae are used for the removal or biotransformation of pollutants (nutrients and xenobiotics) from waste water and CO_2 from polluted air. Microalgae can also be used as bioindicators of various pollutants including pesticides and herbicides.

Advantages of microalgae over other methods

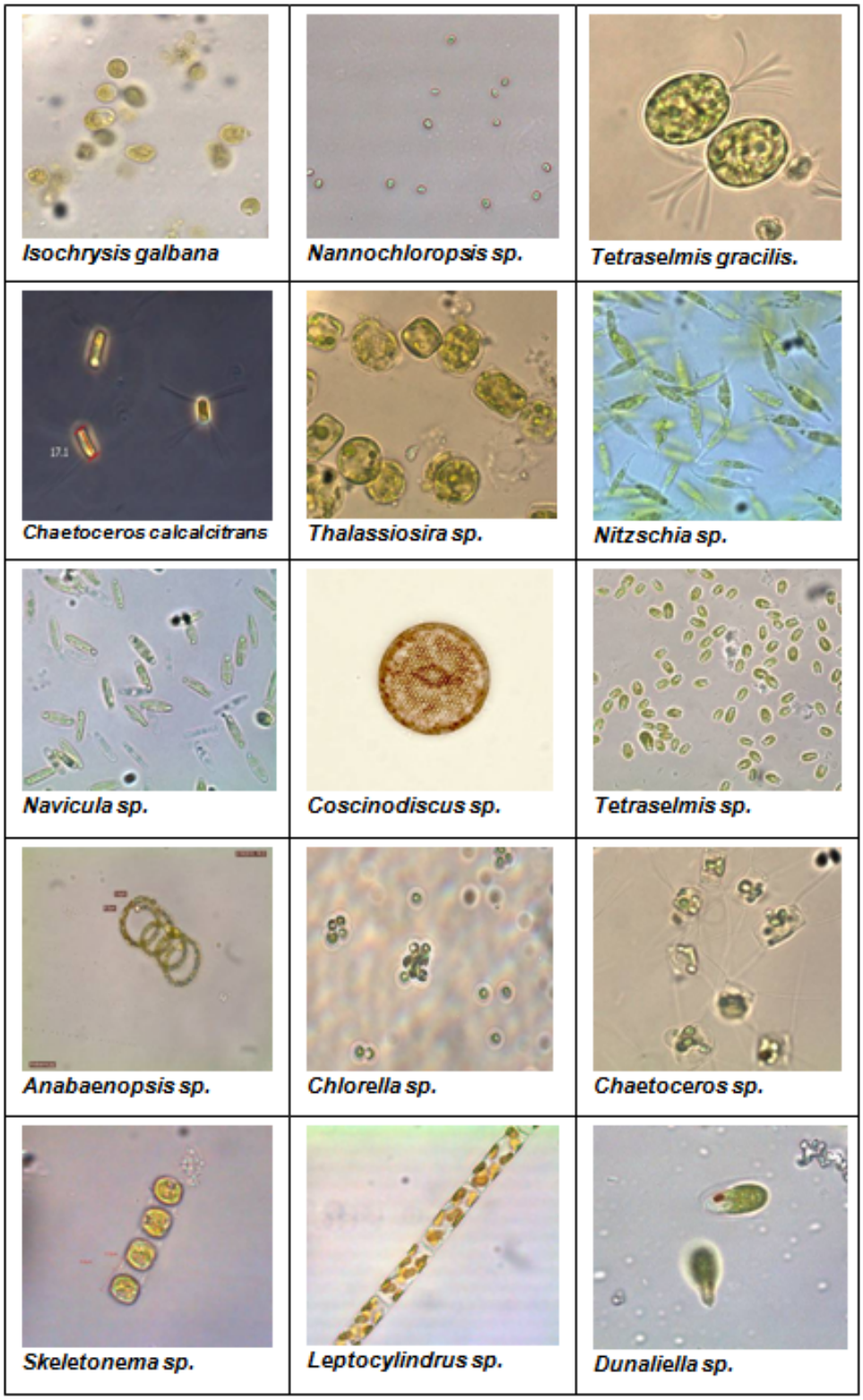
- tackle more than one problem at a time
- natural hence environmentally safe
- more selective and specific in treatments
- no sludge generation
- easy to handle; no need of specialists for operating
- no foul smell
- Oxygenation of environment
- CO_2 removal
- Co-production of biofuels, biofertilizer, etc.

Microalgae in Transgenics

Microalgae represent a much simpler system for genetic manipulations compared with higher plants and other complex eukaryotic hosts. Through metabolic engineering microalgae could be transformed into green cell factories for the production of many heterologous proteins, high quality mammalian proteins (hormones or anibodies) and many other industrially valuable compounds. *Chlamydomonas reinhardtii,* a chlorophyte, is the first and best studied transformation system (for producing antigenic protein by chloroplast transformation) (Rosa Leo Banares, 2004). *Phaeodactylum, Arthrospira, Chlorella, Navicula, Thalassiosira* etc. are some of the other successfully transformed microalgae. Modified organisms are used not only for the production of special compounds but also for bioremediation, Moreover controlled and contained growth of microalgae in photobioreactors minimizes the ethical issues related to GMOs.

Conclusion and future perspectives

Microalgae have been explored for millennia for various purposes like, Arthrospira (as food) in Africa and Nostoc (as fertilizer) in China. Their countless applications range from nutrition and bioactive compounds to cosmetics. Present world is facing challenges like pollution, global warming, water scarcity, health disorders and demand for novel rich nutritional sources. Microalgae can be a unique solution for all such harms and problems. Carbon dioxide - neutral fuel production and discovery of new drugs seems to be the most promising ones. In addition these rich microalgae can be cultured in saline, hyper saline or any other unused water bodies which can save a large volume of fresh water. Among the known 30,000 species (world wide) very few have been explored and cultivated in an extensive way. A country like India can explore marine microalgal diversity and exploit its coastal waters for their production shows great prospective. The genetic modification of microalgae is also a challenging approach. Rather new technical approaches should be developed in mass culture, in designing photobioreactors and in processing methods which will support the extensive production systems. (Spolaore 2006).



Some important microalgal isolates of CMFRI from Indian coast

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Bioprospecting

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Marine Microbes as a Source of Antimicrobial Compounds

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Introduction

Earth is a blue planet; oceans cover 70% of its surface, and in terms of phyla, the diversity of the oceans is about double that of the land. Environments such as the deep sea floor, once thought barren, are now known to be equally or more biologically diverse than tropical rainforests. It has been known for at least 40 years that microorganisms could be recovered from the sea. An impressive number of modern drugs have been isolated from microorganisms, mainly based on their use in traditional medicine. In the past century, however, an increasing role has been played by microorganisms in the production of antibiotics and other drugs (Fenical, 1993). The importance of terrestrial bacteria and fungi as sources of valuable bioactive metabolites is very well established for more than half a century. As a result, over 120 of the most important medicines (penicillins, cyclosporin A, adriamycine, etc.) in use today are obtained from terrestrial microorganisms (Alanis, 2005). For more than two decades, there has been an ongoing quest to discover new drugs from the sea. Most efforts have been directed towards chemical studies of marine invertebrates (Chin et al., 2006). Although these studies have indeed proven that marine invertebrates are an important source of new biomedical leads, a fact well demonstrated by the number of compounds currently in clinical trials, it has proven notoriously difficult to obtain adequate, reliable supplies of these compounds from nature. Because of these problems, a new avenue of study focusing on marine microorganisms has been gaining considerable attention (Faulkner, 2002). At first sight thus, the expectable enormous biodiversity of marine microorganisms might have been the reason for the interest in their study. Although marine microorganisms are not well defined taxonomically, preliminary studies indicate that the wealth of microbial diversity in the world's oceans, make this a promising frontier for the discovery of new medicines (Blunt et al., 2004). Marine bacteria are most generally defined by their requirements of seawater, or more specifically sodium for growth. In the case of marine fungi, which in general do not display specific ion requirements, obligate marine species are generally considered to be those that grow and sporulate exclusively in a marine habitat. Although such definitions can prove useful, they tend to select for a subset of the microorganisms that can be isolated from any one environment. This problem is compounded in the case of near - shore or estuarine samples where a large percentage of the resident microbes are adapted to varying degrees of marine exposure. For the purpose of microbial drug discovery, it seems only logical to study all microbes that can be isolated from the marine environment. Based on the species studied, most of the new compounds

reported from marine microorganisms were obtained from species that can, in principle, be isolated from both land and sea. Although these facultative marine species are clearly a good source of novel metabolites, their ecological roles and degrees of adaptation to the marine environment is largely unknown. Screening of marine bacteria isolated from the surface of marine algae and invertebrates has shown that a high percentage produce antimicrobial metabolites. Marine microbial floras have an unrivalled capacity to synthesize bioactive secondary metabolites with a wide spectrum of bioactivities. Historically, microorganisms have provided the source for the majority of the drugs in use today. As new chemical entities are likely to be discovered from novel microbes, marine microorganisms are a likely target for improved technological platforms in the search and discovery of novel bioactive compounds. The first antibiotic from marine bacterium was identified and characterized in 1966 (Burkholder et al., 1966). In addition, bacteria in biofilms formed on the surface of marine organisms have been documented to contain a high proportion of antibiotic producing bacteria than some other marine environment (Lemos et al., 1985; Anand et al., 2006). Marine epiphytic bacteria, associated with nutrient rich algal surfaces and invertebrates, have also been shown to produce antibacterial secondary metabolites, which inhibit the settlement of potential competitors (Bernan et al., 1997). A number of surface associated marine bacteria have also been found to produce antibiotics (Hans et al., 2004). A *Bacillus sp* isolated from a marine worm in Papua New Guinea produced a novel cyclic decapeptide antibiotic, loloatin B, which inhibit growth of MRSA (methicillin resistant *Staphylococcus aureus*) and VRE (Vancomycin resistant *Enterococcus*) (Gerard et al., 1999). The marine bacterium *Alteromonas rava* was found to produce a new antibiotic thiomarinol (Shiozawa et al*.,* 1993). Antibiotics from marine microorganisms have been reported, including loloatins from *Bacillus.* Agrochelin and sesbanimides from *Agrobacterium* (Acebal et al., 1999), pelagiomicins from *Pelagiobacter variabilis* (Imamura 1997), pyrones from *Pseudomonas* (Singh et al., 2003). Screening of seaweed and invertebrate-associated bacteria has shown their bioactivities (Chakraborty et al., 2010), and that over 25% of these isolates can produce compounds capable of killing methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE; Mearns-Spragg et al., 1997). This is a much higher proportion than found with free-living or soil-associated bacteria.

Antimicrobials from microbes, a brief history

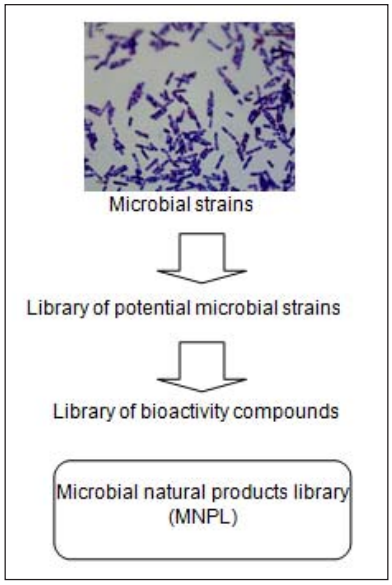
It has been argued that because of the high dilution effect of seawater, marine-derived bioactive compounds may have evolved great potency. This theory was supported in 2004 with the report of a first-in-class antimicrobial compound, was described from a marine isolate *Verrucosispora*. Renewed interest in marine microorganisms and their ability to produce antimicrobials has resulted in numerous reports of novel antimicrobial compounds. The period of antimicrobial drug discovery from the early 1940s to the 1960s is referred to as the Golden Age. During this time, the industrialization of penicillin production created the expertise and facilities to make significant quantities of antimicrobial compounds by fermentation. The clinical use of antibiotics heralded a health care miracle; deaths due to bacterial infections were significantly reduced, resulting in increases in life expectancy. The majority of compounds that were discovered during this period were isolated from soil bacteria, most notably the filamentous *Actinobacteria*. Microorganisms are a prolific source of structurally diverse bioactive metabolites and have yielded some of the most important products of the pharmaceutical industry. Microbial secondary metabolites are now being used for applications other

than antibacterial, antifungal and antiviral infections. It was during 1928s when Alexander Fleming (Fleming, 1929) began the microbial drug era when he discovered in a Petri dish seeded with Staphylococcus aureus that a compound (penicillin) produced by a fungus/mold killed the bacteria. Later, penicillin was isolated as a yellow powder and used as a potent antibacterial compound during the Second World War. Following this extraordinary discovery be Flemming, the antibiotics chloramphenicol and streptomycin, were isolated. Naturally occurring antibiotics are produced by fermentation, an old technique that can be traced back almost 8000 years. Owing to technical improvements in screening programs, and separation and isolation techniques, the number of natural compounds discovered exceeds 1 million (Ecker et al. 2005). Among them, 50–60% are produced by plants (alkaloids, flavonoids, terpenoids, steroids, carbohydrates, etc.) and 5% have a microbial origin. Of all the reported natural products, approximately 20–25% show biological activity, and of these approximately 10% have been obtained from microbes. Furthermore, from the 22 500 biologically active compounds that have been obtained so far from microbes, 45% are produced by bacteria or bacteria-like microbes, 38% by fungi and 17% by others (Berdy, 2005). The increasing role of microorganisms in the production of antibiotics and other drugs for treatment of serious diseases has been dramatic. However, the development of resistance in microbes to various life-thretening diseases and in aquaculture has become a major problem and requires much research effort to combat it. The emergence of antibiotic resistance in the 1970s coincided with a high rediscovery rate of the major antimicrobial classes; the low-hanging fruit had apparently all been picked. Antimicrobial development after the Golden Age was characterized by semi-synthetic modifications of compounds that were already clinically proven. The poor antimicrobial discovery rate from microbes, coupled with the availability of chemically synthesized small molecule libraries, led to the abandonment of microbial screening programmes in the majority of pharmaceutical companies. To date, small chemical libraries have failed to deliver a new antimicrobial compound to the clinic, prompting many to speculate that the withdrawal of microbial screening was premature, exacerbating the threat of antibiotic resistant bacteria.

Microbial natural products

Microbial natural products that have reached the market without any chemical modifications are a testimony to the remarkable ability of microorganisms to produce drug-like small molecules. Although still in clinical trails, a feature example of this is salinosporamide A (NPI-0052), a novel anticancer agent found in the exploration of new marine environments (Fenical et al. 2009). In 2008, over 1000 marine natural products were reported (Blunt et al 2010). However, out of the 19 microbial-derived drugs reported in 2008, no natural products from marine microbes were present, signifying the novelty of their systematic exploration (Ganesan, 2008). Currently, >30 compounds of marine microbial origin are in clinical or preclinical studies for the treatment of different types of cancer (Simmons et al 2005) clearly demonstrating that marine microorganisms have become an essential resource in the discovery of new antibiotic leads. The evolution of marine microbial natural product collections and development of high-throughput screening methods have attracted researchers to the use of natural product libraries in drug discovery. These libraries include subsections of crude extracts, pre-fractionated extracts (automated HPLC-MS fractionation) and purified natural products. A research group in Ireland has developed a two-dimensional chromatographic strategy that includes a protocol to generate purified marine natural product libraries that are accurately characterized by

mass during production to expedite dereplication of known compounds and identification of novel chemotypes. Although the biosynthetic and regulative crosstalk of secondary metabolite biosynthesis is complex within and between microorganisms, all levels can be influenced by imitating natural environmental changes. Development and testing of new culture media for the maximum expression of secondary metabolites is important as chemical diversity in the construction process of an marine natural products library.



Schematic representation of Bioprospecting antibacterial molecules from microbial flora

An optimization of 'one strain, many active compounds' can be used together with 'fingerprint' methods (HPLC and nuclear magnetic resonance) including tandem analytical techniques such as MS/MS, GC-EI/MS, HPLC-SPE-NMR, LC-MS-MS and LC-NMR for the optimization/selection of culture media for high-throughput fermentation of novel strains. Tormo et al. (2003) developed a method for the selection of production media for bacterial strains based on their metabolite HPLC profiles, that yielded the highest metabolite diversity and least overlapping HPLC profiles were selected for large-scale fermentation. Targeted high-throughput screening methods are important for the speed and accuracy of identification of novel antimicrobials. From these evaluation models, many crude extracts or purified compounds were obtained as positive hits. In addition for evaluation purposes, it is worthy to note that these screening assays also provide mode of action hypothesis from the crude extracts.

Antibacterial molecules from natural sources

Drugs of natural origin have been classified as (i) original natural products, (ii) products derived or chemically synthesized from natural products or (iii) synthetic products based on natural product structures. Evidence of the importance of natural products in the discovery of leads for the development of drugs for the treatment of human diseases and aquaculture are provided by the fact that close to half of the best selling pharmaceuticals and antibiotics in 1990-2000 were either natural products or their derivatives (Cragg et al 1997). In this regard, of the 25 top-selling drugs reported in 1997, 42% were natural products or their derivatives and of these, 67% were antibiotics. Today, the structures of around 140 000 secondary metabolites have been elucidated. Applications of chemically synthesized natural metabolites include the use of a natural product derived from plant salicyclic acid derivatives present in wintergreen and meadowsweet to relieve pain and suffering. Synthetic salicylates were produced initially by Bayer in 1874, and later in 1897, Arthur Eichengrun at Bayer discovered that an acetyl derivative (aspirin), reduced acidity, bad taste and stomach irritation. These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization (WHO) that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care (Farnsworth et al 1985). The alkaloid quinine, the active constituent of *Cinchona succirubra*, has been known for centuries by South American Indians to control malaria. During the twentieth century, massive programs to synthesize quinoline derivatives, based on the quinine prototype, were carried out. The first of the new quinolones to be

used clinically as an antibacterial agent was nalidixic acid (Topliss et al. 2002). The compound 7-chloro-1, 4-dihydro-1-ethyl-4-oxoquinolone-3-carboxylic acid was obtained as a side product during purification of chloroquine and found to have antibacterial activity against Gram-negative bacteria and was shown to be an inhibitor of DNA gyrase. Its discovery led to a whole series of synthetic quinolone and fluoroquinolone antibiotics (pefloxacin, norfloxacin, ciprofloxacin, levofloxacin, ofloxacin, lomefloxacin, sparfloxacin, etc.), which have been very successful in medicine and have achieved major commercial success. Secondary metabolites have exerted a major impact on the control of infectious diseases and other medical conditions, and the development of pharmaceutical industry. Their use has contributed to an increase in the average life expectancy in the world. In 2000, the market for major antiinfectives from bacteria and other natural sources was US$55 billion and in 2007 it was US$66 billion.

Various classes of antibiotics/drugs from microbial flora (upto 2000) (Barber, 2001).

Sl. No.	Antibiotics/drugs	Market share (US billion $)
1	Antiviral compounds	10.2
2	Penicillins	8.2
3	Cephalopsporins	9.9
4	Beta lactam antibiotics	1.5
5	Quinolines	6.4
6	Other antibacterials	6.0
7	Tetracyclines	1.5

Two antivirals that are chemically synthesized today were originally isolated from marine organisms. They are acyclovir (active against the herpes virus by inhibition and inactivation of DNA polymerase) and cytarabine (active against non-Hodgkin's lymphoma). Both compounds are nucleoside analog drugs, originally isolated from sponges (Rayl, 1999). Other antiviral applications of natural compounds are related to human immunodeficiency virus (HIV) treatment. Furthermore, reports have been published on natural product inhibitors of HIV integrase obtained from among the marine ascidian alkaloids; that is, the lamellarins (produced by the mollusk *Lamellaria* sp.), and from terrestrial plants (Baccharis genistelloides and Achyrocline satureioides). The most consistent anti-HIVactivity was observed with extracts prepared from several Baccharis species (Robinson et al 1996).

Antibiotics from marine microbes

During recent decades, we have seen an increasing number of reports on the progressive development of bacterial resistance to almost all available antimicrobial agents. In the 1970s, the major problem was the multidrug resistance of Gram-negative bacteria, but later in the 1980s the Gram-positive bacteria became important, including methicillin-resistant staphylococci, penicillin-resistant pneumococci and vancomycin-resistant enterococci (Moellering, 1998). In the past, the solution to the problem has depended primarily on the development of novel antimicrobial agents. However, the number of new classes of antimicrobial agents being developed has decreased dramatically in recent years. The conventionally used antibiotics/drugs become resistant to most of the natural antimicrobial agents that have been developed over the past 50 years (Hancock, 2007)

thereby limiting the effectiveness of current antimicrobial drugs. In 2004, more than 70% of pathogenic bacteria were estimated to be resistant to at least one of the currently available antibiotics (Katz et al 2006). The so-called 'superbugs' (organisms that are resistant to most of the clinically used antibiotics) are emerging at a rapid rate. S. aureus, which is resistant to methicillin, is responsible for many cases of infections each year (Balaban et al 2005). The incidence of multidrug-resistant pathogenic bacteria is increasing. The Infectious Disease Society of America (IDSA) reported in 2004 that in US hospitals alone, around 2 million people acquire bacterial infections per year (dedicated website: http://www.idsociety.org/Content.aspx). There are also other examples of Gram-positive (Enterococcus and Streptococcus) and Gram-negative pathogens (Klebsiella, Escherichia, Enterobacter, Serratia, Citrobacter, Salmonella and Pseudomonas) (Cragg & Newman, 2001). Among them, Pseudomonas aeruginosa accounts for almost 80% of these opportunistic infections. They represent a serious problem in patients hospitalized with cancer, cystic fibrosis and burns, causing death in 50% of cases. Other infections caused by Pseudomonas species include endocarditis, pneumonia and infections of the urinary tract, central nervous system, wounds, eyes, ears, skin and musculoskeletal system (Levin, & Bonten, 2004). In addition to the antibiotic-resistance problem, new families of anti-infective compounds are needed to enter the marketplace at regular intervals to tackle the new diseases caused by evolving pathogens. At least 30 new diseases emerged in the 1980-2000s and they are growing in incidence. Emerging infectious organisms often encounter hosts with no prior exposure to them and thus represent a novel challenge to the host's immune system. Several viruses responsible for human epidemics have made a transition from animal host to humans and are now transmitted from human to human. HIV, responsible for the acquired immunodeficiency syndrome (AIDS) epidemic, is one example. Although it has not been proven, it is suspected that severe acute respiratory syndrome (SARS), caused by the SARS coronavirus, also evolved from a different species (Kremer et al 2000). One additional reason for developing new antibiotics is related to their own toxicity. As with other therapeutic agents, the use of antibiotics may also cause side effects in patients. Some side effects are more severe and, depending on the antibiotic, may disrupt the hearing function (aminoglycosides), kidneys (aminoglycosides and polypeptides) or liver (rifampin).

New molecules as a solution towards multiresistant antibiotic and drug molecules

In recent times, several scientific groups are making concerted efforts to find novel antimicrobial agents as a solution towards multiresistant antibiotic and drug molecules. Novel glycylcyclines (modified Tetracyclines) developed to treat tetracycline-resistant bacteria. These show potent activity against a broad spectrum of Gram-positive and Gram-negative bacteria, including strains that carry the two major tetracycline-resistance determinants, involving efflux and ribosomal protection. Two of the glycylcyline derivatives, DMG-MINO and DMG-DMDOT, have been tested against a large number of clinical pathogens isolated from various sources. The spectrum of activity of these compounds includes organisms with resistance to antibiotics other than tetracyclines; for example, methicillin- resistant staphylococci, penicillin-resistant S. pneumoniae and vancomycin-resistant enterococci (Sum, 2006). Tigecycline was approved by the FDA in 2005 as an injectable antibiotic (Bacque et al 2005). A new glycopeptide antibiotic, teicoplanin, was developed against infections with resistant Gram-positive bacteria, especially bacteria resistant to the glycopeptide vancomycin. In another instance, the approach involved the redesign of a mixture of two compounds, called streptogramin, into a new mixture, called pristinamycin, to allow administration of the drug parenterally

and in higher doses than the earlier oral preparation (Bacque et al 2005). The two components of streptogramin, quinupristin and dalfopristin, were chemically modified to allow intravenous administration. The new combination, pristinamycin, was approved by the FDA for use against infections caused by vancomycin-resistant Enterococcus faecium. Among the novel class of antimicrobial agents used in treating resistance to Gram-positive infections, we can also mention the cyclic lipopeptide antibiotic daptomycin produced by Streptomyces roseosporus. This compound was approved in 2003 by the FDA for skin infections resulting from complications following surgery, diabetic foot ulcers and burns (LaPlante et al 2004). Telithromycin, a macrolide antibiotic, is the first orally active compound of a new family of antibacterials named the ketolides. It shows potent activity against pathogens implicated in community acquired respiratory tract infections, irrespective of their â-lactam, macrolide or fluoroquinolone susceptibility (Leclercq, 2001).

Aquaculture grade antimicrobial chemicals from marine microbes

Disease caused by bacterial pathogens has been widely recognized as a major cause of economic loss in many commercially cultured fish and shellfish species in India, with mortality of larval stages in hatcheries and the growing stages in different mariculture systems. Pathogenic vibrios are involved in significant mortalities in the larviculture and growout phases of famed finfish and shellfishes. In an attempt to control the proliferation of pathogenic vibrios, the prophylactic and therapeutic use of antibiotics has been practiced in commercial hatcheries, creating more serious problem of antibiotic resistance among the microflora in the environment. With safety concerns about synthetic antibiotics, considerable interest has arisen in finding alternative natural sources (Gomez-Gil et al., 2000). Screening and development of aquaculture-grade chemicals from bacterial flora could be a highly promising approach to produce these bioactive moleules. Members of the genus *Pseudomonas* and *Bacillus* either free living or associated with marine flora are common beneficial bacterial candidates, and are known to produce a wide range of secondary metabolites (Raaijmakers et al., 1997) inhibiting a wide range of pathogenic bacteria (Rengpipat et al., 1998). The metabolites 6-oxo-de-O-methyllasiodiplodin, (E)-9-etheno-lasiodiplodin, lasiodiplodin, de-O-methyllasiodiplodin, and 5-hydroxy-de-O-methyllasiodiplodin, were isolated from the mycelium extracts of a microbe obtained from South China Sea (Yang *et al*., 2006). Studies conducted at CIBA, Chennai isolated two bacteria, *Pseudomonas* sp. PM 11 as potential candidate probionts from a pool of bacteria isolated from gut of farm reared sub-adult shrimp (Alavandi *et al*, 2004). Marine bacterial strain, *Pseudomonas* I-2, producing inhibitory compounds against shrimp pathogenic vibrios including *Vibrio harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus* was reported by Chaitanya *et al* (2002). Bioactive compounds were isolated from a marine bacterium *Bacillus circulans* (Chakraborty *et al*., 2010). Labda-14-ene-3a,8a-diol and labda-14-ene-8a-hydroxy-3-one were found to be inhibitory to the growth of *Vibrio parahaemolyticus* with minimum inhibitory concentrations of 30-40 µg/mL (Chakraborty *et al.*, 2010), and their structures have been elucidated by ^{1}H NMR and ^{13}C NMR spectra, including 2D NMR. Several bacterial flora were isolated from marine ecosystem (*Bacillus subtilis*, *Bacillus amyloliquifaciens*, *Pseudomonas putida,* and *Pseudomonas aeroginosa*) with potential activities (> 20 mm inhibition zone) against pathogenic Vibrios (Chakraborty et al., 2010). The antibacterial component in the $CHCl_3$ fraction of *P. aerogenosa* was found to be N-substituted methyl octahydro-1-phenazinecarboxylate. The other important antibacterial molecules were found to be propyl 2-oxoacetate and phenethyl 2-oxoacetate.

About 4530 bacterial isolates were purified from seaweeds and sediments, and 23 isolates (*B. subtilis* MTCC 10402, 10403 & 10407, *B. amyloliquifaciens* 10456, *P. putida* MTCC 10458, *P. aeroginosa* MTCC 10610) were found to be potential against pathogenic *Vibrios*. *N*-substituted phenazinecarboxylate, propyl/phenethyl 2-oxoacetates were the major antibacterial molecules in bacteria.

Future perspectives

The ability of marine microorganisms to produce novel antimicrobial compounds has been well demonstrated, and clearly they have a future role in the fight against antibiotic-resistant pathogens. Ongoing research efforts to isolate and screen new marine microorganism species should be accompanied by efforts to understand their ecology. Extensive culture-dependent and -independent surveys of marine microorganisms should be prioritized to determine the extent to which marine diversity differs, e.g. is the isolation of rare microorganisms' genera from the sea merely due to the fact that terrestrial-to-sea input skews the species distribution. The isolation of seawater-obligate microorganisms has proved that marine adaptation has occurred in this lineage, but so far this property has only been identified at the genus and species level, an indication that marine adaptation is a comparatively recent evolutionary event. If such adaptation is rare within the microorganisms, it is reasonable to expect that seawater-obligate strains will represent species that have no terrestrial counterparts, and thus they are unlikely to have been previously screened for antimicrobial compounds. This raises the intriguing possibility that there are antimicrobial compounds unique to marine species. Whole-genome analysis of the genus *Salinispora* indicates hat differences in secondary metabolite biosynthetic genes may be a driver of speciation, supporting the hypothesis that new species will produce new compounds. Further analysis is needed to determine whether this property will hold as more species are described. Finally, if antimicrobial compounds are to make it from the ocean to the clinic, big pharma must re-engage in drug discovery from microbes. Currently, small pharmaceutical and biotechnology companies have been, or are currently engaged in antimicrobial discovery from marine microorganisms

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26

Fatty acids from marine fish and their implications in health and diseases

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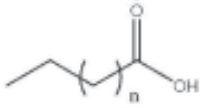
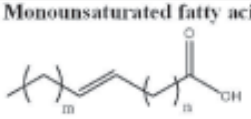
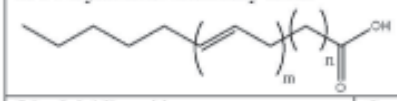
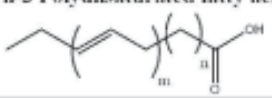
Fatty acids and their classification

Fatty acids are carboxylic acids with long hydrocarbon chains (usually C_{12-22}). Dietary fats are used to build every cell in the body and cell membranes are made of a variety of individual fatty acids. The essential fatty acids from marine fish have protective mechanisms against coronary heart disease, which became apparent in the investigations of the health status of Greenland Eskimos who consumed diets very high in fat from seals, whales, and fish, and yet had a low rate of coronary heart disease events. This paradox was explained by the fact that Eskimos consumed contained large quantities of the very-long-chain and highly polyunsaturated fatty acids with C_{20-22} carbons and 5-6 olefinic bonds, which are abundant in marine fish, and are scarce or absent in land animals and plants. Classification of fatty acids is based on to denote hydrocarbon chain length and number and positions of olefinic bonds. However, the most accepted system of classification is based on the number of olefinic bonds. Saturated fatty acids (SFAs) donot possess olefinic bonds in hydrocarbon chain. Examples of SFAs are lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, and lignoceric acid (Table 1). Monounsaturated fatty acids (MUFAs) possess one double bond, the typical examples being myristoleic acid, palmitoleic acid, elaidic acid, oleic acid, erucic acid, and nervonic acid. Fatty acids with e" 2 double bonds are termed as polyunsaturated fatty acids (PUFAs). The tetrahedral bond angles on carbon results in a molecular geometry for saturated fatty acids that is relatively linear. Olefinic bonds in hydrocarbon chain of unsaturated fatty acids results in kinks in their structure results in weak stacking. PUFAs are broadly divided into two major families' viz., ù-3 and ù-6 PUFAs (otherwise termed as n-3 and n-6 PUFAs). However, ù-3 fatty acids are found to be abundantly available in marine sources particularly fish and phytoplanktons. These fatty acids affect many physiological processes including cognitive function, visual acuity, immunosuppressive, and anti-thrombic activities along with having major role on glucose and lipid metabolism. Table 1 illustrates the details regarding the differential changes of fatty acids and their structures including their abbreviated formulae, molecular formulae, and molecular weight.

Biosynthetic route of fatty acids

Fatty acid synthesis is a metabolic process to combine eight C_2 – moieties (-$CH_3C(=O)$ group from $CH_3COSCoA$) to synthesize saturated fatty acid with C_{16}-moiety ($C_{16}H_{32}O_2$) , which thereafter modified to form homologous fatty acid analogues. These modifications include: elongase-catalyzed

Table 1. Nomenclature of fatty acids

			Abbreviated nomenclature		Molecular formulae	Molecular weight
Saturated fatty acids 	**m**	**n**	**With respect to -COOH group**	**With respect to n (or ω)-group**		
Butyric acid	0	1	4:0	4:0	$C_4H_8O_2$	88.11
Caproic acid	0	3	6:0	6:0	$C_6H_{12}O_2$	116.16
Caprylic acid	0	5	8:0	8:0	$C_8H_{16}O_2$	144.21
Capric acid	0	7	10:0	10:0	$C_{10}H_{20}O_2$	172.26
Undecanoic acid	0	8	11:0	11:0	$C_{11}H_{22}O_2$	186.29
Lauric acid	0	9	12:0	12:0	$C_{12}H_{24}O_2$	200.32
Tridecanoic acid	0	10	13:0	13:0	$C_{13}H_{26}O_2$	214.34
Myristic acid	0	11	14:0	14:0	$C_{14}H_{28}O_2$	228.37
Pentadecanoic acid	0	12	15:0	15:0	$C_{15}H_{30}O_2$	242.4
Palmitic acid	0	13	16:0	16:0	$C_{16}H_{32}O_2$	256.42
Heptadecanoic acid	0	14	17:0	17:0	$C_{17}H_{34}O_2$	270.45
Stearic acid	0	15	18:0	18:0	$C_{18}H_{36}O_2$	284.48
Arachidic acid	0	17	20:0	20:0	$C_{20}H_{40}O_2$	312.53
Heneicosanoic acid	0	18	21:0	21:0	$C_{21}H_{42}O_2$	326.53
Behenic acid	0	19	22:0	22:0	$C_{22}H_{44}O_2$	340.58
Tricosanoic acid	0	20	23:0	23:0	$C_{23}H_{46}O_2$	354.61
Lignoceric acid	0	21	24:0	24:0	$C_{24}H_{48}O_2$	368.64
Monounsaturated fatty acids 						
Myristoleic acid	2	7	$14:1\Delta^{9}$	14:1n9	$C_{14}H_{26}O_2$	226.36
Cis-10-Pentadecenoic acid	2	8	$15:1\Delta^{10}$	15:1n10	$C_{15}H_{28}O_2$	240.36
Palmitoleic acid	4	7	$16:1\Delta^{9}$	16:1n9	$C_{16}H_{30}O_2$	254.41
Cis-10-Heptadecenoic acid	5	8	$17:1\Delta^{10}$	17:1n10	$C_{17}H_{32}O_2$	268.43
Elaidic acid	7	7	$18:1\Delta^{9}$	18:1n9 trans	$C_{18}H_{34}O_2$	282.46
Oleic acid	7	7	$18:1\Delta^{9}$	18:1n9 cis	$C_{18}H_{34}O_2$	282.46
Cis-11-eicosenoic acid	7	9	$20:1\Delta^{11}$	20:1n11	$C_{20}H_{38}O_2$	310.51
Erucic acid	7	11	$14:1\Delta^{13}$	22:1n13	$C_{22}H_{42}O_2$	338.57
Nervonic acid	7	13	$14:1\Delta^{15}$	24:1n15	$C_{24}H_{46}O_2$	366.62
n-6 Polyunsaturated fatty acids 						
Linolelaidic acid	2	6	$18:2\Delta^{9,12}$	18:2n6 trans	$C_{18}H_{32}O_2$	280.45
Linoleic acid	2	6	$18:2\Delta^{9,12}$	18:2n6 cis	$C_{18}H_{32}O_2$	280.45
γ-Linolenic acid	3	3	$18:2\Delta^{6,9,12}$	18:3n6	$C_{18}H_{30}O_2$	278.43
Cis-11,14-Eicosadienoic acid	2	8	$20:2\Delta^{11,14}$	20:2n6	$C_{20}H_{36}O_2$	308.5
Cis-8,11,14-Eicosatrienoic acid	3	5	$20:2\Delta^{8,11,14}$	20:3n6	$C_{20}H_{34}O_2$	306.48
Arachidonic acid	4	2	$20:2\Delta^{5,8,11,14}$	20:4n6	$C_{20}H_{32}O_2$	304.47
Cis-13,16-Docosadienoic acid	2	10	$22:2\Delta^{13,16}$	22:2n6	$C_{22}H_{40}O_2$	336.55
n-3 Polyunsaturated fatty acids 						
Linolenic acid	3	6	$18:2\Delta^{9,12,15}$	18:3n3	$C_{18}H_{30}O_2$	278.43
Cis-11,14,17-Eicosatrienoic acid	3	8	$20:2\Delta^{11,14,17}$	20:3n3	$C_{20}H_{34}O_2$	306.48
Cis-5,8,11,14,17-Eicosapentaenoic acid	5	2	$20:2\Delta^{5,8,11,14,17}$	20:5n3	$C_{20}H_{30}O_2$	302.45
Cis-4,7,10,13,16,19-Docosahexaenoic acid	6	1	$22:2\Delta^{4,7,10,13,16,19}$	22:6n3	$C_{22}H_{32}O_2$	328.49

chain elongation to synthesize fatty acids with longer hydrocarbon chain, e.g., stearic acid ($C_{18}H_{36}O_2$), arachidic acid ($C_{20}H_{40}O_2$), and so on. These SFAs, on desaturation yield unsaturated fatty acid analogues. In general, fatty acid synthesis takes place in cytoplasm of liver, adipose, central nervous system, and lactating mammary gland tissues of human. Glycolytic breakdown of glucose y**ields acetyl CoA through** pyruvate ($CH_3COCOOH$) by aerobic glycolysis that is starting material for fatty acid synthesis. Acetyl CoA serves as substrate to synthesize citrate that transported out of mitochondria to cytosol and generates acetyl CoA. The overall reaction of anabolism of fatty acids to form unsaturated faty acids is as follows:

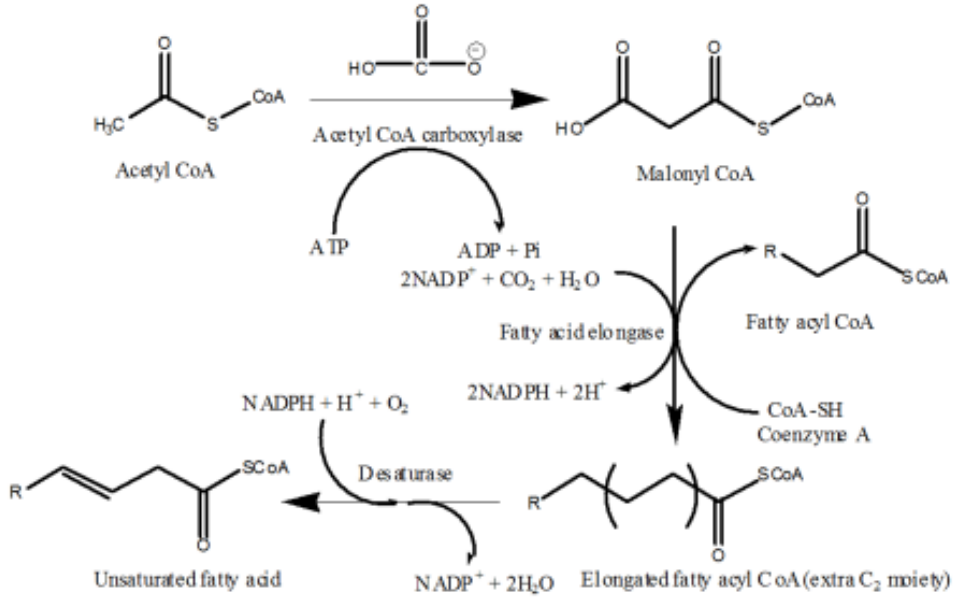


Figure 1. Synthesis of unsaturated fatty acids from acetyl CoA

Fatty acids are stored in adipocytes as triacylglycerol that must be hydrolyzed to release free fatty acids.

Polyunsaturated fatty acids and their importance in health and disease

To prevent cancer

Among dietary factors postulated to influence cancer development are long chain polyunsaturated ù-3 fatty acids, found in fish. Earlier studies revealed inverse relation between marine fatty acid consumption and mortality rates of prostate (Hebert et al., 1998) and breast cancer (Hebert et al., 1996). The mechanisms proposed how the intake of marine fatty acids might lower the risk of cancer is the inhibition of eicosanoid biosynthesis from AA, a ù-6 fatty acid. Prostaglandins converted from AA by the cyclooxygenase-2 enzyme, notably PGE_2, have been linked to carcinogenesis viz., mammary tumor development, proliferation of breast and prostate cancer (Erickson, 1986). Tumor cells typically produce large amounts of AA-derived PGE2, which may impede immune system function, possibly through their role in the generation of suppressor T cells (Erickson, 1986). Marine fatty acids were reported to inhibit cyclooxygenase-2 and the oxidative metabolism of AA to PGE2. EPA and DHA also inhibit lipoxygenases which metabolize AA to HETEs and leukotrienes. 12-HETE

has been linked to the suppression of apoptosis, stimulation of angiogenesis, stimulation of tumor cell adhesion, and expression of the invasive phenotype. It is apparent that both EPA and DHA can inhibit the biological activity of eicosanoids and androgens (Liang et al., 1992), which are known to have a stimulating effect on cell growth and uncontrolled cell proliferation (Ghosh & Myers, 1997). It is well established that in animal models and in human cancer cell lines, EPA and DHA were found to suppress cell growth. However, because intakes of fish and marine fatty acids are highly correlated, it is difficult to disentangle the effect of fatty acids from the effect of fish *per se*.

To combat atherosclerosis and cardiovascular diseases

Eating ù-3 fatty acids abundantly available in marine fish were reported to protect human beings from heart failure (*European Heart Journal*. doi:10.1093/eurheartj/ehp111). Researchers in the USA and Sweden followed 39,367 Swedish men, aged between 45-79, from 1998 to 2004. They recorded details of the men's diet and tracked the men's outcome through Swedish inpatient and cause-of-death registers. PUFAs in the diet have long been considered essential to the growth and proper nutrition of humans and other vertebrates. It was reported that atherosclerosis and thrombosis represent essential fatty acid deficiencies, but rather that the polyunsaturated fat may affect these pathological processes through other mechanisms. There is evidence from epidemiology that marine n-3 PUFA is associated with a reduced risk of coronary heart disease. This was originally found in Greenland Eskimos with an extremely high intake of n-3 PUFA (10–14 g/day) and later also reported in several other populations (Schmidt et al., 2005; Kris-Etherton et al., 2002) including Western populations with an average intake of marine n-3 PUFA below 0.2–0.4 g/day. Recently, a meta-analysis was published on fish consumption and CHD mortality from 13 cohort studies including a total of 222,364 individuals with an average of 11.8 years of follow-up (He et al., 2004). Fish consumption was inversely related with fatal CHD and sudden cardiac death (He et al., 2004).

Estimation of fatty acids in laboratory

Broadly fatty acid estimation is divided under the broad categories, viz., (1) lipid extraction and acid-catalyzed transesterification of fatty acid to methyl esters (FAMEs) and *N*-acyl pyrrolidides; and (3) gas-liquid chromatography and gas chromatography-mass spectrometry (GC/MS) analysis of FAMEs. Below are illustrated the details under each head.

Lipid extraction

Lipid from the crude sardine oil was extracted by using $CHCl_3$-CH_3OH-H_2O (Bligh, & Dyer, 1959). In brief, about 10 g tissue together with chloroform methanol mixture (2:1) ratio is homogenized, and $CHCl_3$-CH_3OH mixture (15 times) was added and mixed (to 1/3rd of the total volume). The resulting solution was filtered, and the filtrate was collected. The process was repeated two more times with rest of the $CHCl_3$-CH_3OH mixture. To the filtrate, add distilled water (20% of the total volume of the filtrate) and leave overnight. The water-soluble residue diffuses away from the solvent and occupies the top position in the separating funnel. Solvent containing lipid (bottom layer) is collected by filtering through anhydrous Na_2SO_4. Evaporate to dryness and make up the volume using $CHCl_3$. On extraction with $CHCl_3$-CH_3OH, lipid (bottom layer) is separated from the sample and is collected by filtering through anhydrous Na_2SO_4. After saponification of the dried extract PUFA is determined using gas chromatograph as illustrated below.

Extraction and derivatization of fatty acids to fatty acid methyl esters (FAME) and *N*-acyl pyrrolidides

The lipid extract thus obtained was saponified with 0.5 N KOH in CH_3OH. After removal of the nonsaponifiable material with *n*-hexane and acidification with 1 N HCl, the saponifiable materials were extracted with petroleum etherdiethyl ether (1:1 v/v) and transesterified to furnish fatty acid methyl esters (FAME) by reaction (30 min under reflux) with a methylating mixture (14% BF_3/ CH_3OH, 5 mL) in a boiling water bath under an inert atmosphere of N_2 (Metcalf, Schimtz, & Pleka, 1966). The FAME thus obtained was cooled to ambient temperature, and distilled water (20 mL) was added. The solution was extracted with *n*-hexane (10 mL X 6), and the upper *n*-hexane layer was removed and concentrated under an inert atmosphere of N_2. The resulting FAME concentrate was reconstituted in petroleum ether, flushed with N_2 in glass vials, and stored in deep freeze (-20^{o}C) until required for GC/GC-MS analyses. Analysis was performed in triplicate.

Gas-liquid chromatography and gas chromatography-mass spectrometry (GC/MS) analysis of fatty acid derivatives

Quantitative and qualitative analyses of FAME obtained by transesterification were performed on gas chromatograph using a flame ionization detector (FID). FAMEs were identified by comparison of retention times with the known standards. In another process, FAMEs were derivatized to *N*-acyl pyrrolidides by condensation of fatty acid methyl ester with a mixture of pyrrolidine (1 mL) and acetic acid (0.1 mL) at 100 ?C under reflux (2 h) for GC-MS analyses (Andersson, 1978). The GC-MS analyses need to be performed by GC interfaced with mass spectrometer for confirmation of fatty acid identification.

Mass Spectroscopic Analyses of FAME Derivatives

The following are the mass spectrometric data of FAME derivatives.

Methyl Palmitate. EI-MS *m/z* (relative intensity, %): 270 (M+, 61.11), 239 (15.74), 227 (31.48), 213 (7.41), 199 (14.81), 185 (12.96), 171 (12.96), 157 (7.41), 143 (31.48), 129 (11.11), 87 (74.07), 74 (*100*), 55 (18.52).

Methyl Oleate. EI-MS *m/z* (relative intensity, %): 296 (M+, 20.00), 111 (76.67), 264 (33.33), 222 (26.67), 180 (18.33), 166 (23.33), 152 (23.33), 123 (23.33), 110 (38.33), 97 (75.00), 83 (70.00), 74 (66.67), 69 (78.33), 55 (*100*).

Methyl Linoleate. EI-MS *m/z* (relative intensity, %): 294 (M+, 52.46), 263 (24.59), 220 (8.20), 178 (13.11), 164 (19.67), 150 (21.31), 136 (18.03), 123 (18.85), 109 (37.70), 95 (70.49), 81 (*100*), 67 (91.80), 55 (50.82).

Methyl Linolenate. EI-MS *m/z* (relative intensity, %): 292 (M+, 16.67), 261 (5.00), 236 (6.67), 173 (6.67), 163 (6.67), 149 (20.00), 135 (20.00), 121 (25.00), 108 (56.67), 95 (58.33), 79 (*100*), 67 (56.67), 55 (35.00).

Methyl Arachidonate. EI-MS *m/z* (relative intensity, %): 318 (M+, 1.82), 290 (1.82), 264 (1.82), 175 (5.45), 150 (7.27), 133 (7.27), 105 (30.91), 91 (70.91), 79 (*100*), 67 (80.00), 55 (49.09).

Methyl Eicosapentaenoate. EI-MS *m/z* (relative intensity, %): 315 (M+, 1.67), 175 (6.67), 161 (8.33), 145 (11.67), 131 (18.33), 119 (31.67), 108 (31.67), 91 (70.00), 79 (*100*), 67 (68.33), 55 (48.33).

Methyl Docosahexaenoate. EI-MS *m/z* (relative intensity, %): 342 (M+, 0.60), 145 (4.20), 131 (6.60), 119 (10.80), 108 (11.40), 91 (28.20), 79 (*100*), 67 (20.40). (Chakraborty et al, 2010).

Mass Spectroscopic Analyses of *N*-Acyl Pyrrolidide Derivatives

The following are the mass spectrometric data of *N*-acyl pyrrolidide derivatives.

1-(Pyrrolidin-1-yl)hexadecan-1-one/Palmitoylpyrrolidine. EI-MS *m*/*z* (relative intensity, %): 309 (M+, 16.00), 294 (2.00), 168 (8.00), 140 (10.00), 126 (16.00), 113 (*100*), 98 (8.00), 70 (12.00), 55 (14.00).

1-(Pyrrolidin-1-yl)octadec-9-en-1-one. EI-MS *m*/*z* (relative intensity, %): 335 (M+, 27.56), 250 (8.62), 236 (10.34), 208 (6.90), 196 (5.17), 182 (12.07), 126 (53.45), 113 (*100*), 98 (18.97), 85 (8.62), 72 (20.69), 55 (27.59).

1-(Pyrrolidin-1-yl)octadeca-9,12-dien-1-one. EI-MS *m*/*z* (relative intensity, %): 333 (M+, 77.97), 290 (10.17), 236 (15.25), 222 (20.34), 182 (16.95), 168 (15.25), 140 (22.03), 126 (44.07), 113 (*100*), 98 (25.42), 70 (42.37), 55 (49.15).

1-(Pyrrolidin-1-yl)octadeca-9,12,15-trien-1-one. EI-MS *m*/*z* (relative intensity, %): 331 (M+, 44.00), 182 (22.00), 168 (24.00), 140 (26.00), 126 (60.00), 113 (*100*), 98 (30.00), 72 (64.00), 55 (42.00).

1-(Pyrrolidin-1-yl)icosa-5,8,11,14-tetraen-1-one. EI-MS *m*/*z* (relative intensity, %): 357 (M+, 18.97), 232 (10.34), 180 (10.34), 126 (13.79), 113 (*100*), 85 (17.24), 70 (22.41), 55 (27.59).

1-(Pyrrolidin-1-yl)icosa-5,8,11,14,17-pentaen-1-one. EI-MS *m*/*z* (relative intensity, %): 355 (M+, 3.85), 286 (7.69), 232 (7.69), 126 (13.46), 113 (*100*), 85 (17.31), 72 (26.92), 55 (21.15).

1-(Pyrrolidin-1-yl)octadeca-9,12-dien-1-one. EI-MS *m*/*z* (relative intensity, %): 381 (M+, 3.91), 312 (7.05), 272 (7.29), 232 (16.22), 218 (15.76), 192 (8.24), 166 (23.67), 153 (22.85), 113 (*100*), 98 (46.62), 72 (21.98) (Chakraborty et al, 2010).

Conclusions

Research on exploring sources long-chain PUFAs, viz., DHA, EPA, and AA for use in nutrition have received considerable attention. These PUFAs, which are usually low in abundance in human, are regarded as essential and must be supplied in diet. The importance of PUFAs in human nutrition has been extensively investigated during the past 20 years. DHA is one of the important PUFAs, which maintains structural and functional integrity in larval cell membranes in addition to the neural development and function, while AA and EPA are involved in, respectively, the production and modulation of eicosanoids. Docosahexaenoic acid (22:6ù-3), which is a vital component of the phospholipids of cellular membranes, especially in the brain and retina, is necessary for their proper functioning. The ù-3 fatty acids favorably affect atherosclerosis, coronary heart disease, inflammatory disease, and perhaps even behavioral disorders. Membrane fluidity is essential for proper functioning of these tissues. In the retina, where ù-3 fatty acids are especially important, deficiency can result in decreased vision and abnormal electroretinogram results. The ù-3 fatty acids are essential fatty acids, necessary from conception through pregnancy and infancy and, undoubtedly, throughout life. AA has been an essential function of producing eicosanoids, making it an essential fatty acid because prostaglandins (PGF2R) are produced from 20:4n6, and has roles in reproduction. AA is the basis for cyclo-oxygenase (COX) action to produce PGF2R. AA, being a major component of phosphoinositol, was reported to have a vital role in the transduction signal mechanism. An imbalance in ù-3/ù-6 ratio can accentuate ù-3 fatty acid deficiency state, as shown by earlier studies. The ratio may have increased in industrialized societies because of increased consumption of vegetable oils

rich in ù-6 fatty acids, ie, linoleic acid (18:2n26), and reduced consumption of foods rich in ù-3 fatty acids. Another important feature of ù-3 fatty acids is their role in the prevention and modulation of certain diseases that are common (Importance of n23 fatty acids in health and disease (*W. E. Connor Am J Clin Nutr* 2000;71(suppl):171S–5S). Below is appended a partial list of diseases that may be prevented or ameliorated with ù-3 fatty acids:

- Coronary heart disease and stroke
- Cancers of the breast, colon, and prostate
- Retinal and brain development);
- Immunostimulant
- Hypertension

The first two functions are extremely important and are related directly or indirectly with other diseases as listed earlier.

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Instrumental Methods in Bioprospecting: Gas Liquid Chromatography

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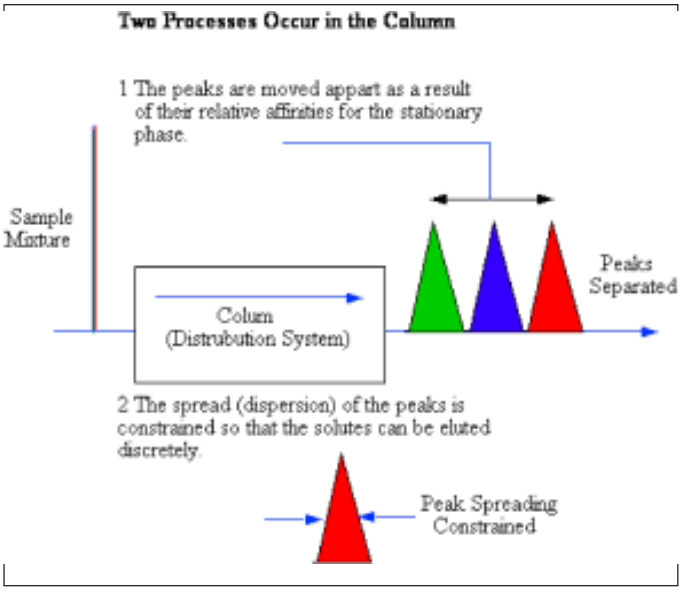
Chromatography: An Overview

Chromatography, although primarily a separation technique, is mostly employed in chemical analysis. Nevertheless, to a limited extent, it is also used for preparative purposes, particularly for the isolation of relatively small amounts of materials that have comparatively high intrinsic value. In a single step process it can separate a mixture into its individual components and simultaneously provide an quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature and can range in complexity from a simple blend of two entantiomers to a multi component mixture containing widely differing chemical species. The first scientist to recognize chromatography as an efficient method of separation was the Russian botanist Tswett, who used a simple form of liquid-solid chromatography to separate a number of plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography for this type of separation (color writing). Although color has little to do with modern chromatography, the name has persisted and, despite its irrelevance, is still used for all separation techniques that employ the essential requisites for a chromatographic separation, viz. a mobile phase and a stationary phase. Today, chromatography is an extremely versatile technique; it can separate gases, and volatile substances by gas chromatography (GC), in-volatile chemicals and materials of extremely high molecular weight (including biopolymers) by liquid chromatography (LC). Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase; *ipso facto* a separation is achieved.

In practice, the distribution system, (that part of the chromatographic apparatus where the solutes are distributed between the phases) can take the form of a column such as a tube packed with particulate matter on which the stationary phase is bonded or coated. The mobile phase (which may be a gas or a liquid) passes under pressure through the column to elute the sample. The column form may also be a long, small-diameter open tube that has the stationary phase coated or bonded to the internal surface. Alternatively, the chromatographic system may take the form of a plate (usually glass) the surface of which is loaded with particulate matter to which the stationary

phase is coated or bonded. The mobile phase (a liquid) is arranged to percolate up the plate (usually by surface tension forces) to elute the sample. The sample is injected into the mobile phase stream just before the front of the columns. The column is designed to allow two processes to take place that will produce the separation. Firstly, as a result of different forces between each molecular type and the stationary phase, each solute is retained to a different extent and, thus, the more weakly held will elute first and the more strongly held elute last. The process is diagramatically depicted below.



Classification of Chromatography

As all chromatographic separations are carried out using a *mobile* and a *stationary* phase, the primary classification of chromatography is based on the physical nature of the *mobile* phase. The mobile phase can be a gas or a liquid which gives rise to the two basic forms of chromatography, namely, gas chromatography (GC) and liquid chromatography (LC).

Table 1 The Classification of Chromatography

Mobile phase	Stationary phase
Gas	Liquid
Gas Chromatography (GC)	Liquid Gas-liquid chromatography (GLC) SolidGas Solid Chromatography (GSC)
LiquidLiquid chromatography (LC)	LiquidLiquid –liquid chromatography (LLC) SolidLiquid solid chromatography (LSC)

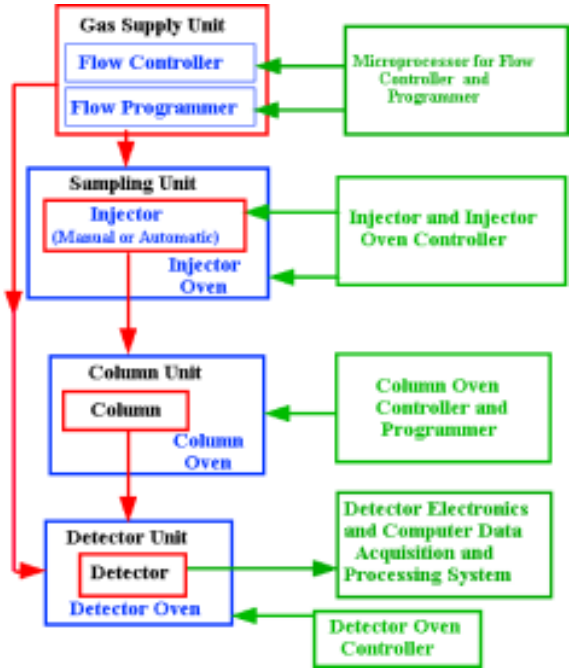
The stationary phase can also take two forms, solid and liquid, which provides two subgroups of GC and LC, namely; gas–solid chromatography (GSC) and gas–liquid chromatography (GLC), together with liquid solid chromatography (LSC) and liquid chromatography (LLC). The different forms of chromatography are summarized in Table 1. Most thin layer chromatography techniques are considered liquid-solid systems although the solute normally interacts with a liquid-like surface coating on the adsorbent or support or, in some cases an actual liquid coating.

Gas Liquid Chromatography

Gas-liquid chromatography (GLC) was in invented by James and Martin and is a chromatography separation technique in which the mobile phase is a gas (usually helium or nitrogen) and the stationary phase is a liquid. In the original columns used by James and Martin, the liquid stationary phase was adsorbed on the surface of an inert support such as Celite (a diatomateous earth) or calcined Celite (a form of brick dust). The support was usually deactivated before use by acid treatment and

subsequent reaction with hexamethyldisilazane. The technique was extensively used for the separation of a wide range of volatile substances including fatty acids.

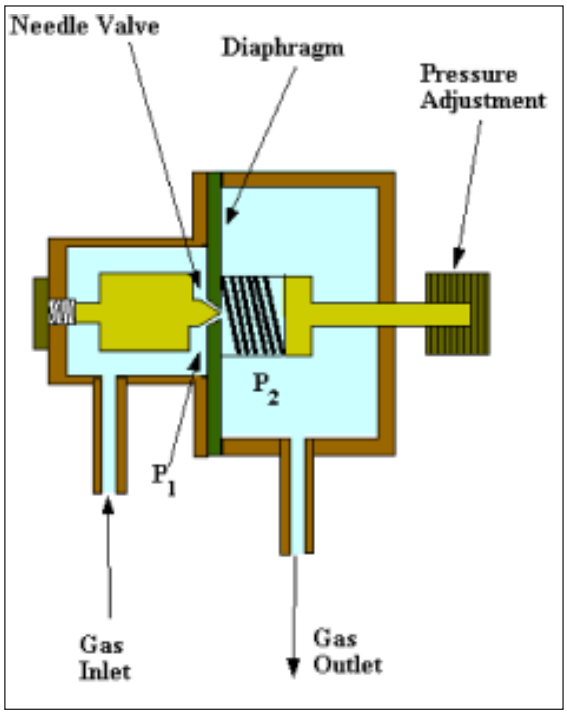
The modern gas chromatograph is a fairly complex instrument mostly computer controlled. The samples are mechanically injected, the analytical results are automatically calculated and the results printed out, together with the pertinent operating conditions in a standard format. However, the instrument has evolved over many years although the majority of the added devices and techniques were suggested or describe in the first three international symposia on gas chromatography held in 1956, 1958 and 1960. The layout of the modern gas chromatograph is shown as a block diagram:



The different components of GLC

Gas supplies

Gases (carrier gas-N_2 or He; and fuel gas-air and H_2) for use with the gas liquid chromatography were originally all obtained from gas cylinders fitted with reducing valves that are set to supply the gas to the instrument at the recommended pressure defined by the manufacturers. The reducing valves on the gas tanks are examples of simple pressure controllers and the flow controllers that are used for detector and column flow control often involve devices based on the same principles. The pressure controller consists essentially of two chambers separated by a diaphragm, in the center of which is a needle valve that is actuated by the diaphragm. The diaphragm is held down by a spring that is adjustable so that the pressure in the second chamber, and thus the outlet flow, can be set at any chosen value. When gas enters the lower chamber, the pressure on the lower part of the diaphragm acts against the spring setting, and opens the valve. Gas then passes into the upper chamber and pressure is built up in the upper chamber to the value that has been set at which time the diaphragm moves downward closing the valve. If the pressure falls in the upper cylinder, the



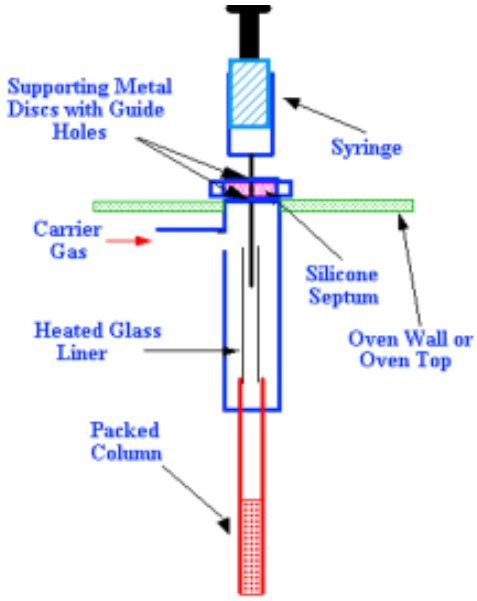
Pressure controller

diaphragm again moves upward due to the pressure in the lower chamber, which opens the valve and the pressure in the upper chamber is brought back to its set value.

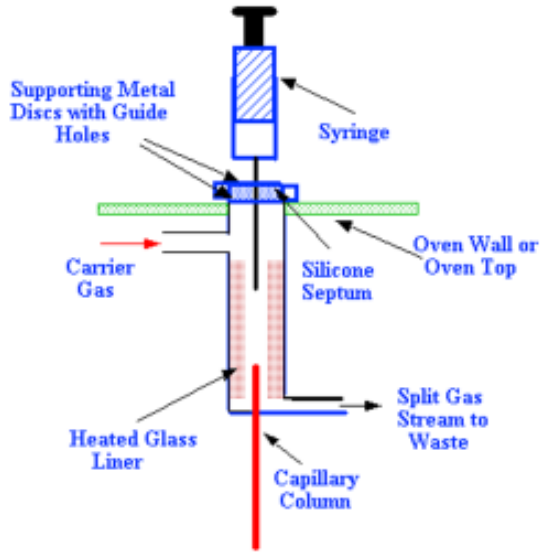
Injectors

The sample is injected by a hypodermic syringe, through a silicone rubber septum directly into the column packing or into a flash heater. An example of a septum injection system used for packed columns is shown in following figure. The silicone septum is compressed between metal surfaces in such a manner that a hypodermic needle can pierce it, but when it is withdrawn the hole is closed as a result of the septum compression and there is no gas leak. The glass liner prevents the sample coming in contact with the heated metal wall and thus, reduces the chance of thermal decomposition. The glass liner can be fitted with a separate heater and the volatilization temperature can, thus, be controlled. By using a syringe with a long needle, the tip can be made to penetrate past the liner and discharge its contents directly into the column packing. This procedure is called 'on-column injection' and, as it reduces peak dispersion on injection and thus, provides higher column efficiencies, is often the preferred procedure.

The basic difference between the two types of injection systems is that the capillary column now projects into the glass liner and a portion of the carrier gas sweeps past the column inlet to waste. As the sample passes the column opening, a small fraction is split off and flows directly into the capillary column, *ipso facto* this device is called a split injector. The split ratio is changed by regulating the portion of the carrier gas that flows to waste which is achieved by an adjustable flow resistance in the waste flow line. This device is only used for small diameter capillary columns where the charge size is critical. Consequently, quantitative analyses carried out using the high efficiency small diameter capillary columns may have limited accuracy and precision, depending on the nature of the sample.



Packed Column Injector



Split Injection System

GLC Columns

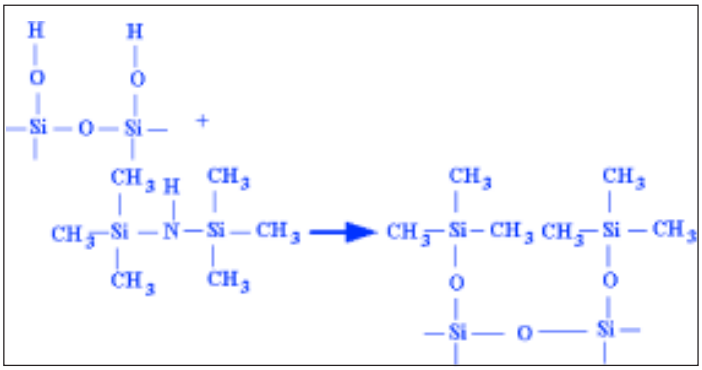
There are two types of columns in common use in GC and they are the conventional packed column and the open tubular column. The former are usually 2 to 4 mm I.D. and 1-4 m long and, packed with a suitable adsorbent, are mostly used for gas analysis. As a result of the simpler injection procedure and the more precise sampling method, the packed column tends to give greater quantitative accuracy and precision. However, despite its problems with sample injection, the open tubular column is seen as the 'state of the art' column and is by far the most popular column system in general use. The length of open tubular or capillary columns range from about 10-100 m and can have internal diameters from 100-500 ìm. The stationary phase is coated on the internal wall of the column as a film 0.2-1 ìm thick.

The Packed GC Column

Packed columns are usually constructed from stainless steel or Pyrex glass. Pyrex glass is favored when thermally labile materials are being separated such as essential oils and flavor components. Longer columns can be U-shaped but columns more than a meter long are usually coiled. Glass columns are sometimes treated with an appropriate silanizing reagent to eliminate the surface hydroxyl groups which can be catalytically active or produce asymmetric peaks.

Supports for GLC

There have been a number of materials used as supports for packed GC columns including, Celite (a proprietary form of a diatomaceous earth), fire-brick (calcined Celite), fire-brick coated with metallic silver or gold, glass beads, Teflon chips and polymer beads. Today however, the vast majority of contemporary packed GLC columns are filled with materials that are either based on of Celtic or polystyrene beads as a support. There are two processes used to modify Celite. One was to crush, blend and press the Celite into the form of a brick and then calcine it at a temperature of about 900ÚC. Under these conditions some of the silica is changed into cristobalite and traces of iron and other heavy metals interact with the silica causing the material to become pink in color. This material is sold under the trade name of Chromosorb P. The second process involves mixing the Celite with sodium carbonate and fluxing the material at 900ÚC. This causes the structure of the Celite to be disrupted and the fragments adhere to one another by means of glass formed from the silica and the sodium carbonate. As the original Celite structure is disrupted, the material exhibits a wide range of pore sizes which differs significantly from the material that was calcined in the absence of sodium carbonate. This materials is sold under the name of Chromosorb W together with two similar materials called Chromosorb G and Chromosorb S. The residual deleterious adsorptive properties of the support are due to silanol groups on the surface and these can be removed by silanization. The support is treated with hexamethyldisilazane which replaces the hydrogen of the silanol group with a trimethylsilyl radical. The reaction proceeds as follows,



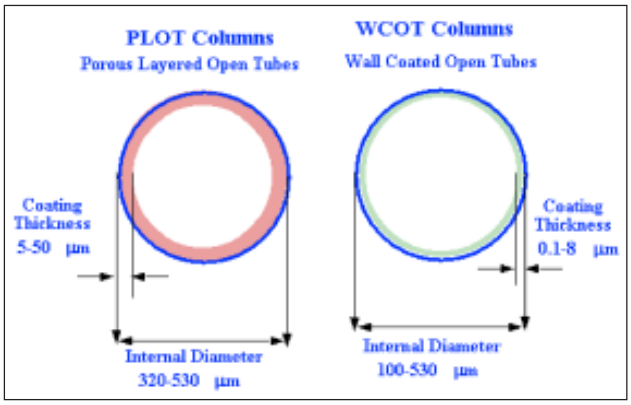
In this way the strongly polar silanol groups are methylated and assume dispersive characteristics that do not produce peak tailing. Although the major contributors to adsorption by the support are the silanol groups, a residual adsorption results from the presence of trace quantities of heavy metals such as iron, which can be largely removed by acid washing prior to silanization.

The Capillary or Open Tubular Column

Capillary columns are fabricated from stainless steel. Metal columns provide the high efficiencies expected from open tubular columns and were used for the analysis of petroleum, fatty acids and fuel oils, etc. Metal columns, however, have some disadvantages as although easily coated with dispersive stationary phases (*e.g*., squalane, Apiezon grease etc.) they are not so easily coated with the more polar stationary phases such as CARBOWAX®. In addition, hot metal surfaces can cause decomposition or molecular rearrangement of many thermally labile materials such as the terpenes contained in essential oils. Metal can also react directly with some materials by chelation and adsorb polar material which results in asymmetric and tailing peaks. Nevertheless, metal columns are rugged, easy to handle and easy to remove and replace in the chromatograph consequently, their use has persisted in many application areas despite the introduction of fused silica columns.

Open Tubular Column Types

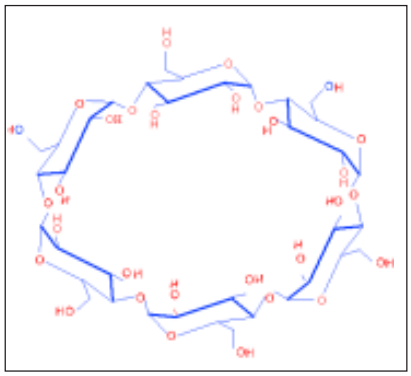
Open Tubular columns are broadly split into two classes, *the wall coated open tubular columns* or WCOT Columns (which have already been described and are by far the mot popular,) and the *porous layer open tubes* or PLOT Columns. The two types of column are shown diagramatically in the following figure. The external diameter of PLOT columns range from 320-530 ìm with a porous layer that can be 5-50 ìm thick.



Open Tubular Column Types

Chiral stationary phases

Modern organic chemistry and pharmaceutical research are becoming increasingly interested in methods of asymmetric syntheses. This enthusiasm has been provoked by the differing physiological activity that has been shown to exist between the geometric isomers of pharmaceutically active compounds. A tragic example being the drug Thalidomide, which was made available as a racemic mixture of N-phthalylglutamic acid imide. The important physiological activity resides in the R-(+)-isomer and it was not found, until too late, that the S-enantiomer was probably tetratogenic and caused serious fetal malformations. The separation and identification of isomers can, clearly, be very important and chromatography can be very effective in the resolution of such mixtures. The use of GC for the separation of asymmetric isomers is not as common as LC, but nevertheless there some very effective optically active stationary phases that



The Structure of Cyclodextrin

can be used in GC for the separation of enantiomers. Some of the more useful GC stationary phases are based on cyclodextrins already described. The columns are usually 30-60 m long 0.25 mm I.D. and have an operating temperature range of 30ÚC to 250ÚC. In order to employ the cyclodextrins as stationary phases for GC the permethylated cyclodextrins are often embedded in a siloxane matrix (e.g. 35% phenyl-65% methyl polysiloxane) which is deposited on the walls of fused quartz capillary tubes.

Derivatization of the base cyclodextrin structure can introduce groups to which only one enantiomer can interact, while the other(s) are partially or wholly entropically hindered from interaction. This increases the differential interaction between the enantiomers and the stationary phase, thus, increasing the separation ratio and hence the resolution.

Column oven and accessories

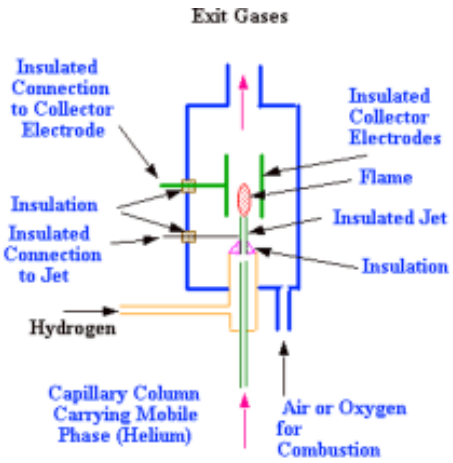
The column oven should operate over a fairly wide temperature range (*e.g.* from 5ÚC to 400ÚC). In practice, however, the maximum oven temperature needed is usually less than 250ÚC, particularly when synthetic stationary phases are being used, as many of them tend to be unstable and either decompose or volatilize at higher temperatures. Similarly, initial temperatures below 50ÚC are also rarely needed. The oven usually has air circulation driven by a powerful fan to ensure an even temperature throughout the oven. The temperature programmer (hardware and software) usually has a range of linear gradients from 0.5ÚC/min. to about 20ÚC/min. Some programmers include nonlinear programs such as logarithmic and exponential, but most GC analyses can be effectively accomplished using linear programs only. The program rate can be changed at any time in the chromatographic development or intermittent isothermal periods can be inserted where necessary in the program. The temperature programming limits are usually the same as those of the oven (*viz*. 5ÚC to 400ÚC).

GC detectors

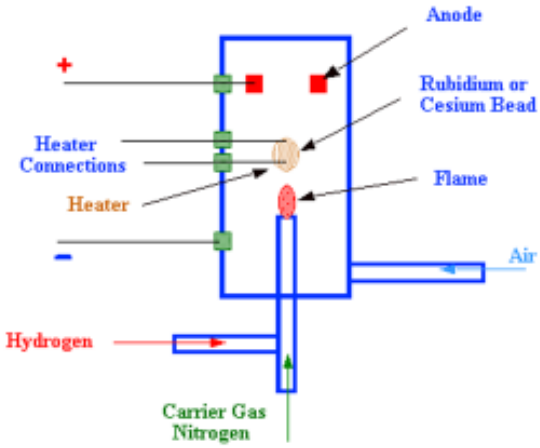
A large number of GC detectors have been developed and made commercially available. The detectors with the highest sensitivity tend to be specific and sense specific types of sample (*e.g*., halogenated substances by the electron capture detector). The detectors with a catholic response are the most popular and the majority of GC separations are monitored by the flame ionization detector (FID). The most commonly used specific detectors are the nitrogen phosphorus detector (NPD) and the electron capture detector (ECD).

Flame Ionization Detector

The FID detector employs hydrogen as the combustion gas which is mixed with the column eluent (helium, nitrogen or other appropriate gas) and burnt at a small jet situated inside a cylindrical electrode. A potential of a few hundred volts is applied between the jet and the electrode and when a carbon containing solute is burnt in the jet, the electron/ion pairs that are formed are collected at the jet and cylindrical electrode. The current is amplified and fed to a recorder or to the A/D converter of a computer data acquisition system. During the process of oxidation, oxidized or partially oxidized fragments of the solute are formed in the flame which is thought to generate electrons by thermionic emission. The background current (ions and electrons from the hydrogen flame alone) is very small (1-2 x 10^{-12} amperes) and consequently, the noise level is also commensurably small (about 10^{-14} amperes).



Flame Ionization Detector



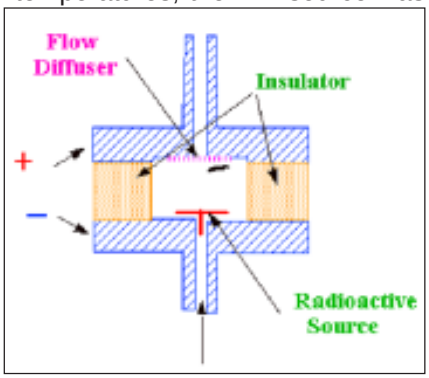
Nitrogen Phosphorus Detector

Nitrogen Phosphorus Detector

The nitrogen phosphorus detector (NPD) is a highly sensitive but specific detector and evolved directly from the FID. It gives a strong response to organic compounds containing nitrogen and/or phosphorus. Although it appears to function in a very similar manner to the FID, in fact, it operates on an entirely different principle. The actual NPD sensor is a rubidium or cesium bead contained inside a small heater coil. A potential is applied between the bead and the anode. The heated alkali bead emits electrons by thermionic emission which is collected at the anode and thus produces an ion current. When a solute containing nitrogen or phosphorus is eluted, the partially combusted nitrogen and phosphorus materials are adsorbed on the surface of the bead. This adsorbed material reduces the work function of the surface and, as consequence, the emission of electrons is increased which raises the anode current. The sensitivity of the NPD is about 10^{-12} g/ml for phosphorus and 10^{-11} g/ml for nitrogen).

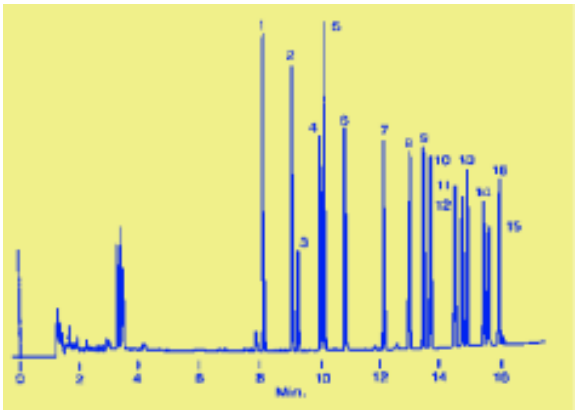
Electron Capture Detector

The electron capture detector contains a low energy â-ray source which is used to produce electrons for capturing by appropriate atoms. Although tritium adsorbed into a silver foil has been used as the â particle source, it is relatively unstable at high temperatures, the Ni^{63} source was found to be preferable. The detector can be used in two modes, either with a constant potential applied across the cell (the DC mode) or with a pulsed potential across the cell (the pulsed mode). In the DC mode, hydrogen or nitrogen can be used as the carrier gas and a small potential (usually only a few volts) is applied across the cell that is just sufficient to collect all the electrons available and provide a small standing current. If an electron capturing molecule (for example a molecule containing a halogen atom which has only seven electrons in its outer shell) enters the cell, the electrons are captured by the molecule and the molecules become charged. The mobility of the



Electron Capture Detector

captured electrons is much smaller than the free electrons and the electrode current falls dramatically. In the inactive period of the wave form, electrons having thermal energy only will attached themselves readily to any electron capturing molecules present in the cell with the consequent production of negatively charged ions. The negative ions quickly recombine with the positive ions (produced simultaneously with the electrons by the â particles) and thus become *unavailable* for collection. Consequently the standing current measured during the potential pulse will be reduced.



The basic electron capture detector consists of a small chamber one or two ml in volume enclosing two metal electrodes. The electrodes may be concentric cylinders or metal discs separated by an insulator. The cell contains the radioactive source, electrically connected to the entrance conduit and to the negative side of the power supply. A gauze "diffuser" is connected to the cell exit and to the positive side of the power supply. The output from the sensor is processed by suitable electronics and the output passed to either a potentiometric recorder of a computer data acquisition system. The electron capture detector is very sensitive, probably the most sensitive GC detector available (*ca*. 10^{-13} g/ml) and is widely used in the analysis of halogenated compounds.

1	2	3	4 Heptachlor
5	6 Aldrin	7 Heptachlor Epox.	8 Endosulphan
9 p,p'-DDE	10 Dieldrin	11 Endrin	12 p,p'-DDD
13 Endosulphan 11	14 p,p'-DDt	15 Endin Aldehyde	16Endosulp. Sulf.

Analysis of chlorinated insecticides

Data acquisition and processing

Originally, analytical results were calculated from measurements made directly on the chromatogram provided by the chart recorder. The output from the detector (which is only rarely the direct output from the detector sensor) is usually in millivolts and is suitable for direct connection to a potentiometric recorder. The output from the detector usually passes directly to a scaling amplifier that modifies the signal to a range that is appropriate for the analog-to-digital (A/D) converter. The output can alternatively pass to a potentiometric recorder and produce the chromatogram in real time. The computer system can also produce a real time chromatogram but, to do so, the data must be processed and the chromatogram presented on the printer.

Quantitative analysis

There are three important stages in a GC analysis,

1. The preparation of the sample.

2. The development of the separation and the production of the chromatogram

3. The processing of the data and the presentation of the results.

Each stage is equally important and if not carried out correctly the results will be neither precise nor accurate. Sample preparation can be very simple involving no more that diluting a known weight of sample with mobile phase or be much more complex including an extraction procedure followed by derivatization and then dilution. Liquid extraction is a clumsy procedure, particularly when used on the micro scale which is often necessary in sample preparation. An alternative procedure is solid phase extraction. The procedure is relatively simple and involves the use of a short tube packed with an appropriate adsorbent such as silica, reversed phase silica or, for some applications, macro porous polymer beads. The adsorbent must be capable of removing the substances of interest from the liquid medium.

Derivatization

GC samples are usually derivatized to render highly polar materials sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition or molecular re-arrangement. Examples of such materials that need to be derivatized are the organic acids, amides, poly hydroxy compounds, amino acids etc. In order to render such materials more volatile, they are either esterified, silanated or acetylated using one of a number of different methods of derivatization. Acids can be esterified by treating them with an appropriate alcohol using an inorganic acid to catalyze the reaction. Hydrochloric acid was popular for this purpose because it's strength was adequate and any excess could be easily removed. Other catalysts that have been found effective are trifluoroacetic acid, dichloroacetic acid, benzene sulphonic acid, *p*-toluene sulphonic acids and suphuryl and thionyl chlorides. A volatile acid is recommended such as hydrochloric acid or thionyl chloride. However, the derivative must be must be sufficiently involatile not to allow loss when removing the excess alcohol and where appropriate the catalyst itself. The Lewis acid boron trifluoride or the equivalent reagent boron trichloride is also very useful for forming ester derivatives. Boron trifluoride is supplied as a 14% solution in methanol. Boron trifluoride catalyzed reactions are very fast and can be complete in a few minutes. The esters can be extracted with *n*-hexane with vigorous shaking. Another popular esterifying reagent is diazomethane. Diazomethane is a yellow gas but is used in the form of an ethereal solution. Its reacts with an organic acid in the following manner,

$$R—COOH + CH_2N_2 \longrightarrow R—COO–CH_3 + N_2$$

When the reaction is complete, the yellow color persists and thus the reagent acts as its own indicator.

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Instrumental Methods in Bioprospecting: Spectroscopy

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Introduction

Spectroscopy is a technique that uses the interaction of energy with a sample to perform an analysis. The data that is obtained from spectroscopy is called a spectrum. A spectrum is a plot of the intensity of energy detected versus the wavelength (or mass or momentum or frequency, etc.) of the energy. A spectrum can be used to obtain information about atomic and molecular energy levels, molecular geometries, chemical bonds, interactions of molecules, and related processes. Often, spectra are used to identify the components of a sample (qualitative analysis). Spectra may also be used to measure the amount of material in a sample (quantitative analysis). Because the response of a compound to electromagnetic (EM) radiation depends on its structure, spectroscopy can be used to educate the structure of unknown chemical products. **E**M radiation behaves both as a particle of light (called a photon) and as a wave moving at the speed of light (*c*; $c = 3 \times 10^8$m/s).

Properties of EM particles and waves

1. **Wavelength (ë):** Distance between two peaks or troughs in a light wave.
2. **Frequency (í):** Number of wave cycles that pass a given point per line. Usually measured in Hertz (Hz; 1 Hz = 1 cycle/second).
3. **Energy of a photon:** E = hí = hc/ë, where h = Planck's constant = 6.6×10^{-34} J/sec.

Types of Spectroscopy

There are several types of spectroscopy, and among all these three are important for bioprospecting.

1. **Nuclear magnetic resonance (NMR) spectroscopy:** Measures interaction of radio waves with atomic nuclei in a magnetic field.
2. **Infrared (IR) spectroscopy:** Measures absorption of infrared light by chemical bonds.
3. **Ultraviolet/Visible (UV/Vis) spectroscopy:** Measures absorption of ultraviolet or visible light by π bonds.

Nuclear magnetic resonance (NMR) spectroscopy

Atomic nuclei have a "spin" associated with them (i.e., they act as if they were spinning about an axis) due to the spin associated with their protons and neutrons. Because nuclei are positively

charged, their spin induces a magnetic field. When a magnetic field is applied to atomic nuclei, the magnetic fields of the nuclei align themselves either parallel or antiparallel to the applied magnetic field. The nuclei have a slight preference for the parallel alignment, as it has a slightly lower energy, but nuclei can flip between the two possible alignments. When EM radiation with energy equal to the energy difference between the two alignments is applied to the nuclei, it induces them to flip from parallel to antiparallel alignment. Rapid flipping between alignments occurs. The nuclei are said to be in resonance, and the energy they emit when flipping from the high to the low energy state can be measured. The energy at which a given nucleus achieves resonance depends on its chemical surroundings. NMR spectra are taken by applying a magnetic field to as ample, irradiating the sample with EM radiation whose energy is varied over a given range, and measuring the energy emitted by flipping nuclei at each energy.

1. The range of radiation energies is generally chosen such that emission from only one type of nucleus (e.g., ^{1}H) in a molecule is seen.
2. NMR spectroscopy does not work for nuclei that have an even number of protons and neutrons—these nuclei have no net spin.
3. NMR spectroscopy is most commonly done on ^{1}H and ^{13}C.

Features of an NMR Spectrum

The distinguished features and terms related to NMR spectrum are as follows:

1. NMR spectra are displayed as plots of intensity of energy emission (due to resonance) versus the energy of the radiation applied to the sample. Peaks in the spectrum represent resonance energies for nuclei in a molecule.
2. **Shielding:** An electron cloud circulates around each nucleus and creates a small magnetic field opposing the applied magnetic field. The electron cloud around each atom depends on the surrounding atoms. As a result, each nucleus experiences a slightly different magnetic field (the sum of the applied field and the field from the electron cloud). For this reason, the energy at which a nucleus achieves resonance depends on its surroundings.
3. **Chemical shift (ä):** The resonance energy for a given nucleus is reported in an NMR spectrum as the difference (in parts/million) between the resonance frequency for a given proton and the resonance frequency for protons in a reference compound, which is usually tetramethylsilane, $(CH_3)_4Si$. Chemical shifts give information about the atomic surroundings of a given nucleus.
4. **Peak intensity:** The area under a peak in an NMR spectrum is proportional to the number of nuclei in a given chemical environment in a molecule (e.g., if the area under a peak is two times the area under another peak, there are twice as many nuclei responsible for the larger peak than for the smaller one). The intensity of an NMR peak gives information about the relative number of a given type of nucleus in a molecule.
5. **Spin-spin splitting:** In ^{1}H NMR, a given hydrogen nucleus interacts with hydrogen nuclei on neighboring carbon atoms such that the peak from that nucleus is split into multiple peaks called a multiplet. Relative intensities of the peaks in a multiplet follow Pascal's triangle. Spin-spin splitting gives information about the hydrogen atoms neighboring a given hydrogen nucleus.

Infrared (IR) spectroscopy: **Theory**

The principles and theory related to IR spectroscopy are detailed below:

1. Covalent bonds are similar to springs—bonded atoms vibrate (i.e., stretch and compress) and bend about their bonds. As a consequence of quantum mechanics, these bonded atoms can vibrate and bend only at frequencies that are integral multiples of a fundamental frequency that depends on the type of bond.
2. Bonds about which vibration and bending occur can absorb light if the frequency of the light wave is the same as the frequency of the movement about the bond. The frequency of light absorbed by these bonds is generally in the infrared region of the EM spectrum.
3. In IR spectroscopy, a chemical sample is irradiated with infrared light over a wide range of frequencies, and the light absorbed by the sample at each frequency is measured.
4. Bonds about which a molecule is symmetric cannot absorb IR light and therefore cannot be detected by IR spectroscopy.

Features of an IR spectrum

The distinguished features and terms related to IR spectrum are as follows:

1) IR spectra are displayed as plots of absorption versus wave number (cm^{-1}, 1/ë), which is another measure of the energy of a light wave (similar to frequency). Peaks in an IR spectrum represent wavelengths at which light was absorbed by the molecules in the sample.
2) Because each functional group that is IR-active has a characteristic set of frequencies at which it absorbs IR light, IR spectroscopy is useful in detecting the presence of specific functional groups in a molecule.
3) Because nearly all organic molecules contain C-C and C-H bonds that absorb IR light in similar ways, IR spectroscopy is most useful in identifying functional groups that contain other bonds besides C-C and C-H bonds.
4) **Fingerprint region:** Region between 1200 cm^{-1} and 1700 cm^{-1} in an IR spectrum; contains complicated absorption peaks that are characteristic of a specific molecule. An unknown compound can be identified with reasonable certainty if its fingerprint region matches that of a known compound.

Ultraviolet/Visible (UV/Vis) spectroscopy : Theory

If a molecule has ð electrons, it can absorb UV or visible light to promote one of those electrons into a higher-energy orbital. UV/Vis spectroscopy generally involves the promotion of the ð electron in the highest-energy occupied orbital to the lowest-energy unoccupied orbital. A UV/Vis spectrum is taken by irradiating a sample with UV/Vis light over a range of wavelengths and measuring the amount of light absorbed at each wavelength.

Features of UV/Vis spectra

The distinguished features and terms related to UV/Vis spectrum are as follows:

1. UV/Vis spectra are displayed as plots of absorption versus wavelength. Peaks represent wavelengths at which light was absorbed by the molecules in the sample.

2. The energy of the UV/Vis light absorbed by a ð electron system in a molecule depends on the nature of the ð system. As a result, the presence of a particular type of ð system in a molecule can be identified by UV/Vis spectroscopy.
3. The energy gap between the highest-energy occupied orbital and the lowest-energy unoccupied orbital decreases as the size of the ð electron system increases. Consequently, the wavelength of UV/Vis light absorbed by a molecule increases as the size of its conjugated ð electron system increases (because the energy of a light wave decreases with increasing wavelength).
4. UV/Vis spectroscopy is generally used on conjugated hydrocarbon systems, but other molecules containing π electron systems, such as carbonyls, are also weak absorbers of UV/Vis light.

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Amino acids from marine fish and their implications in health and diseases

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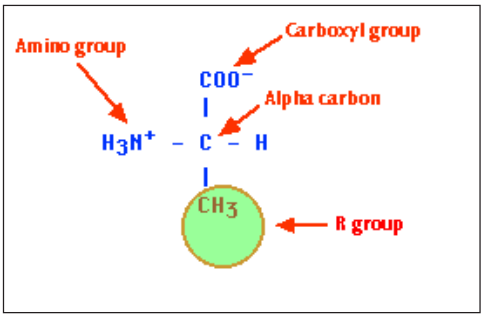
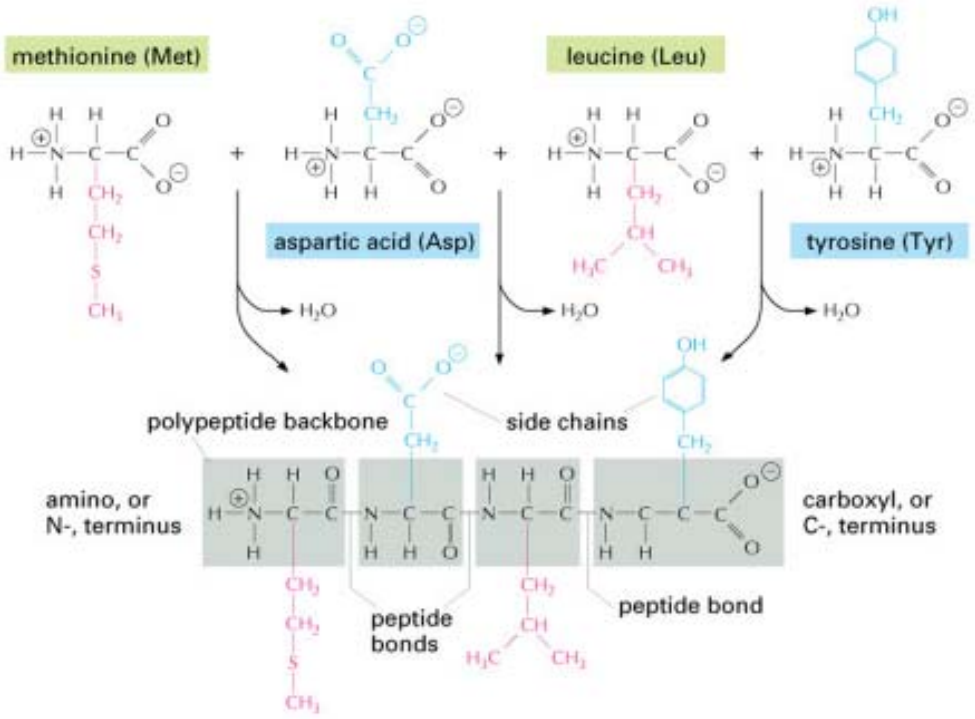
An **amino acid** is any molecule that contains both amino and carboxylic acid functional groups. Amino acid is any one of a class of simple organic compounds containing carbon, hydrogen, oxygen, nitrogen, and in certain cases sulfur. These compounds are the building blocks of proteins. Amino acids are the building blocks (monomers) of protein, and are utilized by every cell in the body for a variety of crucial functions. The shape and other properties of each protein is dictated by the precise sequence of amino acids in it. Normally, we obtain them from our food sources, particularly those high in protein; the body breaks these proteins down into their constituent parts, and then our cells use these to build the specific types of protein each of them needs. Amino acids form short polymer chains called peptides or polypeptides which in turn form structures called proteins. Each amino acid has at least one carboxyl (COOH) group, which is acidic, and one amino (NH_2) group, which is basic.

Each amino acid consists of an alpha carbon atom to which is attached

- A hydrogen atom
- An amino group (hence “amino” acid)
- A carboxyl group (-COOH). This gives up a proton and is thus an acid (hence amino “acid”)
- One of 20 different “R” groups. It is the structure of the R group that determines which of the 20 it is and its special properties. The amino acid shown here is alanine.



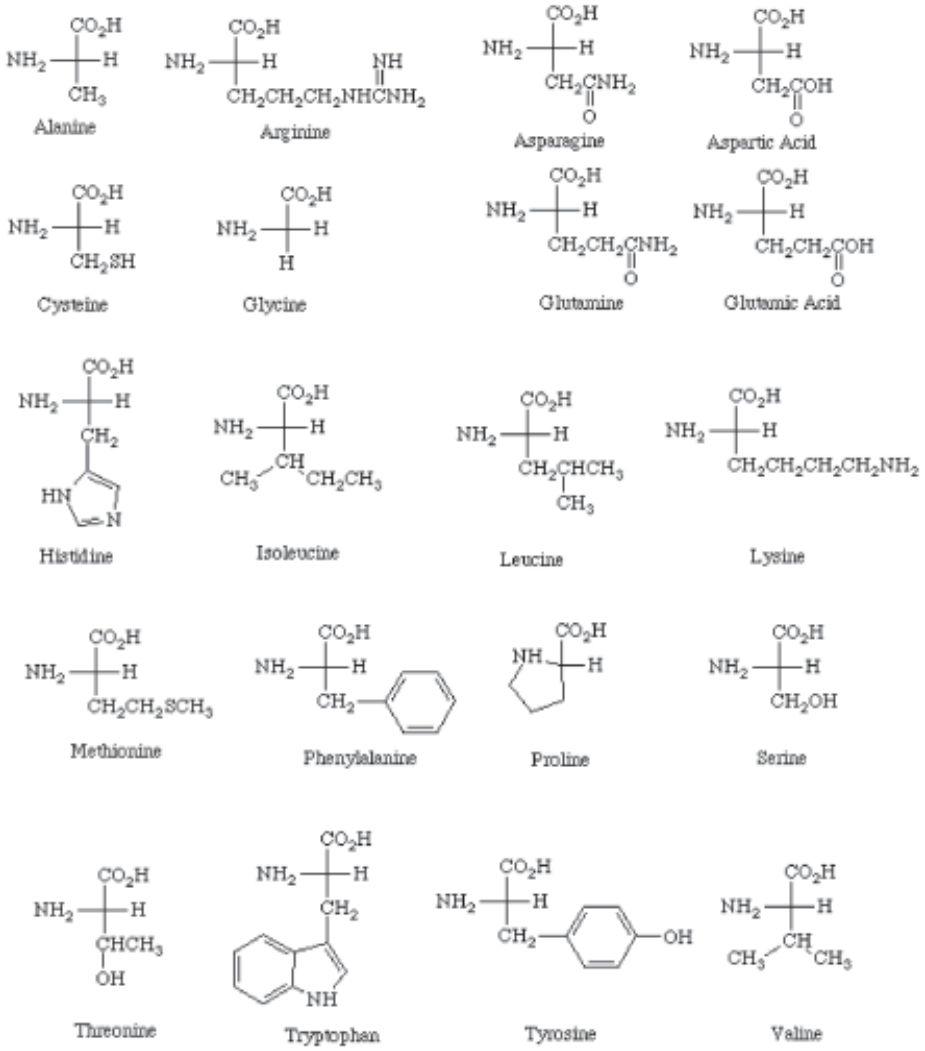
Amino acids join together in long chains, the amino group of one amino acid linking with the carboxyl group of another. The linkage is known as a peptide bond, and a chain of amino acids is known as a polypeptide. Each type of protein differs in its amino acid sequence. Thus the sequential position of the chemically distinct side chains gives each protein its individual properties. The two ends of each polypeptide chain are chemically different: the end that carries the free amino group (NH_3^+, also written NH_2) is called the amino, or N-, terminus; and the end carrying the free carboxyl group ($C00^-$, also written COOH) is the carboxyl, or C-, terminus. The amino acid sequence of a protein is always presented in the N to C direction, reading from left to right.



There are two types of amino acids: essential and nonessential. Essential ones are defined as those which the body cannot manufacture on its own and must obtain from food sources (or supplements); nonessential ones, on the other hand, can be produced by our own bodies from an available source of nitrogen and a carbon skeleton, but can also be consumed as supplements. The essential amino acids are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The nonessential amino acids are arginine, alanine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, proline, serine, and tyrosine. However, cysteine can partially meet the need for methionine (they both contain sulfur), and tyrosine can partially substitute for phenylalanine. Semi-essential amino acids are ones that can sometimes be made internally if conditions are right. Histidine is considered semi-essential because the body does not always require dietary sources of it. Other amino acids, such as carnitine, are used by the body in ways other than protein-building and are often used therapeutically.

The molecular structures of the major amino acids are listed below:

Leucine, isoleucine, and valine are called branched-chain amino acid (BCAAs) because human beings cannot survive unless these amino acids are present in the diet. The combination of these three amino acids makes up approximately one-third of skeletal muscle in the human body. In addition to their role in protein and enzyme synthesis, amino acids are extremely crucial for good health. Amino acids contribute significantly to the health of the nervous system, muscular structure, hormone production, vital organs and cellular structure. Some affects low levels of the essential amino acids result in hormonal imbalances, irritability, low concentration, and depression.



Essential amino acids and their importance

Isoleucine : Isoleucine belongs to a special group of amino acids called branched-chain amino acids (BCAAs), which are needed to help maintain and repair muscle tissue. Leucine and valine are other two branched-chain amino acids. Isoleucine is an essential amino acid that is not synthesized by mammalian tissues. Isoleucine is needed for hemoglobin formation and also helps to maintain regular energy levels. Isoleucine is important for stabilizing and regulating blood sugar and energy levels and is required through the diet as it cannot be produced by our bodies.

Leucine : Leucine is a member of the branched-chain amino acid family, along with valine and isoleucine. The branched-chain amino acids (BCAAs) are found in proteins of all life forms. Leucine ties glycine for the position of second most common amino acid found in proteins with a concentration of 7.5 percent on a molar basis compared to the other amino acids. Leucine is necessary for the optimal growth of infants and for the nitrogen balance in adults. It functions on balancing blood sugar level in the body. It also promotes in the development of the muscle tissue. It modulates the

level of hormone production and energy regulation. It also provides support by preventing the breakdown of muscles. Deficiency in leucine may include dizziness, irritation, headache, fatigue, etc.

Lysine : Lysine is an essential amino acid that has a net positive charge at physiological pH values making it one of the three basic (with respect to charge) amino acids. Lysine is an essential amino acid because it cannot be synthesized in the body and its breakdown is irreversible. It is an essential building block for all protein, and is needed for proper growth and bone development in children. Lysine helps the body absorb and conserve calcium and it plays an important role in the formation of collagen.

Methionine : Methionine is an important amino acid that helps to initiate translation of messenger RNA by being the first amino acid incorporated into the N-terminal position of all proteins. Methionine supplies sulfur and other compounds required by the body for normal metabolism and growth. Methionine reacts with adenosine triphosphate to form S-adenosyl methionine. S-adenosyl methionine is the principal methyl donor in the body and contributes to the synthesis of many important substances, including epinephrine and choline. It helps in breaking down of fatty acids, and hence it prevents in building up fatty elements on the artery walls. It also works significantly in the normal detoxification of liver. It is essential in promoting energy production as well as in muscle building. Methionine, one of the essential amino acids, functions like an effective anti-oxidant by supplying sulfur for inactivating free radicals.

Phenylalanine : Phenylalanine is an essential amino acid that is also one of the aromatic amino acids that exhibit ultraviolet radiation absorption properties with a large extinction coefficient. Phenylalanine is part of the composition of aspartame, a common sweetener found in prepared foods (particularly soft drinks, and gum). Phenylalanine plays a key role in the biosynthesis of other amino acids and some neurotransmitters.

Threonine : This amino acid is perfect in assisting protein balance in the body. Additionally, it helps in the development of collagen and maintaining elasticity in the skin. It also functions of liver. It functions well in reducing liver fat. In addition to other essential amino acids, threonine promotes well balancing of immune system in terms of antibody production and thymus growth.

Tryptophan : Tryptophan is an essential amino acid formed from proteins during digestion by the action of proteolytic enzymes. Tryptophan is also a precursor for serotonin (a neurotransmitter) and melatonin (a neurohormone). This is an essential ingredient for the formation of vitamin B3. It is responsible for the production of serotonin which is exclusively important for balancing nerve and brain functioning. It is beneficial for controlling hyperactivity among children. It aids in alleviating stress. It works effectively as an appetite suppressant. It also promotes in reducing weight.

Valine : Valine is a branched-chain amino acid (BCAA) that is closely related to leucine and isoleucine both in structure and function. Valine is a constituent of fibrous protein in the body. As a branched-chain amino acid (BCAA), valine has been found useful in treatments involving muscle, mental, and emotional upsets, and for insomnia and nervousness. Valine may help treat malnutrition associated with drug addiction.

Histidine : Histidine is one of the basic (with reference to pH) amino acids due to its aromatic nitrogen-heterocyclic imidazole side chain. Histidine is the direct precursor of histamine; it is also an important source of carbon atoms in the synthesis of purines. Histidine is needed to help grow and

repair body tissues, and to maintain the myelin sheaths that protect nerve cells. It also helps manufacture red and white blood cells, and helps to protect the body from heavy metal toxicity. Histamine stimulates the secretion of the digestive enzyme gastrin.

Non-essential amino acids

Alanine : Alanine is one of the simplest of the amino acids and is involved in the energy-producing breakdown of glucose. L-alanine is created in muscle cells from glutamate in a process called transamination. Alanine comes from the breakdown of DNA or the dipeptides, anserine and carnosine, and the conversion of pyruvate, a compound in carbohydrate metabolism. Alanine is used by the body to build proteins. Alanine is vital for the production of protein, essential for proper function of the central nervous system and helps form neurotransmitters. Alanine is necessary for the promotion of proper blood glucose levels from dietary protein.

Arginine : Arginine is a complex amino acid that is often found at the active (or catalytic) site in proteins and enzymes due to its amine-containing side chain. Arginine is involved in multiple areas of human physiology and metabolism. Arginine plays an important role in cell division, the healing of wounds, removing ammonia from the body, immune function, and the release of hormones. Arginine has a number of functions in the body such as assisting in wound healing, hormone production, immune function and removal of excess ammonia.

Asparagine : Asparagine is the ß-amide of aspartic acid synthesized from aspartic acid and ATP (adenosine triphosphate). Asparagine is one of the principal and frequently the most abundant amino acids involved in the transport of nitrogen. Asparagine is very active in converting one amino acid into another (amination and transamination) when the need arises. Asparagine serves as an amino donor in liver transamination processes.

Aspartic acid : Aspartic acid is alanine with one of the â hydrogens replaced by a carboxylic acid group. Aspartic acid is a part of organic molecules containing an amino group, which can combine in linear arrays to form proteins in living organisms. Although aspartic acid is considered a non-essential amino acid, it plays a paramount role in metabolism during construction of other amino acids and biochemicals in the citric acid cycle. Among the biochemicals that are synthesized from aspartic acid are asparagine, arginine, lysine, methionine, threonine, isoleucine, and several nucleotides.

Cysteine : Cysteine is a naturally occurring hydrophobic amino acid which has a sulfhydryl group and is found in most proteins. Cysteine is one of the key components in all living things. N-acetyl cysteine (which contains cysteine) is the most frequently used form of cysteine. N-acetyl-L-cysteine (NAC) helps break down mucus and detoxify harmful substances in the body. Both cysteine and NAC have been shown to increase levels of the antioxidant glutathione.

Cystine : Cystine is the product of an oxidation between the thiol side chains of two cysteine amino acids. As such, cystine is not considered one of the 20 amino acids. This oxidation product is found in abundance in a variety of proteins such as hair keratin, insulin, the digestive enzymes chromotrypsinogen A, papain, and trypsinogen where it is heavily involved in stabilizing the tertiary structure of these macromolecules.

Glutamine : Glutamine is one of the twenty amino acids generally present in animal proteins. Glutamine is the most abundant amino acid in the body. Over 61% of skeletal muscle tissue is

glutamine. It contains two ammonia groups, one from its precursor, glutamate, and the other from free ammonia in the bloodstream. Glutamine is involved in more metabolic processes than any other amino acid. Glutamine is converted to glucose when more glucose is required by the body as an energy source. Glutamine assists in maintaining the proper acid/alkaline balance in the body, and is the basis of the building blocks for the synthesis of RNA and DNA.

Glutamic acid : Glutamic acid is biosynthesized from a number of amino acids including ornithine and arginine. When aminated, glutamic acid forms the important amino acid glutamine. Because it has a carboxylic acid moiety on the side chain, glutamic acid is one of only two amino acids (the other being aspartic acid) that has a net negative charge at physiological pH. This negative charge makes glutamic acid a very polar molecule and it is usually found on the outside of proteins and enzymes where it is free to interact with the aqueous intracellular surroundings. On a molar basis, glutamic acid is incorporated into proteins at a rate of 6.2 percent compared to the other amino acids.

Glycine : Glycine is the simplest amino acid and is the only amino acid that is not optically active (it has no stereoisomers). The body uses it to help the liver in detoxification of compounds and for helping the synthesis of bile acids. It has a sweet taste and is used for that purpose. Glycine is essential for the synthesis of nucleic acids, bile acids, proteins, peptides, purines, adenosine triphosphate (ATP), porphyrins, hemoglobin, glutathione, creatine, bile salts, one-carbon fragments, glucose, glycogen, and l-serine and other amino acids.

Proline : Proline is a non-essential amino acid that is involved in the production of collagen and in wound healing. Proline is the precursor for hydroxyproline, which the body incorporates into collagen, tendons, ligaments, and the heart muscle. Proline plays important roles in molecular recognition, particularly in intracellular signalling. Proline is an important component in certain medical wound dressings that use collagen fragments to stimulate wound healing.

Serine : The methyl side chain of serine contains a hydroxy group making this one of two amino acids that are also alcohols. Serine plays a major role in a variety of biosynthetic pathways including those involving pyrimidines, purines, creatine, and porphyrins. Serine has sugar-producing qualities, and is very reactive in the body. It is highly concentrated in all cell membranes, aiding in the production of immunoglobulins and antibodies.

Tyrosine : Tyrosine is metabolically synthesized from phenylalanine to become the para-hydroxy derivative of that important amino acid. Tyrosine is a precursor of the adrenal hormones epinephrine, norepinephrine, and the thyroid hormones, including thyroxine. L-tyrosine, through its effect on neurotransmitters, is used to treat conditions including mood enhancement, appetite suppression, and growth hormone (HGH) stimulation.

Hydroxyproline : Hydroxyproline is derived from the amino acid proline and is used almost exclusively in structural proteins including collagen, connective tissue in mammals, and in plant cell walls. An unusual feature of this amino acid is that it is not incorporated into collagen during biosynthesis at the ribosome, but is formed from proline by a posttranslational modification by an enzymatic hydroxylation reaction. Non-hydroxylated collagen is commonly termed pro-collagen.

Non protein amino acids

In humans, non-protein amino acids also have important roles as metabolic intermediates, such as in the biosynthesis of the neurotransmitter gamma-aminobutyric acid. These class of amino acids are described in detail.

Carnitine : Carnitine is a non-essential amino acid produced in the liver, brain and the kidneys from the essential amino acids methionine and lysine. Carnitine is a nutrient responsible for the transport of long-chain fatty acids into the energy-producing centers of the cells (known as the mitochondria). Carnitine is recommended as a daily supplement to help maintain blood lipid profile and promote fatty acid utilization within heart muscle.

Carnosine : Carnosine is a dipeptide composed of the covalently bonded amino acids alanine and histidine and is found in the brain, heart, skin, muscles, kidneys and stomach. Carnosine is one of the most important and potent natural antioxidant agents which act as universal antioxidants both in the lipid phase of cellular and biological membranes and in the aqueous environment protecting lipids and water-soluble molecules like proteins (including enzymes), DNA and other essential macromolecules from oxidative damage mediated by reactive oxygen species and lipid peroxides.

Creatine : Creatine is a natural derivate of an amino acid and is synthesized in the liver, kidneys and pancreas out of arginine, methionine and glycine. Creatine functions to increase the availability of cellular ATP, adenosine triphosphate. Creatine works by acting on mechanisms of ATP by donating a phosphate ion to increase the availability of ATP. Creatine is stored in muscle cells as phosphocreatine and is used to help generate cellular energy for muscle contractions.

Citrulline : Citrulline is a precursor to arginine and is involved in the formation of urea in the liver. Arginine is a contributing member of the various amino acids found in the urea cycle, which is responsible for detoxifying ammonia. Citrulline supports the body in optimizing blood flow through its conversion to l-arginine and then nitric oxide (NO).

Gamma-aminobutyric acid : Gamma-aminobutyric acid (GABA) is a non-essential amino acid formed from glutamic acid with the help of vitamin B6. GABA (gamma-aminobutyric acid) is found in almost every region of brain, and is formed through the activity of the enzyme glutamic acid decarboxylase (GAD). GABA serves as a inhibitory neurotransmitter to block the transmission of an impulse from one cell to another in the central nervous system.

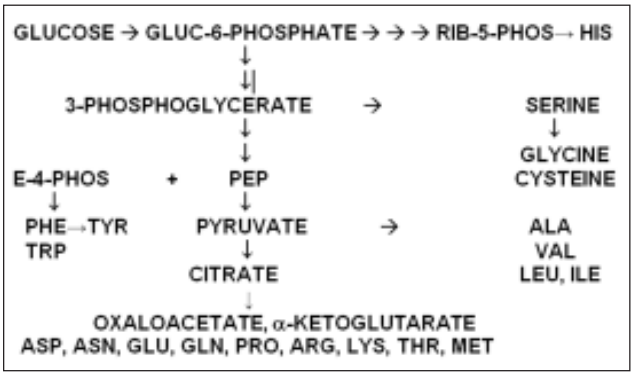
Glutathione : Glutathione (GSH) is a tripeptide composed of three different amino acids: glutamate, cysteine and glycine that have numerous important functions within cells. Glutathione plays a role in such diverse biological processes as protein synthesis, enzyme catalysis, transmembrane transport, receptor action, intermediary metabolism, and cell maturation. Glutathione acts as an antioxidant used to prevent oxidative stress in most cells and help to trap free radicals that can damage DNA and RNA.

Ornithine : Ornithine plays an important role in the urea cycle and is the precursor of the amino acids citrulline, glutamic acid, and proline. Another primary role of ornithine is being an intermediate in arginine biosynthesis, although this is due to its participation in the urea cycle (responsible for the production of urea). Ornithine is not directly incorporated into proteins and enzymes and does not have a codon in the genetic code.

Taurine - Taurine is a non-essential sulfur-containing amino acid that functions with glycine and gamma-aminobutyric acid as a neuroinhibitory transmitter. Taurine is the body's water soluble anti-oxidant, and inhibitory neurotransmitter. The major antioxidant activity of taurine derives from its ability to scavenge the reactive oxygen species hypochlorite. Taurine plays an important role in numerous physiological functions.

Metabolism of amino acids

In plants, nitrogen is first assimilated into organic compounds in the form of glutamate, formed from alpha-ketoglutarate and ammonia in the mitochondrion. In order to form other amino acids, the plant uses transaminases to move the amino group to another alpha-keto carboxylic acid. For example, aspartate aminotransferase converts glutamate and oxaloacetate to alpha-ketoglutarate and aspartate. Other organisms use transaminases for amino acid synthesis too.



Transaminases are also involved in breaking down amino acids. Degrading an amino acid often involves moving its amino group to alpha-ketoglutarate, forming glutamate. In many vertebrates, the amino group is then removed through the urea cycle and is excreted in the form of urea. However, amino acid degradation can produce uric acid or ammonia instead. For example, serine dehydratase converts serine to pyruvate and ammonia. Nonstandard amino acids are usually formed through modifications to standard amino acids. For example, homocysteine is formed through the transsulfuration pathway or by the demethylation of methionine via the intermediate metabolite S-adenosyl methionine, while hydroxyproline is made by a posttranslational modification of proline. Microorganisms and plants can synthesize many uncommon amino acids. For example, some microbes make 2-aminoisobutyric acid and lanthionine, which is a sulfide-bridged derivative of alanine. Both of these amino acids are found in peptidic lantibiotics such as alamethicin. While in plants, 1-aminocyclopropane-1-carboxylic acid is a small disubstituted cyclic amino acid that is a key intermediate in the production of the plant hormone ethylene.

The health benefits of amino acids

Amino acids are needed to build the various proteins used in the growth, repair, and maintenance of body tissues. Amino acids play innumerable roles in human health and disease. Alanine is necessary for the promotion of proper blood glucose levels from dietary protein. Alanine stimulates lymphocyte production and may help people who have immune suppression. Alanine strengthens the immune system by producing antibodies. L-arginine is used by the immune system to help regulate the activity of the thymus gland, which is responsible for manufacturing T lymphocytes. The body uses arginine to produce nitric oxide. Nitric oxide is an endogenous messenger molecule involved in a variety of endothelium-dependent physiological effects in the cardiovascular system. In the central nervous system, asparagine is needed to maintain a balance, preventing over nervousness or being overly calm. Aspartic acid can help protect the liver from some drug toxicity

and the body from radiation. Carnosine is the water-soluble counterpart to vitamin E in protecting cell membranes from oxidative damage. L-carnosine supports healthy aging and cellular rejuvenation by its effects on two mechanisms: glycosylation and free radical damage. Cysteine strengthens the protective lining of the stomach and intestines, which may help prevent damage caused by aspirin and similar drugs. The health benefits of glutamine include immune system regulation, nitrogen shuttling, oxidative stress, muscle preservation, intestinal health, injuries, and much more. Glycine is an inhibitory amino acid with important functions centrally and peripherally. Glycine may be indicated to help alleviate the symptoms of spasticity. Histidine is known to be vital in the maintenance of the myelin sheaths surrounding nerves, particularly the auditory nerve and is used to treat some forms of hearing disability. Isoleucine is necessary for the optimal growth of infants and for nitrogen balance in adults. Leucine is used as a source for the synthesis of blood sugar in the liver during starvation, stress, and infection to aid in healing. Lysine is used in managing and preventing painful and unsightly herpes sores caused by the herpes simplex virus (HSV). Methionine is both an antioxidant and lipotrope, meaning it helps remove fat from the liver. Phenylalanine is used to treated depression, rheumatoid arthritis and osteoarthritis, menstrual cramps, Parkinson's disease, vitiligo, and cancer. Proline is an important component in certain medical wound dressings that use collagen fragments to stimulate wound healing. Serine is needed for the metabolism of fats and fatty acids, muscle growth, and a healthy immune system. Taurine helps regulate the contraction and pumping action of the heart muscle and it helps regulate blood pressure and platelet aggregation. Threonine may enhance immunity by assisting in the production of agents that fight viral infections. L-theanine reduces stress and anxiety without the tranquilizing effects found in many other calming supplements. Tryptophan is important for the production of serotonin. Increasing tryptophan may help to normalize sleep patterns. Tyrosine may act as an adaptogen, helping the body adapt to and cope with the effects of physical or psychological stress by minimizing the symptoms brought on by stress. As a branched-chain amino acid (BCAA), valine has been found useful in treatments involving muscle, mental, and emotional upsets, and for insomnia and nervousness. Creatine supplements fuels and enhances short bursts of high-energy exercise. Creatine prevents the body from relying solely on the process of glycolysis. Citrulline supports the body in optimizing blood flow through its conversion to l-arginine and then nitric oxide (NO). GABA has been used in the treatment of depression, manic-depressive (bipolar) disorder, seizures, premenstrual dysphoric (feeling depressed) disorder, and anxiety. Glutathione are necessary for supporting the immune system, glutathione is required for replication of the lymphocyte immune cells.

Suggested Reading

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Instrumental Methods in Bioprospecting: High Pressure Liquid Chromatography

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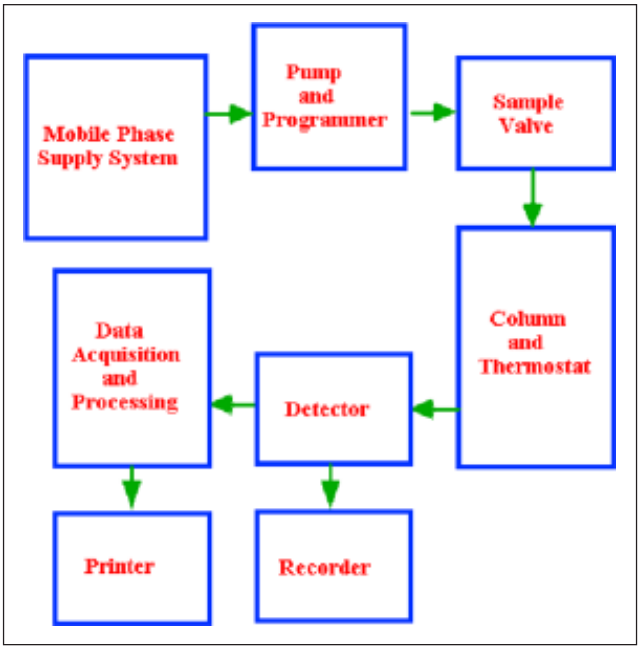
Liquid chromatography (LC) was the first type of chromatography to be discovered and, in the form of liquid-solid chromatography (LSC) was originally used in the late 1890s by the Russian botanist, Tswett to separate and isolate various plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography (color writing) for this type of separation. In the late 1930s and early 1940s Martin and Synge introduced a form of liquid-liquid chromatography by supporting the stationary phase, in this case water, on silica gel in the form of a packed bed and used it to separate some acetyl amino acids. Martin and Synge suggested the use of small particles and high pressures in LC to improve the separation which proved to the critical factors that initiated the development of high performance liquid chromatography (HPLC). The statement made by Martin in 1941 contains all the necessary conditions to realize both the high efficiencies and the high resolution achieved by modern LC columns. Despite his recommendations, however, it has taken nearly fifty years to bring his concepts to fruition. The major impediment to the development of LC was the lack of a high sensitive detector and it was not until the refractive index detector was developed by Tiselius and Claesson in 1942 could the technique being effectively developed. The contemporary chromatograph, however, is a very complex instrument operating at pressures up to 10,000 PSI providing flow rates ranging from a few microliters per minute to 10-20 ml/minute depending on the type of LC that is carried out. Modern detectors can detect solutes at concentration levels of $1x10^{-9}$ g/ml and an analysis can be completed in a few minutes with just a few micrograms of sample.

Modern High Pressure Liquid Chromatography (HPLC)

HPLC is liquid chromatography which has been optimized to provide rapid high resolution separations. The basic liquid chromatograph consists of five basic units as follows. A block diagram of the basic liquid chromatograph is shown in the following figure.

1. Mobile phase supply system and gradient mixers.
2. HPLC high pressure pumps and sample valves.
3. HPLC columns with inert packing materials.
4. High sensitivity low dispersion HPLC detectors.
5. High speed data acquisition systems.



The Basic Liquid Chromatograph

Mobile Phase Supply System and HPLC Gradient Mixers

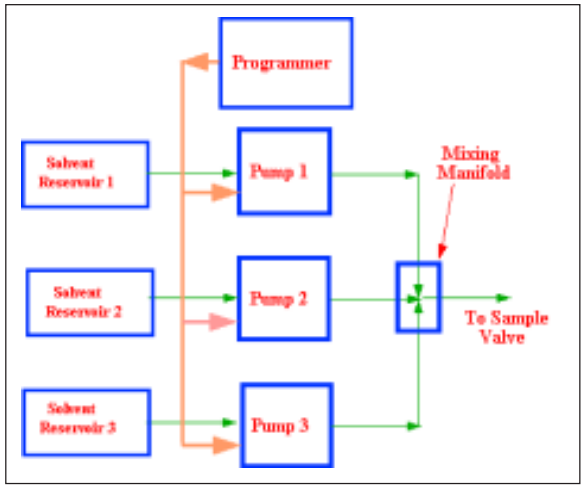
HPLC gradient mixers provide a very precise control of solvent composition to maintain a reproducible gradient profile. The mobile phase supply system consists of number of reservoirs (200-1,000 ml). At least two reservoirs would be necessary and are usually constructed of glass or stainless steel and contain an exit port open to air. Each reservoir is usually fitted with a gas diffuser through which helium can be bubbled. Many solvents and solvent mixtures (particularly aqueous mixtures) contain significant amounts of dissolved nitrogen and oxygen from the air. These gasses can form bubbles in the chromatographic system that cause both serious detector noise and loss of column efficiency. As helium is very insoluble in most solvents, it purges the oxygen and nitrogen from the solvent but does not produce bubbles in the system itself. Applying a vacuum to the reservoir is not a permanent solution to dissolved air as, on releasing the vacuum to allow the solvent to pass to the pump, air again dissolves in the solvent. The solvent is filtered through a stainless steel or sintered glass filter to remove any solid contaminants. Depending on the type of solvent programmer that is employed, the supply from each reservoir may pass either to a pump or to a valve blending device. Solvent reservoirs are not usually thermostatted but, when necessary, the solvent can be brought to the column temperature by the use of an appropriate heat exchanger.

The Gradient Programmer

High Pressure Gradient Programmer

There are two basic types of solvent programmer. In the first, the solvent mixing occurs at high pressure and in the second the solvents are premixed at low pressure and then passed to the pump. Theoretically, there can be any number of solvents involved in a mobile phase program, however, most LC analyses require only two solvents, nevertheless, up to four solvents can be accommodated.

The layout of a high pressure gradient system is shown in the following figure and includes, as an example, provision for three solvents to be mixed by appropriate programming.



High Pressure Gradient Programmer

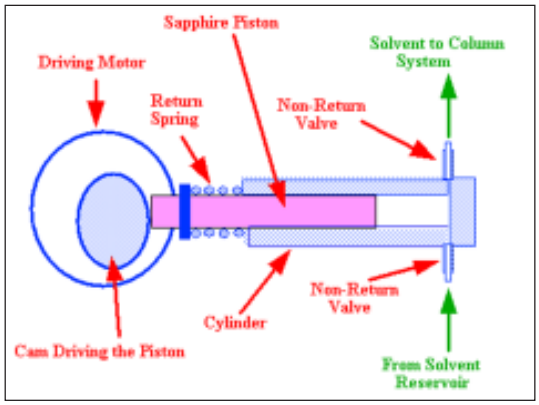
Solvent passes from each reservoir directly to a pump and then to a mixing manifold from which it passes to the sample valve and column. The pumps control the actual program and are usually driven by stepping motors. The volume delivery of each solvent is controlled by the speed of the respective pump which is precisely determined by the frequency of its power supply. The controlling frequency can be generated either by external oscillators or, if the chromatograph is computer controlled, directly from the computer itself.

HPLC Pumps

Because of the small particles used in modern HPLC, LC pumps need to operate reliably and precisely at pressures of 10,000 PSI or at least 6,000 PSI. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, SS cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical proposes HPLC pumps should have flow rates that range from 0-10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. There are a number of different types of pumps that can provide the necessary pressures and flow-rates required by the modern liquid chromatograph. In the early years of the LC renaissance, there were two types of pump in common use; they were the pneumatic pump, where the necessary high pressures were achieved by pneumatic amplification, and the syringe pump, which was simply a large, strongly constructed syringe with a plunger that was driven by a motor. Today the majority of modern HPLCs are fitted with reciprocating pumps fitted with either pistons or diaphragms.

Single Piston Reciprocating Pump

The single piston reciprocating pump was the first of its type to be used with high efficiency LC columns (columns packed with small particles) and is still very popular today. It is simple in design and relatively inexpensive. A diagram of the single piston pump is shown in the figure.



Single Piston Reciprocating Pump

Most pistons of modern LC pumps are made of synthetic sapphire to reduce wear and extend the working life of the pump. The cylinder is usually made of stainless steel and is attached to two non-return valves in line with the inlet and outlet connections to the pump. The piston is driven by a

stainless steel cam which forces the piston into the cylinder expressing the solvent through the exit non-return valve. After reaching the maximum movement, the piston follows the cam and returns as a result of the pressure exerted by the return spring. During this movement the cylinder is loaded with more solvent through the inlet non-return valve. The shape of the cam is cut to provide a linear movement of the piston during expression of the solvent but a sudden return movement on the refill stroke. In this way the pulse effect that results from the refill action is reduced.

Rapid Refill Pump

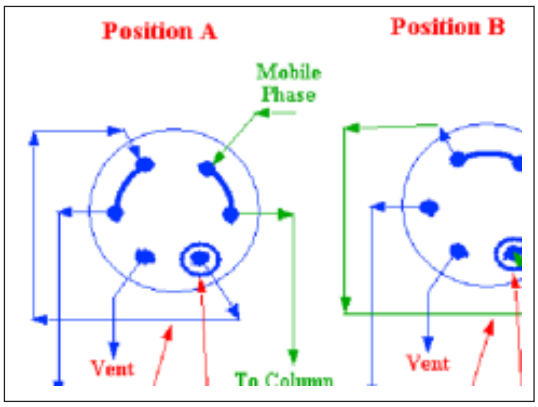
In order to avoid the refill pulses resulting from a single piston pump, a number of rapid refill systems have been developed. The designs have ranged from cleverly designed actuating cams to drive the piston rapidly in the refill mode to electronically operated piston movements.

Diaphragm Pump

The unique property of the reciprocating diaphragm pump is that the actuating piston does not come into direct contact with the mobile phase and thus, the demands on the piston-cylinder seal are not so great. The diaphragm has a relatively high surface area and thus, the movement of the diaphragm is relatively small and consequently the pump can be operated at a fairly high frequency.

HPLC Sample Valves

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 PSI. For analytical HPLC, the sample volume should be selectable from sub- micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. The higher the operating pressure the tighter the valve seating surfaces must be forced together to eliminate any leak. It follows that any abrasive material, however fine, that passes into the valve can cause the valve seating to become scored each time it is rotated which will ultimately lead to leaks. This will cause the sample size to vary between samples and eventually affect the accuracy of the analysis. In LC, the sample valve contains an extra loading port and behaves like an internal loop valve. The basic difference between this type of valve and the normal external loop sample valve is the introduction of an extra port at the front of the valve. This port allows the injection of a sample by a syringe directly into the front of the sample loop. Position (A) shows the inject position. Injection in the front port causes the sample to flow into the sample loop. The tip of the needle passes through the rotor seal and, on injection, is in direct contact with the ceramic stator face. After injection, the valve is rotated to position (B) and the mobile phase flushes the sample directly onto the column. The sample is actually forced out of the beginning of the loop so it does not have to flow through the entire length of the loop. This type of injection system is ideally suited for quantitative LC, and is probably by far the most popular injection system in use.



External Loop Sample Valve

HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary pahse and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary pahse than those that interact more strongly with the mobile phase. In particular optically pure compounds can be used to make Chiral HPLC stationary phases.

Liquid Chromatography Stationary Phases

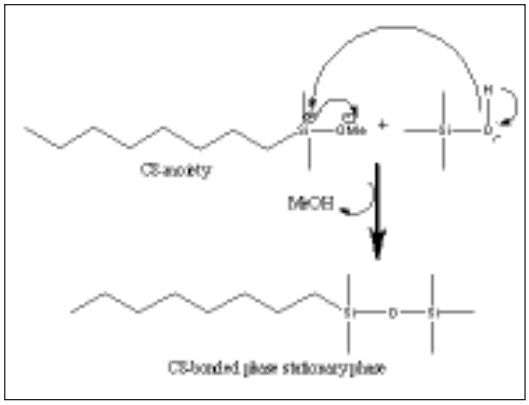
Traditionally the stationary phase used in LC has been silica gel which separates solutes largely on the basis of polarity, although, due to its unique structure, silica gel also exhibits strong exclusion characteristics. The bonded phases were introduced to provide a material that would separate solutes by dispersive interactions and also to provide some semie polar stationary phases. The bonded phases were also based on silica gel. More recently, polymeric stationary phases were introduced to provide materials that were insoluble in water and that were stable at extremes of pH.

The Structure of Silica Gel

The matrix of the primary silica gel particle consists of a core of silicon atoms joined together with oxygen atoms by siloxane bonds (silicon-oxygen-silicon bonds). On the surface of each primary particle some residual, uncondensed hydroxyl groups from the original polymeric silicic acid remain. There are three types of hydroxyl group. The first is a single hydroxyl group attached to a silicon atom which has three siloxane bonds joining it to the gel matrix. The second is one of two hydroxyl groups attached to the same silicon atom which, in turn, is joined to the matrix by only two siloxane bonds. These twin hydroxyl groups are called Geminal hydroxyl groups. The third is one of three hydroxyl groups attached to a silicon atom which is now only joined to the silica matrix by only a single siloxane bond.

Bonded Phases

Bonded phases are formed by reacting the surface hydroxyl groups with an appropriate reagent to chemical link an organic moiety to the silica surface. The nature of the organic moiety will determine the type of interaction that will take place between the solute and the surface. The most efficient bonded phase has the maximum surface coverage. It is understood, that due to stearic hindrance from the bonded moiety itself, only a proportion of the silanol groups can be bonded and there is little that can be done to avoid this problem. However, there are other reasons for incomplete silanization of the silica. Incomplete silanization can result from the reagent molecule being excluded from the smaller pores of the silica. Exclusion can be a particular problem when bonding relatively large molecular weight materials such as long chain hydrocarbons onto the silica surface. It is therefore, important to choose a silica gel that has a relatively large pore size (*e.g.*, a mean pore diameter of



150Å) which may limit the surface area to between 150 and 250 sq.m per gram and thus, reduce the retentive capacity of the stationary phase. The solvents normally used in bonded phase synthesis are aromatic hydrocarbons *e.g.,* toluene that boils at 110ÚC or mixed xylenes that boil 138-140^{Ú}C. The procedure varies a little depending on the size of the batch and the type of silanizing reagent. A method of synthesis of bonded phase for the alkoxysilane reagents is illustrated below. The most reactive alkoxy reagents are the methoxy and ethoxysilanes and their reaction with a hydroxyl group is accompanied by the release of methanol or ethanol.

The final capping process is also the same as that employed in the method using the chlorosilanes reagents, utilizing hexamethyldisilazane as the capping reagent. The alkoxy-silanes are almost as readily available as the chlorosilanes and are easier and more pleasant to handle.

LC Mobile Phases

The choice of phase system can be very complex, particularly if multicomponent mixtures are to be separated. In the first instance the type of stationary phase needs to be chosen and this choice must be based on the interactive character of the solutes to be separated. If the solutes are predominantly dispersive then the stationary phase must also be dispersive (a reversed phase) to promote dispersive interaction with the solutes and provide adequate retention and selectivity. If the solutes are strongly polar then a polarizable stationary phase (one containing aromatic rings or cyano groups) would be appropriate to separate the solutes by polar and induced polar interactions. If the solutes are weakly polar then a strong polar stationary phase would be required (such as silica gel) to separate the solute by polar interactions.

Column Ovens

The effect of temperature on LC separations is often not nearly so profound as its effect in GC separations, but can be critical when closely similar substances are being separated. In LC a change in temperature will change the free energy of the solute in both phases, (generally in a commensurate manner) and so the net change in the free energy difference with temperature, which controls the magnitude of the absolute retention, can be relatively small. Its effect on relative retention, however, can be very significant and, in fact, be the determining factor in achieving a satisfactory resolution. An increase in temperature will increase the diffusivity of the solute in both phases and thus increase the dispersion due to longitudinal diffusion and decrease dispersion due to resistance to mass transfer.

HPLC Detectors

A large number of LC detectors have been developed over the past thirty years based on a variety of different sensing principles. However, only about twelve of them can be used effectively for LC analyses and, of those twelve, only five are in common use. The dominant detectors used in LC analysis are the UV detector (fixed and variable wavelength), photo diode array detector, the

electrical conductivity detector, the fluorescence detector and the refractive index detector. These detectors are employed in over 95% of all LC analytical applications.

The UV Detector

The UV detector is by far the most popular and useful LC detector that is available to the analyst at this time. Although the UV detector has some definite limitations (particularly for the detection of non polar solutes that do not possess a UV chromaphores) it has the best combination of sensitivity, linearity, versatility and reliability of all the LC detectors so far developed. Multi-Wavelength UV detectors utilize a single of wavelengths to detect the solute. Most multi wavelength UV detectors can also provide a UV spectrum of the eluted solute if appropriately arranged.

Electrical Conductivity Detectors

The electrical conductivity detector can only detect those substances that ionize and consequently, are frequently used in the analysis of inorganic acids, bases and salts. It has also found particular use in the detection of organic acids and bases that are frequently required in environmental studies and in biotechnology applications. The sensor is the simplest of all the detectors consisting of only two electrodes situated in a suitable flow cell.

Fluorescence Detector

The fluorescence detector is one of the most sensitive LC detectors and for this reason is often used for trace analysis. Unfortunately, although the detector is very sensitive, its response is only linear over a relatively limited concentration range. In fact, the response of the detector can only be assumed to be linear over a concentration range of two orders of magnitude. Unfortunately, the majority of substances do not naturally fluoresce which is a serious disadvantage to this type of detector. It follows, that in many instances fluorescent derivatives must be synthesized to render the substances of interest detectable.

Refractive Index Detector

The refractive index detector is one of the least sensitive LC detectors. It is very sensitive to changes in ambient temperature, pressure changes, flow-rate changes and can not be used for gradient elution. Despite these many disadvantages, this detector is extremely useful for detecting those compounds that are nonionic, do not adsorb in the UV, and do not fluoresce.

HPLC Data Acquisition

The output from the detector, usually in millivolts, is passed to a scaling amplifier that converts the signal to a voltage that is acceptable to the analog to digital (A/D) converter The A/D converter changes the voltage output to a binary number which is temporarily stored in a register. This process is continuously repeated at a defined rate, called the 'sampling rate'. The current binary number, stored in the register is regularly sampled by the computer and stored (usually on hard disk). On completion of the analysis the computer accesses all the data from store, calculates the retention report, compares peak heights or peak areas to provide the quantitative analysis according to the processing program that is used and finally prints out the results in tabulated form. Modern data processing software often includes routines that can process chromatograms where the components of the sample are incompletely resolved. The routines deconvolute the individual peaks from the

composite envelope and calculate the area of the individual de-convoluted peaks. Such algorithms can be used very effectively on peaks that are entrained in the tail of a major peak but are not so accurate for composite envelopes containing many unresolved peaks.

Conclusions

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, food component analyses, process control, forensic analysis, environmental monitoring, and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster analysis times with a constant incorporation of new highly selective column packings.

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Bioactive compounds and nutraceuticals from marine organisms

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Marine flora and fauna and their potential use

Ocean harbors a large biodiversity of marine fauna and flora with about 5,000 species of sponges, 9,000 species of annelids, and 66,535 species of molluscs. Among the mollusks, 15,000 species of bivalves and 600 species of cephalopods have been reported to occur. Marine biotechnology is the science in which marine organisms are used in full or partially to make or modify products, to improve plants or animals or to develop microorganisms for specific uses. With the help of different molecular and biotechnological techniques, humans have been able to elucidate many biological methods applicable to both aquatic and terrestrial organisms. Only 10% of over 25,000 plants have been investigated for biological activity. The marine environment may contain over 80% of world's plant and animal species. In recent years, many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, sea hares, nudibranchs, bryozoans, sea slugs and marine organisms. The search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites, many of which are endowed with pharmacodynamic properties. The deep knowledge about nerve transmission has been learnt using squid and its giant nerve axons and the mesenteries of vision have been unraveled using the eyes of horseshoe crabs, sharks and skates. The surf clam is proving an excellent model for the cell cycle and its regulation while the sea urchin is a model for understanding the molecular basis of cellular reproduction and development. The objective of this review is to highlight some of the recent developments and findings in the area of marine biotechnology with special reference to the biomedical potential of marine natural products. Therefore, this rich diversity of marine organisms represents an enormous resource for the discovery of potential compounds with valuable pharmaceutical and biomedical potential (Kamboj, 1999). These bioactive compounds belong to different chemical classes' viz., terpenoids, steroids/sterol glycosides, phenolics, amino acids, fatty alcohol esters, glycolipids etc. Of the few bio-evaluated, some showed interesting biological activity with respect to antibacterial, antioxidant, and anti-inflammatory properties. It is well known that during the past 20 years, pharmaceutical industry has been relatively successful in containing problems due to single resistant determinants.

Introduction to Marine Natural Products (MNPs)

Natural products have long been used as foods, fragrances, pigments, insecticides, medicines, etc. Due to their easy accessibility, terrestrial plants have served as the major source of medicinally

useful products, especially for traditional or folk medicine. About 25% of all pharmaceutical sales are drugs derived from plant natural products and an additional 12% are based on microbially produced natural products. The marine environment covers a wide thermal range (from the below freezing temperatures in Antarctic waters to about 350°C in deep hydrothermal vents), pressure range (1-1000 atm), nutrient range (oligotrophic to eutrophic) and it has extensive photic and non-photic zones. This extensive variability has facilitated extensive speciation at all phylogenetic levels, from microorganisms to mammals. Despite the fact that the biodiversity in the marine environment far exceeds that of the terrestrial environment, research into the use of marine natural products as pharmaceutical agents is still in its infancy. This may be due to the lack of ethno-medical history and the difficulties involved in the collection of marine organisms. But with the development of new diving techniques, remote operated machines, etc., it is possible to collect marine samples and during the past decade, over 5000 novel compounds have been isolated from shallow waters to 900-m depths of the sea.

Marine Bacteria as a Source of MNP

Microorganisms continue to be a productive and successful focus for much marine natural products research. Symbiotic marine bacteria from corals may be responsible to produce secondary metabolites displaing antibacterial properties. Zhang et al (2005) highlighted the importance of marine microbes as potential source of novel bioactive compounds. The putative probionts belonging to *Bacillus, Pseudomonas,* and *Micrococcus* sp. are the major candidate species from marine ecosystem (Gatesoupe, 1999). In marine *Pseudomonas aeruginosa* culture supernatants, two compounds of the chemical group diketopiperazines (DKPs), *viz.,* cyclo (ÄAla-L-Val) and cyclo (L-Pro-L-Tyr), respectively were identified capable of activating an N-acylhomoserine lactone (AHL) biosensor. Thallusin, isolated from marine bacterium (Japan), is a potent differentiation inducer. An analogue of the tambjamine alkaloids has been isolated from the marine bacterium *Pseudoalteromonas tunicate*. Cell-free culture supernatant of the psychrophilic aerobic bacterium *P. haloplanktis* obtained from seawater contained a diketopiperazine. The diastereoisomeric quinolinones were isolated from *P. janczewskii* derived from surface water (German Bight, Helgoland Island). Both compounds were cytotoxic to a range of human tumour cell lines, with strongly cytotoxic to SKOV-3 cells (human ovarian carcinoma). Use of fluorescent Pseudomonads as biocontrol agent was reported. Torrento and Torres (1996) reported the in vitro inhibition of *V. harveyi* by a *Pseudomonas* species isolated from the the aquatic environment. Gram *et al.* (1999) demonstrated the protection of rainbow trout administered with *P. fluorescens* AH2 when challenged with *V. anguillarum.* Another study by Smith and Davey (1993) demonstrated that bathing Atlantic salmon in a strain of *P. fluorescens* reduced subsequent mortality from stress-induced furunculosis. Specific inhibition of *V. harveyi* by *Pseudomonas aeruginosa* has been reported earlier by Torrento and Torres (1996). Pridmore et al. (1996) reported that variacin, a bacteriocin produced by *Micrococcus varians*, inhibited other Gram-positive bacteria. It was reported to be a new lanthionine-containing bacteriocin, variacin, displaying a broad host range of inhibition against Gram-positive food spoilage bacteria, has been identified from two strains of *Micrococcus varians* isolated from meat fermentations. Variacin, like lacticin 481, contains lanthionine and beta- methyllanthionine residues. El-Shafei (1997) observed that the introduction of *Micrococcus* to fungal cultures resulted in lysis and inhibition of fungal growth, and attributed this to the production of mycolytic enzymes. A *Bacillus* sp, which enables them to digest

plant cellulose since fish do not produce the enzyme was reported by Saha et al. (2006). An improvement in survival of trout was observed by means of enhanced adhesion in the intestines by *B*. *animalis* (Ibrahim et al. 2004).

MNPs from Marine Algae

The fact that microalgae/cyanobacteria in general and marine forms in particular are one of the richest sources of known and novel bioactive compounds including toxins with wide pharmaceutical applications is unquestionable. Among the five divisions of microalgae, studies of biomedical natural products have been concentrated on only two divisions, i.e., Cyanophyta (blue-green algae) and Pyrrophyta (dinoflagellates). Although several metabolites have been isolated from cyanophytes, most of them are isolated from fresh water species, which are cultured easily in comparison to marine organisms. Lyngbyatoxin-A and debromoaplysiatoxin are two highly inflammatory but structurally different metabolites isolated from toxic strains of *Lyngbya mausculata* collected in Hawaii, and anatoxin-a from *Anabaena ciecinalis*. Some of the marine cyanobacteria appear to be potential sources for large-scale production of vitamins of commercial interest such as vitamins of the B complex group and vitamin-E. The carotenoids and phycobiliprotein pigments of cyanobacteria have commercial value as natural food colouring agents, as feed additives, as enhancers of the color of egg yolks, to improve the health and fertility of cattle, as drugs and in the cosmetic industries. Some anti-HIV activity has been observed with the compounds extracted from *Lyngbya lagerhaimanii* and *Phormidium tenue*. More than 50% of the 100 isolates from marine sources are potentially exploitable bioactive substances. The substances tested for were either the ones that killed cancer cells by inducing apoptotic death.

MNPs from seaweeds

Seaweeds are abundant in the intertidal zones and in clear tropical waters. However, they have received comparatively less bioassay attention. Seaweeds, popularly known as green algae, are widely distributed in both inter-tidal and deep-water regions of the seas. These seaweeds are of immense pharmaceutical and agricultural value. A wide range of compounds, particularly terpenes, polyphenolic compounds and steroids, have been reported from various seaweeds (Blunt, Copp, Munro, Northcote, & Prinsep, 2006), amongst which terpenoid compounds represent a major share. For example, Caulerpa brownii from Australia was reported to yield a number of bioactive novel diterpenoids and terpenoid esters (Handley & Blackman, 2005). Capisterones A and B are triterpenesulphate esters that were isolated from the tropical green alga, Panicillus capitatus, and were found to exhibit potent antifungal activity against the marine algal pathogen Lindra thallasiae (Puglisi, Tan, Jensen, & Fenical, 2004). Monocyclic diterpenes have been purified from the Tasmanian green alga Caulerpa trifaria (Handley & Blackman, 2000). The green alga, Caulerpa racemosa, was reported to yield a bioactive sesquiterpene acid (Anjaneyulu, Prakash, & Mallavadhani, 1991). Halitunal, a novel antiviral diterpene aldehyde has been isolated from the marine alga, Halimeda tuna (Koehn, Gunasekera, Neil, & Cross, 1991). 2-Hydroxy-10-methylzeatin has been purified from seaweeds, NIO-143, and the absolute configuration of the said cytokinin has been determined by spectroscopic procedures (Farooqi, Shukla, Shukla, & Bhakuni, 1990). Kahalalide F, a cytotoxic, antiviral and antifungal cyclic depsipeptide, was isolated from a Hawaiian species of *Bryopsis* sp. (Hamann & Scheuer, 1993). A method to purify labdane diterpenois as major constituents of

dichloromethane-soluble fraction green alga *Ulva fasciata* has been illustrated. Antimicrobial assay showed that the compounds labda-14-ene-3a,8a-diol (ULV2) and labda-14-ene-8a-hydroxy-3-one (ULV4) were inhibitory to the growth of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* with minimum inhibitory concentrations of 30 ìg/ml by ULV2, and 40 ìg/ml by ULV4, respectively against the former and 30 ìg/ml by ULV2, and 80 ìg/ml by ULV4, respectively, against the latter (Chakraborty et al., 2010). Two new guaiane sesquiterpene derivatives, guai-2-en-10a-ol (G1) and guai-2-en-10a-methanol (G2), were chromatographically purified as major constituents of the $CHCl_3/CH_3OH$ (1:1, v/v) soluble fraction of *Ulva fasciata*. Acetylation of G2 furnished guai-2-en-10a-methyl methanoate (G3) with acetyl group at C_{11} position. Compounds G2 and G3 exhibited significant inhibition to the growth of *Vibrio parahaemolyticus* with minimum inhibitory concentrations of 25 and 35 mg/mL, respectively (Chakraborty et al., 2010). The antiinflammatory agent produced by *Ulva lactuca* was identified as 3-O-b-glucopyranosylstigmasta-5,25-diene (Awad, 2000). A survey of the metabolites of U. lactuca led to the proposal that 4-hydroxybenzoic acid is the most likely biosynthetic precursor of 2,4,6-tribromophenol, an antibacterial compound (Flodin & Whitfield, 1999). Two new antimicrobial terpenes, taxifolione and 7,7-didehydro-6-hydroxy-6,7-dihydrocaulerpenyne, were purified from Caulerpa taxifolia, a tropical green alga from Cap Martin, France (Guerriero et al., 1993). Neomeris annulata, from Kwajalein Atoll, was reported to possess three brominated sesquiterpenes, shown to deter fish feeding (Paul, Cronan Jr., & Cardellina II, 1993).

Metabolites from molluscs

More than 2600 scientific studies over the last 20 years testify to the important contribution of toxins extracted from marine mollusks to medicine and cellular biology. To date, only 100 out of a potential 50,000 toxins have been extracted and analyzed. The *Conus* species have evolved deadly nerve toxins and small, conformationally constrained peptides of 10-30 amino acids. Some of the conotoxins block channels regulating the flow of potassium or sodium across the membranes of nerve or muscle cells; others bind to N-methyl-D-aspartate receptors to allow calcium ions into nerve cells; and some are specific antagonists of acetylcholine receptors responsible for muscle contraction. Thus, conotoxin are valuable probes in physiological and pharmacological studies. Bivalve mollusks and cephalopods are widely used in different parts of the world for various studies, but only recently they have been recognized as potential sources for bioactive compounds. Preliminary studies indicated marine bivalves and cephalopods as rich sources of structurally diverse compounds with antibacterial potential (Chandran *et al.,* 2009). In the marine environment, where all surfaces are constantly exposed to the threat of surface colonisation, invertibrates like bivalve mollusks and cephalopods remain relatively free of biofouling. It is apparent that these sedentary organisms control fouling epibionts by effective antimicrobial mechanisms (Tincu & Taylor, 2004). Therefore these marine invertebrates appeared to offer a source of potential antimicrobial compounds (Bansemir *et al*., 2006; Mayer *et al.,* 2007). There is evidence that bivalve mollusks are useful in the treatment of inflammatory joint diseases (Couch *et al.,* 1982; Miller *et a*l., 1993). Nonsteriodal anti-inflammatory drugs (NSAIDs), viz., aspirin and ibuprofen, are often used for inflammatory conditions. However, most of these medications can produce the unfortunate side effects, which may lead to stomach ulcer if taken frequently. Therefore exploring the bivalve mollusks for their anti-inflammatory and antioxidant activities and development of product therefrom may significantly reduce adverse side effects resulting from taking NSAIDs. There are reports of dried flesh of the New Zealand mussel

Perna canaliculus possessig polyunsaturated fatty acids (PUFAs) with possible anti-inflammatory effects (Croft, 1979; Zwar, 1994; Gibson & Gibson, 1981). The anti-inflammatory, antioxidant, and anti-prostaglandin activities were reported in green lipped mussels of New Zealand (Couch *et al.,* 1982; Miller *et a*l., 1993). Neosurugatoxin isolated from *Babylonia japonica* is useful in characterizing two classes of acetylcholine receptors. Dolastatin, a cytotoxic peptide from *Dolabella auricularia* is an antineoplastic substance. Ulapualide-A, a sponge-derived macrolide isolated from the nudibranch *Hexabranchus sanguineus* exhibits cytotoxic activity against L 1210 murine leukemia cells and antifungal activity, which exceeds that of clinically useful amphotericin-B. Chromodorolide-A isolated from *Chromocloris cavae* exhibits *in vitro* antimicrobial and cytotoxic activities. Onchidal from *Onchidella bieyi* is a useful probe for identifying the active site residues that contribute to binding and hydrolysis of acetyl cholinesterase. A team from the University of Melbourne extracted the conotoxin from a cone-shell snail. It not only inhibits pain as being 10,000 times more powerful than morphine, but also accelerates the recovery of injured nerves. The absolute stereochemistries of membrenones A–C, -dihydropyrone-containing polypropionates isolated from the skin of the Mediterranean mollusc *Pleurobranchus membranaceus*, have been determined by stereocontrolled syntheses of the enantiomers. The first synthesis of siphonarin-B has confirmed the absolute stereochemistry of the metabolite isolated from the molluscs *Siphonaria zelandica* and *S. atra*. Bursatellanin-P, a 60-kDa protein was purified from the purple ink of the sea hare *Bursatella leachii*. The protein exhibited anti-HIV activity. The first total syntheses of aplyolides B–E, ichthyotoxic macrolides isolated from the skin of sea hare *Aplysia depilans*, have been reported confirming the absolute stereochemistry reported for the metabolites. Cephalopods, gastropods, and bivalve mollusks constitute a major share of marine fauna, and were reported to possess structurally diverse anti-stress metabolites with respect to antibacterial, antioxidant, and anti-inflammatory properties (Chandran *et al.* 2009). A product (Cadalmin™ GMe) developed by CMFRI containing 100% natural anti-inflammatory ingredients was prepared from green mussel *Perna viridis* to combat joint pain and inflammatory diseases (Chakraborty *et al.,* 2010a; Chakraborty *et al*. 2010b). Polysaccharides, lysolecithin, and phenolic components in *P. viridis* were found to competitively inhibit inflammatory COX and LOX in an inflammation and oxidative stress reaction (Chakraborty et al., 2010b).

Metabolites from Sponges

Approximately 10,000 sponges have been described in the world and most of them live in marine waters. A range of bioactive metabolites has been found in about 11 sponge genera. Three of these genera (*Haliclona*, *Petrosia* and *Discodemia*) produce powerful anti-cancer, anti-inflammatory agents, but their cultivation has not been studied [61]. The discovery of spongouridine, a potent tumor-inhibiting arabinosyl nucleoside in Caribbean sponge *Cryptotethia crypta*, focused attention on sponges as a source of biomedically important metabolites. The compound manoalide from a Pacific sponge has spawned more than 300 chemical analogs, with a significant number of these going on to clinical trials as anti-inflammatory agents. An aminoacridine alkaloid, dercitin, has been isolated from the deep-water sponge, *Dercitus* spp. that possesses cytotoxic activities in the low nanomolar concentration range and in animal studies, prolongs the life of mice-bearing ascitic P388 tumours, and is also active against B16 melanoma cells and small cell Lewis lung carcinoma. Halichondrin-B, a polyether macrolide from Japanese sponge *Theonella* spp., has generated much interest as a potential anticancer agent [14,63]. The theopederins are structurally related to

mycalamide-A from marine sponge, *Mycale* spp. collected in New Zealand and onnamide-A from marine sponge, *Theonella* spp. collected in Okinawa, which show *in vitro* cytotoxity and *in vivo* antitumour activity in many leukemia and solid tumour model systems. Isoquinolinequinone metabolite cribostatin from the Indian Ocean sponge *Cribrochalina* spp. shows selective activity against all nine human melanoma cells in National Critical Technologies (NCT) panel. Spongstatin, a macrocytic lactone from the Indian Ocean collection of *Spongia* spp., is the most potent substance known against a subset of highly chemoresistant tumour types in the NCT tumour panel. Two new -pyrones (herbarin) along with a new phthalide, herbaric acid, were isolated from two cultured strains of the fungus *Cladosporium herbarum* isolated from the sponges *Aplysina aerophoba* and *Callyspongia aerizusa* collected in the French Mediterranean and in Indonesian waters, respectively.

Conclusions

"Poison kills the poison," the famous proverb is the basis for researchers in finding the biomedical metabolites from living organisms. Sea has got plenty of metabolites and other resources in living or dead form. Sponges (37%), coelenterates (21%) and microorganisms (18%) are the major sources of biomedical compounds followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%) bryozoans (1%), etc. The main emphasis is given in the search of drugs for deadly human diseases as cancer and AIDS. The scientists at different parts of the world have extracted various drugs for such diseases in recent years.

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Protein Chemistry and its application in Fish

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Proteins are macromolecules made of amino acids arranged in a linear chain and folded into a globular form. The amino acids in a polymer are joined together by the peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. The sequence of amino acids in a protein is defined by the sequence of a gene, which is encoded in the genetic code. Shortly after or even during synthesis, the residues in a protein are often chemically modified by post-translational modification, which alters the physical and chemical properties, folding, stability, activity, and ultimately, the function of the proteins.

The conformation of the protein is extremely important to exhibit its biological role. Conformation is nothing but the spatial arrangement of the protein molecule in which it exhibits its biological activity. The extent to which proteins fold into a defined structure varies widely.

Like other biological macromolecules such as polysaccharides and nucleic acids, proteins are essential parts of organisms and participate in virtually every process within cells. Many proteins are enzymes that catalyze biochemical reactions and are vital to metabolism. Being part of the muscular or contractile system, proteins have structural or mechanical functions. Proteins also play role in cell signaling, immune responses, cell adhesion, and the cell cycle. Proteins are also necessary in animals' diets, since animals cannot synthesize all the amino acids they need and must obtain essential amino acids from food. Through the process of digestion, animals break down ingested protein into free amino acids that are then used in metabolism.

Proteins were first described by the Gerhardus Johannes, a Dutch chemist and the name was given by the Swedish chemist Jöns Jakob Berzelius in 1838. It was understood that protein was the most important nutrient for maintaining the structure of the body. But their role as important agents of biological reactions i.e as enzymes was established in the year 1926 when James B. Sumner showed that the enzyme urease was in fact a protein. Insulin was the first protein to be sequenced by Frederick Sanger and the first protein structures to be solved were hemoglobin and myoglobin, by Max Perutz and Sir John Cowdery Kendrew, respectively, in 1958. Ever since lots of studies are going on to establish the never ending science of proteins in a living system. The three-dimensional structures of both proteins were first determined by x-ray diffraction analysis in 1962.

The basic understanding of proteins is extremely important to understand its heterogeic functions in the system. Beside, primary structure is responsible for the folding leading to different higher levels of structure. The primary structure of protein refers to the linear sequence of the different

amino acids in a peptide chain. In the primary structure, the amino acids are is held together by covalent or peptide bonds, which are made during the process of protein biosynthesis or translation. A specific sequence of nucleotides in DNA is transcribed into mRNA, which is read by the ribosome in a process called translation. The sequence of a protein is unique to that protein, and defines the structure and function of the protein. Post-translational modifications such as disulfide formation, phosphorylations and glycosylations contribute to the ultimate primary structure. The secondary structure refers to highly regular local sub-structures. These secondary structures are defined by patterns of hydrogen bonds between peptide groups. Two main types of secondary structure, the alpha helix and the beta strand represent a way of saturating all the hydrogen bond donors and acceptors in the peptide backbone. Some parts of the protein are ordered but do not form any regular structures. Tertiary structure refers to three-dimensional structure of a single protein molecule. The alpha-helices and beta-sheets are folded into a compact globule through the non-specific hydrophobic interactions such as salt bridges, hydrogen bonds, and the tight packing of side chains and disulfide bonds. Quaternary structure on the other hand is a larger assembly of several protein molecules or polypeptide chains. The quaternary structure is stabilized by the same non-covalent interactions and disulfide bonds as the tertiary structure.

Proteins, particularly enzymes are purified from different sources for possible commercial exploitation. Besides clinically also the techniques are applied in order to understand a particular clinical manifestation or physiological response to a condition. Proteins may be purified from other cellular components using a variety of techniques such as ultracentrifugation, precipitation, electrophoresis, and chromatography; the advent of genetic engineering has made possible a number of methods to facilitate purification.

It is not always possible to use enzymes isolated from nature to solve a given industrial problem. Many industrial processes use conditions that put extreme demands on the enzyme used. Examples of such conditions are very high temperature, extreme high or low pH, or high concentrations of detergents. Characteristics like temperature stability, solvent resistance, or even substrate specificity is among the parameters routinely altered in our enzyme products. The protein optimization platform utilizes several advanced technologies including Protein modeling, Protein engineering, Directed Molecular Evolution and High Throughput Screening.

Since a typical enzyme often is built up of more than 300 amino acids with the 20 possible amino acids occupying different positions, the number of combinations that can be made is almost endless. The simplest scenario is when the structure of an enzyme is known a protein chemist can work out for specific changes in the enzyme which will produce the desired product. These changes are then introduced one by one into the enzyme by our protein engineering experts and the altered proteins are purified and tested. A more complicated situation arises when the structure of the enzyme is known, but the computational analysis is unable to suggest specific changes, but merely suggest regions of the enzymes where introduction of amino acid changes hypothetically can confer a desired change. In this case it is often not sufficient to make single enzyme variants, but instead create libraries of enzyme variants in which the diversity introduction is targeted towards the regions suggested by the analysis. Such enzyme libraries can contain from a few hundred enzyme variants and up to millions or tens of millions of different enzymes. In these cases it is no longer possible to purify and analyze each individual member.

Protein modeling means combining knowledge of protein structure with the wealth of sequence information as such information are tools for understanding the biological activity and provides to alter or improving the natural activity of enzymes. On the basis of the three-dimensional structure of an enzyme it is possible to extract precise information about the enzyme properties and function. As the three-dimensional structure of an enzyme is so closely linked to its properties, it is a key point in enzyme optimization to be able to reveal this structure. At This information has been used to develop new enzymes with properties that can be of industrial benefit. X-ray crystallography and NMR techniques are handy in these types of studies..

An enzyme consists of several hundreds of amino acids linked together in a delicate three-dimensional structure. This structure determines the properties of the enzyme such as reactivity, stability and specificity. The locations and position if individual amino acids are available or can be predicted and it is possible to predict precisely to change one or few of the amino acids in t he enzyme so that the enzyme will function better in a desired application. This provides a way to engineer an enzyme to fit the needs of customers, and optimize its function. Using this information it is possible to construct the slightly altered enzymes by modifying the gene encoding of the enzyme. Engineered organisms then produce the modified enzyme that is tested to evaluate whether the predictions have been correct. By protein engineering it is possible to make enzymes with high activity or better kinetics namely enzymes that can withstand high temperatures, extreme pH and enzymes that work on several new substrates.

Directed Molecular Evolution techniques offer to scientifically alter the DNA make up so that new traits are produced which in turn leads to the production of enzymes or proteins with some changes in its activity or selectivity. Such modifications, known as mutations and recombination respectively, are completely natural and occur spontaneous within all living organisms. Directed molecular evolution is a highly efficient method for making improved enzyme solutions. By forcing enzymes to evolve in the laboratory, many new enzyme products have been discovered.

Proteomics is one of the most emerging fields of science today. Proteome is defined to mean proteins expressed by a genome and proteomics classically understood as the methodology used to study a proteome of an organism under defined conditions. Nowadays proteomics, however, is understood more broadly including also analysis of post-translational modifications of proteins, protein-protein interactions, and protein structures. Proteomics is an ever increasing area with potential commercial applications used for large-scale study of proteins, particularly their structures and functions. Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. The term “proteomics” was first coined in 1997 is the entire complement of proteins including the modifications made to a particular set of proteins, produced by an organism or system. By definition it the systematic study of all of the proteins in a cell, tissue, or organism. At its simplest level, proteomics is the study of protein expression in a proteome, or trying to understand the relative levels (amounts) of each protein within the mixture. Proteomics attempts to characterize proteins, compare variations in their expression levels in normal and disease states, study their interactions with other proteins, and identify their functional roles.

The term proteome was coined in 1994 to describe all of the proteins in a given cell, tissue, or organism. Proteomes are extremely complex and differ among individuals, cell types, and within the

same cell depending on cell activity, stimuli, and disease. There are estimated to be between one and ten million different proteins in the human body. Relatively few of these proteins have been identified.

Proteomics is being developed for use in cancer diagnosis and treatment. A protein pattern or array from blood or a cancer cell eventually may be the primary means of diagnosing cancer. Proteomics technology for cancer diagnosis and treatment identifies biomarkers namely proteins and protein patterns in blood, urine, and tissue that can be used to detect early cancers, treatment response, the likelihood of relapse after treatment. It can also be used to develop better cancer treatments, predict the effects of various treatments and develop individualized therapies for each patient

The advancements in proteomics has been made possible by the development of new technologies including high-resolution mass spectrometry (MS) that can sort out thousands of proteins and protein fragments on the basis of their molecular weight and electrical charge, sophisticated artificial intelligence computer programs that can learn to identify the specific patterns of a few proteins present in a huge protein array, laser capture microdissection microscopes that use low-energy laser beams and special transfer film to lift single cells from a tissue, to collect and analyze all of the proteins in the cell by (MS) and computer technology

A mass spectrometer consists of an ionization source that removes electrons from (ionizes) the proteins and protein fragments in a sample so that they all have a positive charge. The mass analyzer measures the mass-to-charge ratio (m/z) of the ionized (charged) proteins and fragments, as gases under a vacuum and a detector determines the number of ions present at each m/z value. The result is a mass spectrum or chart with a series of spikes or peaks, each representing a charged protein fragment from the sample. The height of each peak represents the amount of that particular protein or fragment that is present in the sample. The size of the peaks and the distance between them is the protein pattern or array of the entire sample.

Laser capture micro-dissection microscopes are useful tool for protein studies in clinical research. This enable to use tissue removed from a patient by a biopsy to isolate pure samples of normal cells, precancerous cells, and tumor cells from a single tissue of a single patient. Analysis of the protein patterns from these cells enable researchers to study patterns that may predict early-stage cancer, how a particular treatment affects the network of proteins in a cell, early signs of cancer drug toxicity, mechanisms of drug resistance, means for reducing side effects of treatment etc.

Unlike the traditional approach of studying individual proteins one at a time, proteomics uses an automated, high-throughput approach. High-throughput refers to the number of items (in this case, proteins) that can be analyzed or studied per unit of time. The development of genomics is much faster than proteomics primarily because proteins are more difficult to work with in a laboratory setting than are nucleic acids. The basic units of protein are related to twenty amino acids, whereas there are only four different nucleotides, the alphabet of DNA. The messenger RNA (mRNA) for some genes can be differentially spliced, meaning that multiple messages can be made from a single gene, resulting in multiple, and distinct protein products. Finally, many proteins are modified once they have been synthesized through post-translational modification. Some of the post-translational modifications include addition of sugar, phosphate, sulfate, lipid, acetyl, or methyl groups and each of these modifications has the ability to change the functional activity of a protein.

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Application of Mass Spectrometry in Bioprospecting

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Bioprospecting is the search in the biosphere, the collection of all living things, for new materials that have commercial value. Biodiversity prospecting or 'bioprospecting' is nothing new. Informal bioprospecting began when prehistoric people noticed that one plant root tasted better than another, or that some plants could be used as medicines. Much later scientists have been able to identify the active ingredients in these plants and extract or replicate that ingredient for widespread use. Alexander Fleming's discovery of penicillin was accidental bioprospecting. The isolation of the active ingredient in willow bark, which had been used by the Greeks since around the fifth century BC to relieve fever and pain gave us aspirin (salicylic acid). In the United States alone, 56% of the top 150 prescribed drugs, with an economic value of $80 billion, are linked to discoveries made in the wild.

The earth is rich in biological material that may not be fully understood. It's often the case that local areas, especially remote ones, use biological cures for disease that aren't lab produced and haven't yet been packaged and patented by some company. Great potential exists in these traditional medicines from small countries, and this has led to increased interest, especially by advanced countries, in finding potential beneficial biological substances, further developing them, and patenting them. This search is often called bioprospecting, but it may also be termed biopiracy by those who disapprove of the occasionally exploitive methods used by large companies desirous of being the first ones to patent a newly discovered biological "cure," which has sometimes been called the scientific equivalent of the gold rush. Bioprospectors often search for useful substances in 'extremophiles' – microorganisms, plants and fungi that grow in extreme environments, such as rainforests, deserts and hot springs.

The ocean is the richest source of chemical diversity in our world, it covers 70% of the earth's surface and hosts in excess of 300,000 described species of plants and animals, as well as very large numbers of microbial species, including bacteria, micro algae and fungi. Since the 1990's, several marine species have been used as a source of unique bioactive compounds, which have been exploited for the development of a range of drug products.

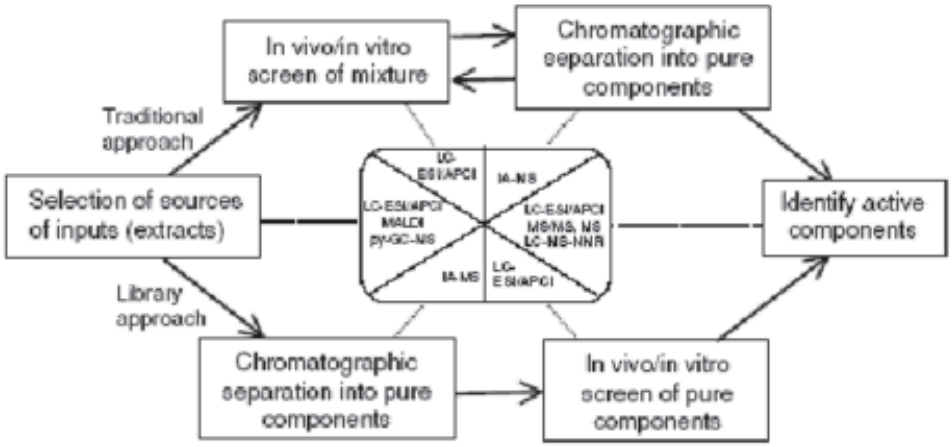
One key factor in the success of any natural product discovery effort is minimizing the time required to identify active compounds from complex natural sources. The use of high throughput and parallel chromatographic techniques has considerably reduced this cycle time. Concomitantly, the broad use of liquid chromatography/mass spectrometry (LC-MS) has significantly improved the efficiency of natural product screening and structure elucidation, which will be the focus of this

chapter. Indeed, mass spectrometry is now a tool that can impact many, if not all, of the stages of lead generation from natural products.

The discovery of novel biologically active compounds derived from nature has evolved from a relatively simple, linear "bioassay guided" process to one that encompasses several different approaches, as shown in Figure . The traditional approach to natural product screening involves selection of source material, extraction and assay of that material, and separation of components using bioassay-guided fractionation, culminating in the isolation and identification of a purified active. Conversely, in the natural product library approach, extracts are semipurified and concentrated prior to screening for activity almost the reverse of the traditional process. Both of these avenues rely on a variety of mass spectrometry techniques, which can have a significant impact on their success.

The demand for new chemical entities (NCEs) in lead discovery is greater now than at any time in the history of the pharmaceutical industry. Natural products have traditionally provided an excellent source of NCEs, often contributing structures that fall outside the diversity space encompassed by synthetic approaches. Natural products represent a diverse range of chemistry classes, including secondary metabolites, peptides, proteins, polysaccharides, and oligonucleotides. Many commercial drugs are either natural products, or have their roots in natural product chemistry, and at present over 60% of small-molecule anticancer leads are natural product–derived . A recent review summarizing the contribution that natural products have made to drug discovery concluded that "natural products play a dominant role in the discovery of leads for the development of drugs."

The role of mass spectrometry in several phases of the natural product discovery process, including (1) selection of source material, (2) screening, (3) dereplication of known compounds, and (4) identification of unknowns is discussed here. It is clear that over the last 10 years mass spectrometry has evolved into an indispensable tool in the generation of lead candidates from nature.



Mass spectrometry

Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample. For large samples such as biomolecules, molecular masses can be measured to within an accuracy of 0.01% of the total molecular mass of the sample *i.e.* within a 4 Daltons (Da) or atomic mass units (amu) error for a sample of 40,000 Da. This is sufficient to allow minor mass changes to be

detected, *e.g.* the substitution of one amino acid for another, or a post-translational modification. For small organic molecules the molecular mass can be measured to within an accuracy of 5 ppm or less, which is often sufficient to confirm the molecular formula of a compound.

Atomic and molecular masses are assigned relative to the mass of the carbon isotope, ^{12}C, whose atomic weight is defined as exactly 12. The actual mass of ^{12}C is 12 daltons, with one dalton is equal to $1.661\ 10^{-24}$ g. The mass of a molecule or an ion can be presented in daltons (Da) or kilodaltons (kDa). Mass spectrometry provides valuable information to a wide range of professionals: physicians, astronomers, and biologists, to name a few. More frequently mass spectrometers are used today in the following areas

- **Biotechnology:** *the analysis of proteins, peptides, oligonucleotides*
- **Pharmaceutical:** *drug discovery, combinatorial chemistry, pharmacokinetics, drug metabolism*
- **Clinical:** *neonatal screening, haemoglobin analysis, drug testing*
- **Environmental:** *PAHs, PCBs, water quality, food contamination*
- **Geological:** *oil composition*
- **Accurate molecular weight measurements:** *sample confirmation, to determine the purity of a sample, to verify amino acid substitutions, to detect post-translational modifications, to calculate the number of disulphide bridges*
- **Reaction monitoring:** *to monitor enzyme reactions, chemical modification, protein digestion*
- **Amino acid sequencing:** *sequence confirmation,* de novo *characterisation of peptides, identification of proteins by database searching with a sequence "tag" from a proteolytic fragment*
- **Oligonucleotide sequencing:** *the characterisation or quality control of oligonucleotides*
- **Protein structure:** *protein folding monitored by H/D exchange, protein-ligand complex formation under physiological conditions, macromolecular structure determination*

The Mass Spectrometer

Mass spectrometers can be divided into three fundamental parts, namely the ionisation source , the analyser , and the detector.

The sample has to be introduced into the ionisation source of the instrument. Once inside the ionisation source, the sample molecules are ionised, because ions are easier to manipulate than neutral molecules. These ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass (m) -to-charge (z) ratios (m/z) . The separated ions are detected and this signal sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of a m/z spectrum .

The analyser and detector of the mass spectrometer, and often the ionisation source too, are maintained under high vacuum to give the ions a reasonable chance of travelling from one end of the instrument to the other without any hindrance from air molecules. The entire operation of the mass spectrometer, and often the sample introduction process also, is under complete data system control on modern mass spectrometers.

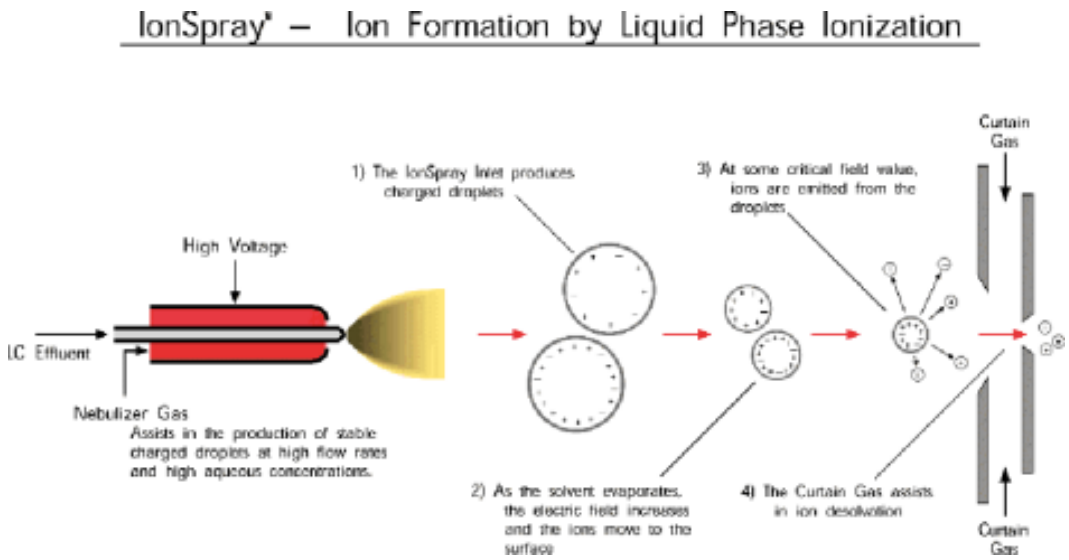
Methods of sample ionisation

Many ionisation methods are available and each has its own advantages and disadvantages . The ionisation method to be used should depend on the type of sample under investigation and the mass spectrometer available.

- Atmospheric Pressure Chemical Ionisation (APCI)
- Chemical Ionisation (CI)
- Electron Impact (EI)
- Electrospray Ionisation (ESI)
- Fast Atom Bombardment (FAB)
- Field Desorption / Field Ionisation (FD/FI)
- Matrix Assisted Laser Desorption Ionisation (MALDI)
- Thermospray Ionisation (TSP)

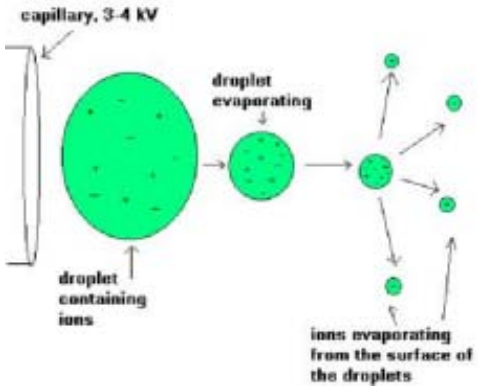
Electrospray ionisation

Electrospray Ionisation (ESI) is one of the Atmospheric Pressure ionisation (API) techniques and is well-suited to the analysis of polar molecules ranging from less than 100 Da to more than 1,000,000 Da in molecular mass.



During standard electrospray ionisation, the sample is dissolved in a polar, volatile solvent and pumped through a narrow,stainless steel capillary (75 - 150 micrometers i.d.) at a flow rate of between 1 µL/min and 1 mL/min. A high voltage of 3 or 4 kV is applied to the tip of the capillary, which is situated within the ionisation source of the mass spectrometer, and as a consequence of this strong electric field, the sample emerging from the tip is dispersed into an aerosol of highly charged droplets, a process that is aided by a co-axially introduced nebulising gas flowing around the outside of the capillary. This gas, usually nitrogen, helps to direct the spray emerging from the capillary tip towards the mass spectrometer. The charged droplets diminish in size by solvent evaporation, assisted by a

warm flow of nitrogen known as the drying gas which passes across the front of the ionisation source. Eventually charged sample ions, free from solvent, are released from the droplets, some of which pass through a sampling cone or orifice into an intermediate vacuum region, and from there through a small aperture into the analyser of the mass spectrometer, which is held under high vacuum. The lens voltages are optimised individually for each sample.



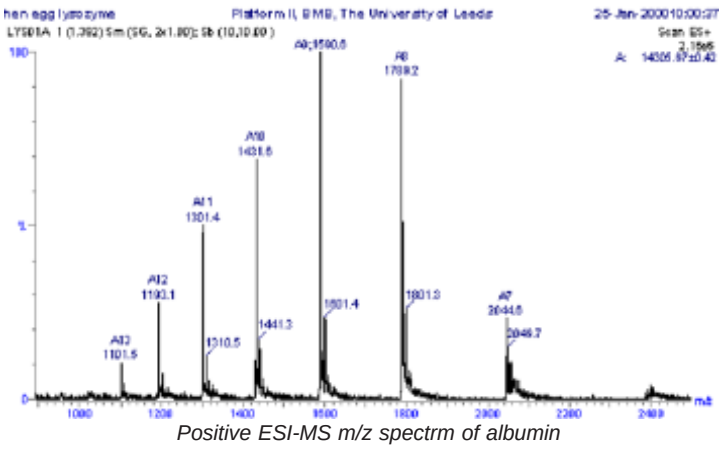
The electrospray ionisation process

Electrospray ionisation are very sensitive analytical techniques but the sensitivity deteriorates with the presence of non-volatile buffers and other additives, which should be avoided as far as possible.

In positive ionisation mode, a trace of formic acid is often added to aid protonation of the sample molecules; in negative ionisation mode a trace of ammonia solution or a volatile amine is added to aid deprotonation of the sample molecules. Proteins and peptides are usually analysed under positive ionisation conditions and saccharides and oligonucleotides under negative ionisation conditions. In all cases, the m/z scale must be calibrated by analysing a standard sample of a similar type to the sample being analysed (e.g. a protein calibrant for a protein sample), and then applying a mass correction.

Electrospray ionisation is known as a “soft” ionisation method as the sample is ionised by the addition or removal of a proton, with very little extra energy remaining to cause fragmentation of the sample ions.

Samples (M) with molecular weights greater than ca. 1200 Da give rise to multiply charged molecular-related ions such as $(M+nH)^{n+}$ in positive ionisation modeand $(M-nH)^{n-}$ in negative ionisation mode. Proteins have many suitable sites for protonation as all of the backbone amide nitrogen atoms could be protonated theoretically, as well as certain amino acid side chains such as lysine and arginine which contain primary amine functionalities.



Positive ESI-MS m/z spectrm of albumin

An example of multiple charging, which is practically unique to electrospray ionisation, is presented in the positive ionisation m/z spectrum of albumin.

Analysis and Separation of Sample Ions

The main function of the mass analyser is to separate , or resolve , the ions formed in the ionisation source of the mass spectrometer according to their mass-to-charge (m/z) ratios. There

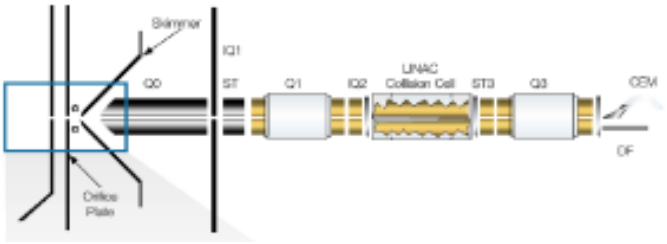
are a number of mass analysers currently available, the better known of which include quadrupoles , time-of-flight (TOF) analysers,magnetic sectors , and both Fourier transform and quadrupole ion traps .

These mass analysers have different features, including the m/z range that can be covered, the mass accuracy, and the achievable resolution. The compatibility of different analysers with different ionisation methods varies. For example, all of the analysers listed above can be used in conjunction with electrospray ionisation, whereas MALDI is not usually coupled to a quadrupole analyser.

Tandem (MS-MS) mass spectrometers are instruments that have more than one analyser and so can be used for structural and sequencing studies. Two, three and four analysers have all been incorporated into commercially available tandem instruments, and the analysers do not necessarily have to be of the same type, in which case the instrument is a hybrid one. More popular tandem mass spectrometers include those of the quadrupole-quadrupole, magnetic sector-quadrupole , and more recently, the quadrupole-time-of-flight geometries.

A tandem mass spectrometer is a mass spectrometer that has more than one analyser, in practice usually two. The two analysers are separated by a collision cell into which an inert gas (e.g. argon, xenon) is admitted to collide with the selected sample ions and bring about their fragmentation. The analysers can be of the same or of different types, the most common combinations being:

- quadrupole - quadrupole
- magnetic sector - quadrupole
- magnetic sector - magnetic sector
- quadrupole - time-of-flight.

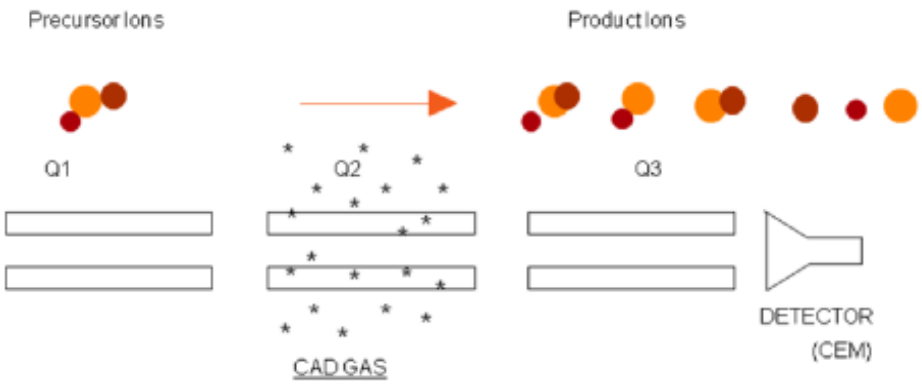


Schematic diagram of a triple quadrupole mass spectrometer (MSMS)

Fragmentation experiments can also be performed on certain single analyser mass spectrometers such as ion trap and time-of-flight instruments, the latter type using a post-source decay experiment to effect the fragmentation of sample ions.

The basic modes of data acquisition for tandem mass spectrometry experiments are as follows:

Product or daughter ion scanning:

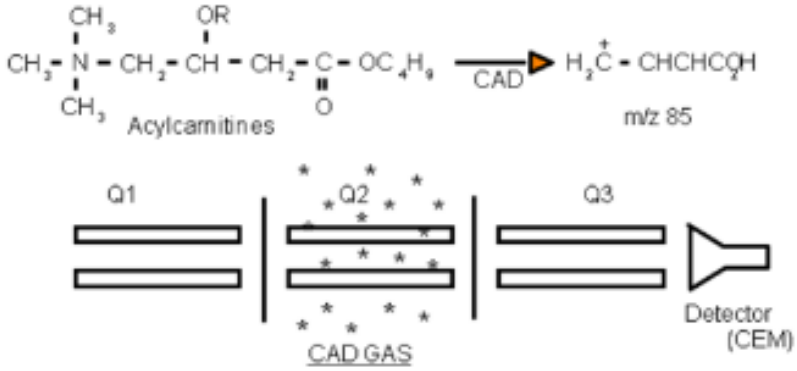


the first analyser is used to select user-specified sample ions arising from a particular component; usually the molecular-related (i.e. $(M+H)^+$ or $(M-H)^-$) ions. These chosen ions pass into the collision cell, are bombarded by the gas molecules which cause fragment ions to be formed, and these fragment ions are analysed i.e. separated according to their mass to charge ratios, by the second analyser. All the fragment ions arise directly from the precursor ions specified in the experiment, and thus produce a fingerprint pattern specific to the compound under investigation.

This type of experiment is particularly useful for providing structural information concerning small organic molecules and for generating peptide sequenceinformation.

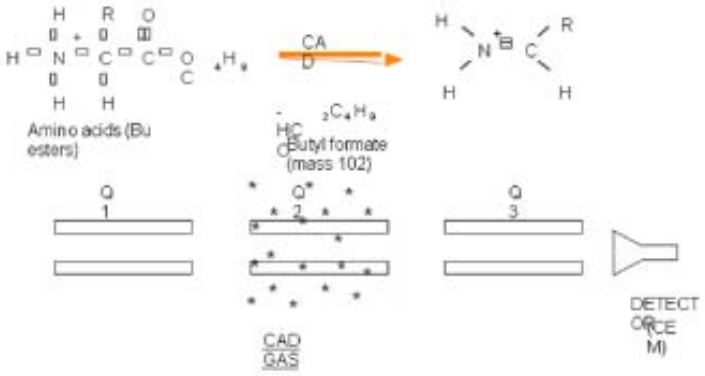
Precursor or parent ion scanning:

the first analyser allows the transmission of all sample ions, whilst the second analyser is set to monitor specific fragment ions, which are generated by bombardment of the sample ions with the collision gas in the collision cell. This type of experiment is particularly useful for monitoring groups of compounds contained within a mixture which fragment to produce common fragment ions, e.g. glycosylated peptides in a tryptic digest mixture, aliphatic hydrocarbons in an oil sample, or glucuronide conjugates in urine.



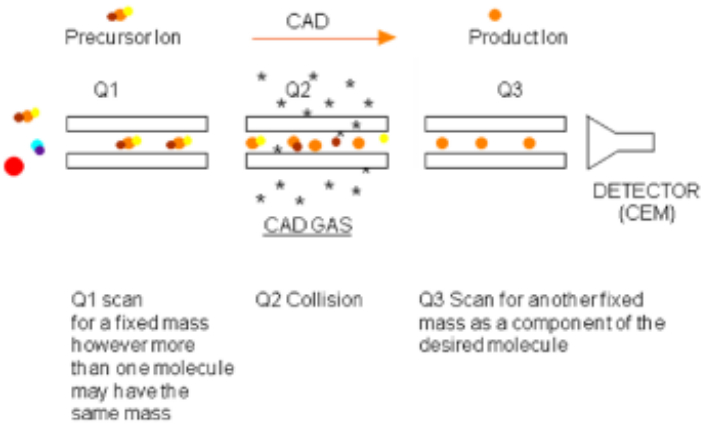
Constant neutral loss scanning:

this involves both analysers scanning, or collecting data, across the whole m/z range, but the two are off-set so that the second analyser allows only those ions which differ by a certain number of mass units (equivalent to a neutral fragment) from the ions transmitted through the first analyser. e.g. This type of experiment could be used to monitor all of the carboxylic acids in a mixture. Carboxylic acids tend to fragment by losing a (neutral) molecule of carbon dioxide, CO_2, which is equivalent to a loss of 44 Da or atomic mass units. All ions pass through the



first analyser into the collision cell. The ions detected from the collision cell are those from which 44 Da have been lost.

Selected/multiple reaction monitoring:



both of the analysers are static in this case as user-selected specific ions are transmitted through the first analyser and user-selected specific fragments arising from these ions are measured by the second analyser. The compound under scrutiny must be known and have been well-characterised previously before this type of experiment is undertaken. This methodology is used to confirm unambiguously the presence of a compound in a matrix e.g. drug testing with blood or urine samples. It is not only a highly specific method but also has very high sensitivity.

Matrix assisted laser desorption ionization

Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) System provides reliable mass information with higher sensitivity and throughput; and need for lesser individual assay optimization for molecular identification and characterization. In addition, the MALDI-TOF MS System has the added potential of detecting submicroscopic deletions within chromosomal DNA. Key features of our MALDI-TOF services are as follows:

- Sensitivity
- Flexibility
- Excellent Reproducibility
- High-throughput
- Accurate
- Fast Turn-Around-Time
- Suitable for large number of samples
- Relative Standard Deviation doesn't vary with concentration

The benefit of MALDI-TOF is that it allows extremely small amount of samples (10-12 to 10-15 moles) to be characterised. Being more accurate, faster analysis times and because it allows high sample throughput, MALDI-TOF system works out to be cheaper for large number of samples. MALDI-TOF system allows analysis of mixtures and different classes of biopolymers, including peptides, oligonucleotides, glycoconjugates, and synthetic polymers.

Key Applications and Services using MALDI-TOF System

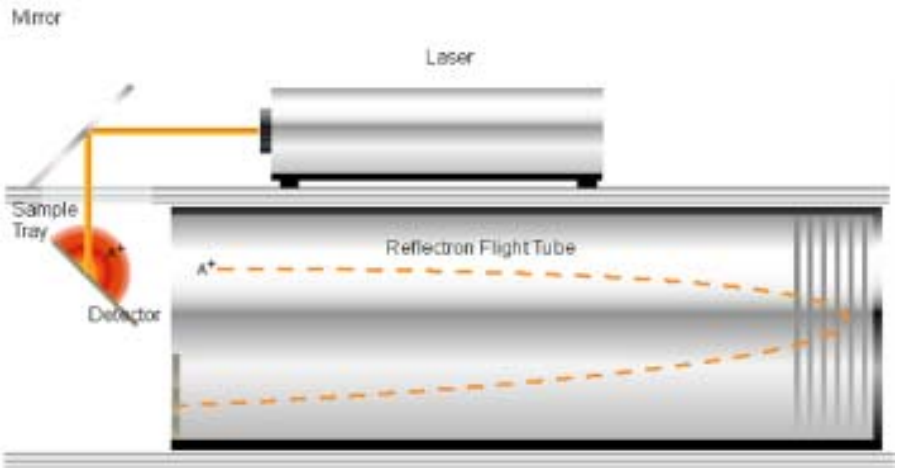
Nutrition and Food Testing Environmental Research

	Nutrition and Food Testing	Environmental Research	Molecular Diagnostics	Biopharmaceutical
Protein Expression	Determination of food adulterants	Environmental bioremediation	Tissue protein profiling Accurate quantitation and differentiation of protein expression between diseased and normal tissues	Identify disease specific proteins
Sequencing	Carcinogen detection.	Detection of isolated bacterial toxins, metabolites and DNA/RNA	Parallel determination of multiple antibiotic resistance Determination of microbial epidemiology on a genetic level	Determination of primary amino acid sequence using MS sequence data. QC tests for verification of primary amino acid sequence.
SNP Genotyping	Identify markers to enhance nutritional quality or aroma generation	Identify enzymes, repeating proteins	Early screening /profiling of complex biomarker patterns for specific diseases	Metabolite identification
Molecular Typing	Characterize food products originating from different geographical locations	Molecular identification of microbes, viruses, haploids causing environmental degradation	Identify hereditary diseases Detect analyte molecules of defined molecular weight	Analysis of spacial distribution of target molecules in tissue samples Measurement of Immunoglobulin characterization
Quantitative Gene Expression Analysis	Identify metabolites produced by spoilage micro-organisms	Create library of microorganism diversity Comparison to reference data set	Sensitive detection of pathogen DNA in human tissues Validation of gene expression data	Compound characterization and impurity detection in APIs

Matrix Assisted Laser Desorption Ionisation (MALDI) deals well with thermolabile, non-volatile organic compounds especially those of high molecular mass and is used successfully in biochemical areas for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. It is relatively straightforward to use and reasonably tolerant to buffers and other additives. The mass accuracy depends on the type and performance of the analyser of the mass spectrometer, but

most modern instruments should be capable of measuring masses to within 0.01% of the molecular mass of the sample, at least up to ca. 40,000 Da.

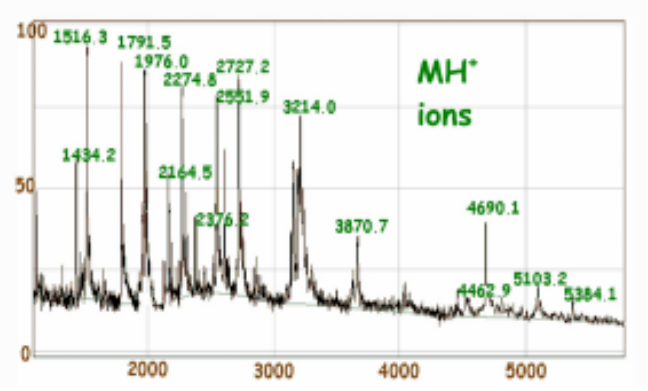
MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionisation. The sample is pre-mixed with a highly absorbing matrix compound for the most consistent and reliable results, and a low concentration of sample to matrix works best. The matrix transforms the laser energy intoexcitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. In this way energy transfer is efficient and also the analyte molecules are spared excessive direct energy that may otherwise cause decomposition. Most commercially available MALDI mass spectrometers now have a pulsed nitrogen laser of wavelength 337 nm. The sample to be analysed is dissolved in an appropriate volatile solvent, usually with a trace of modifier. The laser is fired, the energy arriving at the sample/matrix surface optimised, and data accumulated until a m/z spectrum of reasonable intensity has been amassed. The time-of-flight analyser separates ions according to their mass(m)-to-charge(z) (m/z) ratios by measuring the time it takes for ions to travel through a field free region known as the flight, or drift, tube. The heavier ions are slower than the lighter ones.



Simplified schematic of MALDI-TOF mass spectrometry

MALDI is also a "soft" ionisation method and so results predominantly in the generation of singly charged molecular-related ions regardless of the molecular mass, hence the spectra are relatively easy to interpret. Fragmentation of the sample ions does not usually occur.

In positive ionisation mode the protonated molecular ions ($M+H^+$) are usually the dominant species, although they can be accompanied by salt adducts, a trace of the doubly charged molecular ion at



MALDI m/z spectrum of a peptide mixture using alpha-cyano-4-hydroxycinnamic acid as matrix

approximately half the m/z value, and/or a trace of a dimeric species at approximately twice the m/z value. Positive ionisation is used in general for protein and peptide analyses.

In negative ionisation mode the deprotonated molecular ions ($M-H^-$) are usually the most abundant species, accompanied by some salt adducts and possibly traces of dimeric or doubly charged materials. Negative ionisation can be used for the analysis of oligonucleotides and oligosaccharides.

Positive or negative ionisation?

If the sample has functional groups that readily accept a proton (H^+) then positive ion detection is used e.g. amines $R\text{-}NH_2 + H^+ = R\text{-}NH_3^+$ as in proteins or peptides.

If the sample has functional groups that readily lose a proton then negative ion detection is used

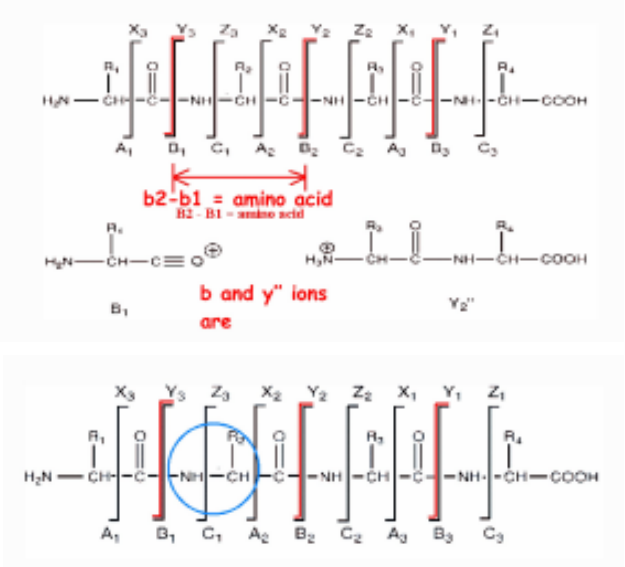
e.g. carboxylic acids $R\text{-}CO_2H = R\text{-}CO_2^-$ and alcohols R-OH = R-O- as in saccharides or oligonucleotides

Uses in Bioprospecting

The most common usage of MS-MS in biochemical areas is the product or daughter ion scanning experiment which is particularly successful for peptide and nucleotide sequencing. Tandem mass spectrometry (MS-MS) is also used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. This information can then be pieced together to generate structural information regarding the intact molecule.

Peptide sequencing

Peptides fragment in a reasonably well-documented manner The protonated molecules fragment along the peptide backbone and also show some side-chain fragmentation with certain instruments. There are three different types of bonds that can fragment along the amino acid backbone: the NH-CH, CH-CO, and CO-NH bonds. Each bond breakage gives rise to two species, one neutral and the other one charged, and only the charged species is monitored by the mass spectrometer. The charge can stay on either of the two fragments depending on the chemistry and relative proton affinity of the two species. Hence there are six possible fragment ions for each amino acid residue and these are labelled as in the diagram, with the a, b, and c" ions having the charge retained on the N-terminal fragment, and the x, y", and z ions having the charge retained on the C-terminal fragment. The most common cleavage sites are at the CO-NH bonds which give rise to the b and/or the y" ions. The mass difference between two adjacent b ions, or y"; ions, is indicative of a particular amino acid residue.



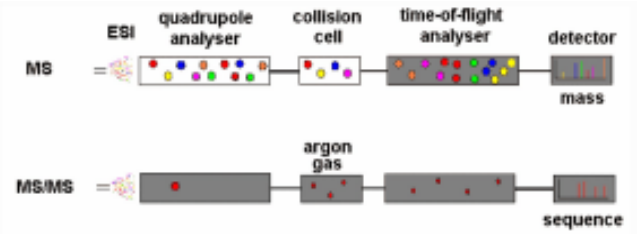
Peptide sequencing by tandem mass spectrometry - backbone cleavages

The extent of side-chain fragmentation detected depends on the type of analysers used in the mass spectrometer. A magnetic sector - magnetic sector instrument will give rise to high energy collisions resulting in many different types of side-chain cleavages. Quadrupole - quadrupole and quadrupole - time-of-flight mass spectrometers generate low energy fragmentations with fewer types of side-chain fragmentations.

Immonium ions (labelled “i”) appear in the very low m/z range of the MS-MS spectrum. Each amino acid residue leads to a diagnostic immonium ion, with the exception of the two pairs leucine (L) and iso-leucine (I), and lysine (K) and glutamine (Q), which produce immonium ions with the same m/z ratio, i.e. m/z 86 for I and L, m/z 101 for K and Q. The immonium ions are useful for detecting and confirming many of the amino acid residues in a peptide, although no information regarding the position of these amino acid residues in the peptide sequence can be ascertained from the immonium ions.

A protein identification study would proceed as follows:

- The protein under investigation would be analysed by mass spectrometry to generate a molecular mass to within an accuracy of 0.01%.
- The protein would then be digested with a suitable enzyme. Trypsin is useful for mass spectrometric studies because each proteolytic fragment contains a basic arginine (R) or lysine (K) amino acid residue, and thus is eminently suitable for positive ionisation mass spectrometric analysis. The digest mixture is analysed - without prior separation or clean-up - by mass spectrometry to produce a rather complex spectrum from which the molecular weights of all of the proteolytic fragments can be read. This spectrum, with its molecular weight information, is called a peptide map. (If the protein already exists on a database, then the peptide map is often sufficient to confirm the protein.) For these experiments the mass spectrometer would be operated in the ”MS” mode, whereby the sample is sprayed and ionised from the nanospray needle and the ions pass through the sampling cone, skimmer lenses, Rf hexapole focusing system, and the first (quadrupole) analyser. The quadrupole in this instance is not used as an analyser, merely as a lens to focus the ion beam into the second (time-of-flight) analyser which separates the ions according to their mass-to-charge ratio.



Q-TOF mass spectrometer operating in MS (upper) and MS/MS mode (lower) modes

- With the digest mixture still spraying into the mass spectrometer, the Q-Tof mass spectrometer is switched into ”MS/MS” mode. The protonated molecular ions of each of the digest fragments can be independently selected and transmitted through the quadrupole analyser, which is now used as an analyser to transmit solely the ions of interest into the collision cell which lies inbetween the first and second analysers. An inert gas such as argon is introduced into the collision cell and the sample ions are bombarded by the collision gas molecules which cause them to fragment. The optimum collision cell conditions vary from peptide to peptide and must be optimised for each one. The fragment (or daughter or product) ions are then analysed by the second (time-of-flight) analyser. In this way an MS/MS spectrum is produced showing all the fragment ions that arise directly from the chosen parent or precursor ions for a given peptide component.

- An MS/MS daughter (or fragment, or product) ion spectrum is produced for each of the components identified in the proteolytic digest. Varying amounts of sequence information can be gleaned from each fragmentation spectrum, and the spectra need to be interpreted carefully. Some of the processing can be automated, but in general the processing and interpretation of spectra will take longer than the data acquisition if accurate and reliable data are to be generated.
- The amount of sequence information generated will vary from one peptide to another, Some peptide sequences will be confirmed totally, other may produce a partial sequence of, say, 4 or 5 amino acid residues. Often sequence "tag" of 4 or 5 residues is sufficient to search a protein database and confirm the identity of the protein.
- This can be achieved by sample clean-up and then MS/MS studies to determine the amino acid sequences of the individual proteolytic peptides contained in the digest mixture, with which further database searching can be carried out.

Mass Spectrometry as a tool For Biomarker Discovery

In MALDI, sample molecules are laser-desorbed from a solid or liquid matrix containing a highly UV-absorbing substance. Both ESI-MS and MALDIMS have made MS increasingly useful for sophisticated biomedical analysis. While MS used to be restricted to the analysis of small organic and inorganic molecules, the development of ESI-MS and MALDI-MS have allowed for such applications as the sequencing and analysis of peptides and proteins, studies of noncovalent complexes and immunological molecules, DNA sequencing, and the analysis of intact viruses.

As you know, the predominant proteomic method of disease biomarker discovery in use today is a combination of 2D-PAGE and MS . In this method, proteins from two distinct samples (i.e., diseased vs. normal, treated vs. control, etc.) are analyzed via 2D-PAGE and their protein expression patterns compared. Protein spots of interest are excised from the gel, proteolytically or chemically digested, and the resultant peptides analyzed by MS to identify the protein.

An excellent example of the use of 2D-PAGE MS analysis for the discovery of potential cancer-related biomarkers is illustrated in the discovery of 93 lung adenocarcinomas and 10 uninvolved lung samples After measuring and comparing the relative abundances of the individual protein spots, those that showed a difference in relative intensity were identified via peptide mapping using MALDI-MS or ESI-MS/M

Purification of Natural Product Libraries

As discussed earlier, natural product discovery has traditionally been performed using the process of bioassay-guided fractionation. In this approach, each sample is extracted, typically with an organic solvent, to produce a small number of extracts. These extracts contain mixtures of compounds that are subsequently submitted for biological screening. Extracts that show activity against the target of interest are then advanced for extensive fractionation and bioassay to determine the identity of the active component(s).

Although bioassay-guided fractionation has been successfully applied for many years, this approach has several limitations. Because natural product extracts often contain complex mixtures, they can create several challenges when submitted for high throughput screening. For example, many extracts contain ubiquitous compounds that may mask or interfere with the biological assays. In addition, the extracts often contain compounds covering a wide range of concentrations. As a result, only a limited

number of the components may be present at the appropriate concentration for the screen of interest. In these cases, minor components with interesting biological activity may be missed.

Modern pharmaceutical screening campaigns can currently screen more than 200,000 compounds from an internal library in a few weeks . These screening efforts are generally optimized to screen relatively pure compounds produced using traditional or combinatorial synthesis. Recent advances in genomics and proteomics have allowed the rapid development of new targets, which has emphasized the need to maintain libraries of diverse, well-characterized, and relatively pure chemistries that can be submitted for high throughput biological screening (HTS). Although the bioassay-guided fractionation process can ultimately generate isolated compounds compatible with HTS, the cost and resources required to generate these libraries is viewed by many as not competitive with approaches such as combinatorial chemistry.

Recently, several approaches have been developed to address these challenges via automated separation of natural product extracts into purified or semipurified fractions. The purpose of these systems is to improve the compatibility of natural product libraries with HTS campaigns by (1) reducing of the number of components in each sample, (2) adjusting the concentrations to levels appropriate for HTS, (3) and eliminating nontarget (e.g., high molecular-weight) compounds that may produce false positives or negatives.

Mass spectrometry has played an important role in the development and implementation of several of these approaches. Significant effort has been made to automate the process of natural-product fractionation prior to screening. These systems can perform automated two-dimensional (2D) preparative LC directly coupled with solid-phase extraction (SPE) under high pressure. Using these systems, large quantities (1–5 g) of crude extract can be intelligently fractionated into several hundred fractions in less than 24 hours. This fractionation is accomplished with preparative LC, followed by polarity adjustment, SPE trapping, washing, and elution. Fraction collection can be triggered with time,

Conclusion:

While owing its origins to J.J. Thomson's discovery of electrons and positive rays in a vacuum tube over a century ago MS did not achieve the status it enjoys in the field of biochemistry until the 1990s. Over the past century the primary use of MS has been the characterization of small organic and inorganic molecules; however, several key developments have made MS one of the (if not the) primary analytical instruments in protein chemistry. These developments have enabled MS with the attributes needed to not only characterize proteins, but also in combination have allowed extremely complex mixtures of proteins to be characterized in an automated, robust, and high throughput fashion. Rightly used, this technique can be used for the development of science, especially in development of medicines for human health and prosperity.

Suggested Reading

Electrospray and MALDI Mass Spectrometry: Fundamentals, Instrumentation, Practicalities, and Biological Applications (Wiley interscience 2nd edition).Richard.B.Cole.

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Mass Spectrometry: Principles and Applications (Wiley interscience, 2007). Edmond de Hoffmann.

Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (1997).John J. Lennon.

Fish Health

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An overview on fish pathogens with special reference to aquaculture

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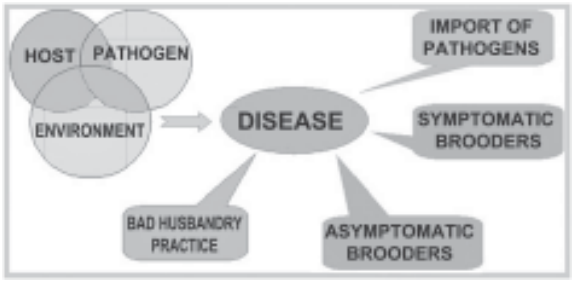
Introduction

50 per cent of global production of aquatic animals for human consumption comes from aquaculture, close to 90 per cent of which is produced by countries in the Asia-Pacific region. With the increase in culture of new aquatic animal species, new and emerging diseases are constantly being discovered.

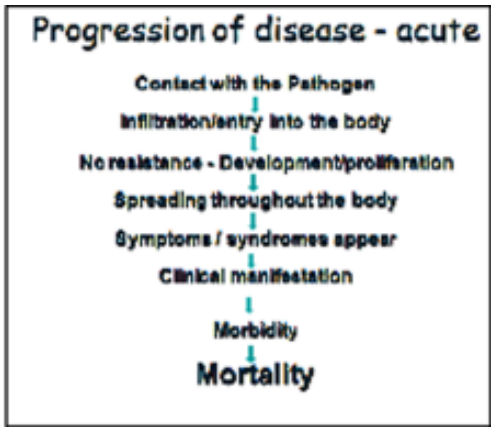
Diseases can be caused by a variety of factors, the most important being pathogens. Other factors contributing towards the development of disease conditions include stress, environmental/ water quality, physical agents, nutritional imbalance, toxins etc or a combination of these. Disease condition what we see is thus a complex situation resulting from the interaction/modification of the primary disease condition by these biotic and abiotic factors. The effect of diseases on animals range from reduced production to mortalities. In nature we are less aware of fish disease problems because sick animals are quickly removed from the population by predators, and moreover fish are much less crowded in natural systems than in captivity. Pathogens are always present in the environment and a delicate balance exists between the host, pathogen and environment (fig.1). Any changes in any of these factors may disturb the equilibrium and may lead to increasing or decreasing levels of disease.

The most obvious sign of disease in any system is the presence of dead or dying animals. However, careful observation can usually tell that fish are sick before they start dying because sick fish often stop feeding and may appear lethargic. Fish that are observed hanging listlessly in edges of the pond, gasping at the surface, abnormalities in the feeding pattern (poor feeding or overfeeding) or rubbing against objects indicate something may be wrong. These behavioral abnormalities indicate that the fish are not feeling well or that something is irritating them. In addition to behavioral changes, there are physical signs that indicate potential disease problems in fish. These include the presence of sores (ulcers or hemorrhages), ragged fins, or abnormal body confirmation (i.e., a distended abdomen or “dropsy” and exopthalmia or “poped up eyes” or bulged eyes). When these abnormalities are observed, the fish should be evaluated for the presence of diseases. Since disease in aquatic system is largely a management problem, the conventional preventive approach - “prevention is better than cure” – is the only viable option. Thus for tackling any disease condition, the first step is the correct diagnosis of the problem at the right time followed by suitable treatment and management schedules. If the diagnosis is faulty the result will be loss of animals along with wasted treatment efforts.



There are two broad categories of diseases that affect fish, infectious and non-infectious diseases. Infectious diseases are caused by pathogenic organisms present in the environment or carried by other fish. Majority of diseases affecting fishes are infectious, caused by opportunist viruses, bacteria, and parasites. These pathogens multiply in vast numbers in the fish, causing massive damage to the organism by depriving it of life-essential substances and/or by producing ichthyotoxic substances. In both cases the health of the fish is affected and results in diseases, unless appropriate treatment is given. In contrast, non-infectious diseases are caused by environmental problems, nutritional deficiencies, or genetic anomalies; they are not contagious and usually cannot be cured by medications.

Based on their nature, diseases can be classified into Acute and Chronic. In the case of chronic infections the development is very slow, usually may or may not show pathology/symptoms and may remain in the host's body for a prolonged period serving as a reservoir/carrier while in acute infections the infection progresses rapidly resulting in mortality.



The time lag between the entry of pathogen into the body and the development of symptoms is known as the incubation period which varies depending upon various biotic and abiotic factors. Healthy fish have the natural ability to defend themselves against infections and even if the pathogens gains entry into the body, a strong immunity/host resistance prevents the pathogen from establishing. Stress, however, slows down the immune system making the fish weak and unable to defend itself. The equilibrium between the host and pathogen is of key importance in the process and if the pathogen dominates, disease symptoms start appearing.

Infectious diseases

Issues regarding aquatic animal health are usually referred to the International Disease Commission (*Office International des Epizootices* [OIE]). Its mission is to inform governments on the occurrence and course of diseases throughout the world and of ways to control these diseases, to co-ordinate studies devoted to the surveillance and control of animal disease, and to harmonize

regulations for trade in animals and animal products among its 158 member countries. In Asia, the OIE and the Network of Aquaculture Centres in Asia-Pacific (NACA) collaborate to create an OIE/ NACA Regional Information System for Aquatic Animal Diseases, aimed at promoting the health of aquatic animals. The *OIE Aquatic Animal Health Code*, first published in 1995, provides international standards for the safe trade of amphibians, crustacean, fish, molluscs and their products. The international fish disease commission has listed more than 17 diseases of concern among the farmed finfishes (OIE, 2006)

OIE Listed diseases in Fishes

- Epizootic haematopoietic necrosis
- Infectious haematopoietic necrosis
- *Oncorhynchus masou* virus disease
- Spring viraemia of carp
- Viral haemorrhagic septicaemia
- Channel catfish virus disease
- Viral encephalopathy and retinopathy
- Infectious pancreatic necrosis
- Infectious salmon anaemia
- Epizootic ulcerative syndrome
- Bacterial kidney disease (*Renibacterium salmoninarum*)
- Enteric septicaemia of catfish (*Edwardsiella ictaluri*)
- Piscirickettsiosis (*Piscirickettsia salmonis*)
- Gyrodactylosis (*Gyrodactylus salaris*)
- Red sea bream iridoviral disease
- White sturgeon iridoviral disease
- Koi herpesvirus disease

Viral Diseases in aquacultured finfishes

Viral diseases have not been considered earlier to be a significant factor in marine and brackish water culture, but there have been many reports indicating the existence of viral diseases which cause severe mortalities in fin fishes. Among the various fish viruses, infectious pancreatic necrosis (IPN) virus which was reported back in 1940 in salmonid hatcheries is very well studied. IPN virus is an RNA virus, affecting young ones of salmonids in hatcheries causing mortality ranging from 60-100%. Other well-characterized fish viruses (e.g., channel catfish virus, *Onchorhynchus masou* virus) can also cause significant losses in aquaculture. Lymphocystis disease has been viewed as a serious problem in sea bass culture. The disease caused by an iridovirus, infects connective tissue cells and is characterized by nodular white swellings (cauliflower like) on fins or body. It is more common in marine and brackish water fishes.

The global expansion of finfish aquaculture has led to the discovery of several new viruses. Many of these are endemic among native populations and opportunistically spill-over to infect fish in

aquaculture facilities. The following are the major emerging fish virus diseases that cause significant losses in aquaculture and are expanding in host or geographic range (Table 1).

Infectious haematopoietic necrosis (IHNV) is an OIE notifiable disease, considered as an important pathogen of farmed rainbow trout (*Oncorhynchus mykiss*) in the USA. Through contaminated eggs, the disease reached out to Europe and Asia, where it emerged to cause severe losses in farmed rainbow trout, an introduced species.

Viral haemorrhagic septicaemia (VHS) is another emerging disease caused by a fish rhabdovirus. Viral haemorrhagic septicaemia virus is also considered as an important cause of mortality in rainbow trout reared in aquaculture.

Spring viraemia of carp (SVC) is caused by a fish rhabdovirus. More recently, SVC has spread out to many geographic regions of the world and has been associated with very large losses in common carp and Koi carp.

Infectious salmon anaemia (ISA) is caused by a virus of the family Orthomyxoviridae. An emerging disease of farmed Atlantic salmon, caused outbreaks with high mortality among Atlantic salmon reared in Norway. The virus was incriminated as the etiological agent of the hemorrhagic kidney disease of farmed Atlantic salmon along the Atlantic coast of Canada and the USA.

Epizootic haematopoietic necrosis is caused by a large DNA virus (EHNV) under the family Iridoviridae. First appeared in Australia where it caused mortalities among cultured rainbow trout and redfin perch.

Red sea bream iridoviral disease is caused by red sea bream iridovirus (RSIV), has a host range of at least 31 species of marine fish. Similar viral diseases were reported from new hosts and other geographic areas of Asia and the etiological agents were novel iridoviruses including: infectious spleen and kidney necrosis iridovirus (ISKNV) from cultured mandarinfish (*Sinaperca chuatsi*) in southern China and sea bass (*Lateolabrax* sp.) iridovirus (SBIV). In addition to causing outbreaks associated with severe necrosis and high mortality in a wide range of cultured marine fish, these viruses have emerged to affect freshwater species such as the African lampeye (*Aplocheilichthys normani*) and dwarf gourami (*Colisa lalia*).

Viral nervous necrosis (VNN) has emerged to become a major problem in the culture of larval and juvenile marine fish worldwide. Initially described as a cause of substantial mortality among cultured barramundi (*Lates calcarifer*) in Australia where the disease was termed vacuolating encephalopathy and retinopathy, the condition was shown to be caused by a small, icosahedral virus. The disease is listed by the Office International des epizooties (OIE) as a major problem in the production of marine fish worldwide during the last decade. To date, the disease has been reported in at least 30 fish species, with the greatest impact being in sea bass and is known to cause the disease in groupers, pleuronectids, snappers, white bass, sea bream, Atlantic halibut, large mouth bass and freshwater aquarium fishes. Adult fish are known to carry the virus which does not produce clinical manifestations in the host. VNN is the first OIE listed viral pathogen reported from India in farmed *Lates calcarifer.*

The disease caused by Koi Herpes Virus (KHV) is one of the most classical examples of an emerging disease of fish. Koi herpesvirus (KHV) is a highly contagious viral disease that cause

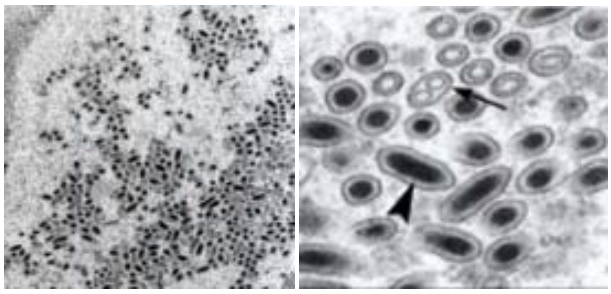
significant morbidity and mortality in common carp (*Cyprinus carpio*) and its ornamental subspecies, the Koi carp. KHV is a member of the genus Cyprinivirus in the family Alloherpesviridae and affects fish of various ages, but fry is more susceptibile. It causes 80-100% mortality in susceptible populations, with clinical signs of disease most commonly being expressed when water temperatures are between 22° and 27 °C. As with other herpes viral infections, KHV is believed to remain in the infected fish for life; therefore, exposed or recovered fish should be considered as carriers of the virus.

Table 1. Emerging viral pathogens of finfish.

Virus	Abbreviation	Genome	Taxonomic classification[1]	Known geographic distribution	OIE listed[2]
DNA viruses					
Epizootic haematopoietic necrosis virus and other ranaviruses	EHNV	dsDNA	*Iridoviridae, Ranavirus*	Australia, Europe, Asia, North America, Africa	Yes
Red sea bream iridovirus	RSIV	dsDNA	*Iridoviridae, Megalocytivirus*	Asia	Yes
Koi herpesvirus	KHV	dsDNA	*Alloherpesviridae, Cyprinivirus*	Asia, Europe, North America, Israel, Africa	Yes
RNA Viruses					
Infectious haematopoietic necrosis virus	IHNV	(−) ssRNA	*Mononegavirales, Rhabdoviridae, Novirhabdovirus*	Europe, North America, Asia	Yes
Viral haemorrhagic septicaemia virus	VHSV	(−) ssRNA	*Mononegavirales, Rhabdoviridae, Novirhabdovirus*	Europe, North America, Asia	Yes
Spring viraemia of carp virus	SVCV	(−) ssRNA	*Mononegavirales, Rhabdoviridae, Vesiculovirus*	Europe, Asia, North and South America	Yes
Infectious salmon anaemia virus	ISAV	(−) ssRNA	*Orthomyxoviridae, Isavirus*	Europe, North and South America	Yes
Viral nervous necrosis virus	VNNV	(+) ssRNA	*Nodaviridae, Betanodavirus*	Australia, Asia, Europe, North America, Africa, South Pacific	No

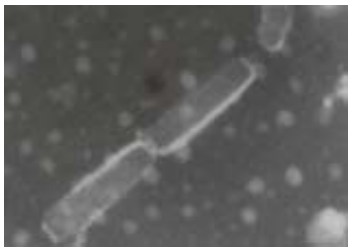
Viral diseases in farmed shrimp

Shrimp is the largest single seafood commodity, accounting for 17% of all internationally traded fishery products and 75% of the production comes from aquaculture. Viral diseases have had a major impact on the shrimp farming industry resulting in major crop failures and economic losses. Since 1981, a succession of new viral pathogens has emerged in Asia and the Americas, causing mass mortalities and threatening the economic sustainability of the industry. Almost all shrimp pathogens exhibit vertical and horizontal transmission. The disease is the result of a massive viral amplification that follows exposure to various biotic and abiotic stress factors. Among the infectious diseases of cultured shrimp, virus-caused diseases stand out as most significant. The pandemics due to the penaeid viruses, WSSV (White Spot Syndrome Virus), TSV (Taura Syndrome Virus) and YHV (Yellow Head Virus), have cost the penaeid shrimp industry billions of dollars in lost crops, jobs, and export revenue. The following are the viruses listed by the OIE as causing notifiable diseases of marine and freshwater shrimp.



White spot syndrome first emerged in China in 1992, was soon after reported in Taiwan and Japan and has since become panzootic throughout the shrimp farming regions of Asia and the America. It is the most devastating disease of farmed shrimp ever reported. White spot syndrome virus (WSSV) is a large, enveloped, ovaloid DNA virus with a

flagellum-like tail and helical nucleocapsid that has been classified as the only member of the new family Nimaviridae, genus Whispovirus. Although first emerged in farmed kuruma shrimp (*Penaeus japonicus*), WSSV has a very broad host range amongst all decapod crustaceans, all of which appear to be susceptible to infection. All farmed marine (penaeid) shrimp species are highly susceptible to white spot disease, with mass mortalities commonly reaching 80–100% in ponds within a period of 3–10 days.



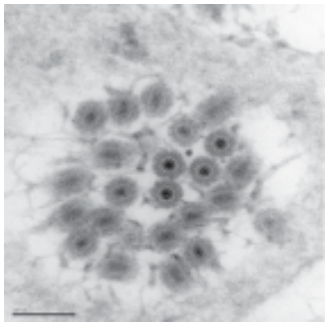
Monodon Baculo Virus (MBV) is the first shrimp virus to be recorded from India. The virus has become enzootic among the shrimp population of the country. It affects the hepatopancreatic tissues and only acute infections are a cause of worry. Non lethal screening of broodstock using PCR can be effectively used as a management strategy to prevent the entry of the virus into the rearing facility

Taura syndrome first emerged in white Pacific shrimp (*P. vannamei*). The disease spread rapidly throughout most shrimp farming regions of Central and South America and has now spread throughout much of Asia. Mortalities in the acute phase can be as high as 95% but surviving shrimp remain infected as a potential source of virus transmission. The susceptible host range of TSV is far more restricted than that of WSSV but includes most farmed marine shrimp species. The rapid spread of TSV has been attributed to the international trade in live shrimp.

Yellow head virus (YHV) is the most virulent of shrimp pathogens, causing total crop loss within several days of the first signs of disease in a pond. It first emerged in black tiger shrimp (*P. monodon*) in Thailand in 1990 and has since been reported in most major shrimp farming countries in Asia. YHV is now considered as a complex of six closely related viruses infecting *P. monodon*. Gill-associated virus (GAV) is a far less virulent virus that emerged to cause mid-crop mortality syndrome in farmed P. monodon in Australia in 1996.

Infectious hypodermal and haematopoietic necrosis was first detected in Hawaii in 1981, causing mass mortalities in blue shrimp (*Penaeus stylirostris*). IHHNV was found to be widely distributed in both *P. stylirostris* and *P. vannamei* shrimp throughout farming regions of the Americas and in the wild shrimp population of the Gulf of California. In Asia, IHHNV is endemic and occurs commonly in *P. monodon* which appears to be the natural host and in which it does not cause disease and has no impact on growth or fecundity.

Infectious myonecrosis is one of the most recent emerging viral diseases of marine shrimp. It first appeared in farmed *P. vannamei* and has subsequently spread throughout coastal regions of north-east Brazil and to Indonesia, Thailand and China Shrimp with the acute form of the disease display various degrees of skeletal muscle necrosis, visible as an opaque, whitish discolouration of the abdomen. Surviving shrimp progress to a chronic phase with persistent low-level mortalities. Several farmed marine shrimp species have been reported to be susceptible to infection but disease has only been reported in white Pacific shrimp. The practice of co-cultivation of white Pacific shrimp and black tiger shrimp is likely to present opportunities for adaptation and further spread of the disease. The trans-continental spread of the disease has certainly been due to the voluminous trade in *P. vannamei* broodstock.



White muscle disease is another emerging infection of the giant freshwater shrimp Macrobrachium rosenbergii. The causative agent is an RNA virus (Machrobrachium noda virus, (*Mr*NV), and the disease can affect larvae, postlarvae and early juvenile stages, causing up to 100% mortalities within 5–7 days of the first gross signs which include a white or milky appearance of abdominal muscle. Adults are resistant to the disease but can be persistently infected and transmit the infection vertically. Marine shrimp (*Penaeus monodon, P. japonicus* and *P. indicus*) have been shown to be susceptible to infection but did not develop disease, while artemia and some species of aquatic insects appear to be vectors.

Table II. Emerging viral pathogens of marine and freshwater shrimp.

Virus	Abbreviation	Genome	Taxonomic classification[1]	Year emerged	Known geographic distribution	OIE listed disease[2]
DNA viruses						
Monodon baculovirus	MBV	dsDNA	*Baculoviridae*	1977	Asia-Pacific, Americas, Africa	No
Baculoviral midgut gland necrosis virus	BMNV	dsDNA	*Baculoviridae*	1971	Asia, Australia	No
White spot syndrome virus	WSSV	dsDNA	*Nimaviridae, Whispovirus*	1992	Asia, Middle-East, Mediterranean, Americas	Yes
Infectious hypodermal and haematopoietic necrosis virus	IHHNV	ssDNA	*Parvoviridae, Densovirus*	1981	Asia-Pacific, Africa, Madagascar, Middle-East, Americas	Yes
Hepatopancreatic parvovirus	HPV	ssDNA	*Parvoviridae, Densovirus*	1983	Asia-Pacific, Africa, Madagascar, Middle-East, Americas	No
RNA viruses						
Yellow head virus	YHV	(+) ssRNA	*Nidovirales, Roniviridae, Okavirus*	1990	East and Southeast Asia, Mexico	Yes
Taura syndrome virus	TSV	(+) ssRNA	*Picornavirales, Dicistroviridae*	1992	Americas, East and Southeast Asia	Yes
Infectious myonecrosis virus	IMNV	(+) ssRNA	Totivirus (unclassified)	2002	Brazil, Indonesia, Thailand, China	Yes
Macrobrachium rosenbergii nodavirus	MrNV	(+) ssRNA	Nodavirus (unclassified)	1995	India, China, Taiwan, Thailand, Australia, Caribbean	Yes
Laem-Singh virus	LSNV	(+) dsRNA	Luteovirus-like (unclassified)	2003	South and Southeast Asia	No
Mourilyan virus	MoV	(−) ssRNA	Bunyavirus-like (unclassified)	1996	Australia, Asia	No

Bacterial diseases

A great number of aquatic bacteria are opportunists and do not cause diseases under normal environmental conditions. However, under the stressful conditions of intensive fish farming, these opportunistic bacteria can cause bacterial diseases. Bacteriae may be the primary cause of disease, or very often may be secondary invaders, taking advantage of a breach in the fish's body covering (skin) or compromised immune system. Opportunistic pathogenic bacteria can proliferate and spread disease throughout the fish's body fluid or tissues if they are absorbed through the gills or gut, or gain entry via the skin. This is known as a systemic infection. Many clinical signs of bacterial diseases of cultured marine finfish are similar. Typical signs of bacterial diseases in fish include: red and inflamed areas on the body and fins, raised scales, skin ulcers, exophtalmos, dropsy or swollen abdomen, fin rot etc. Additionally, affected fish may be lethargic

and anorexic. There may be lesions or haemorrhages in organs and/or a build-up of bloody fluid in the abdominal cavity (ascites).

The majority of bacterial infections are caused by Gram-negative organisms including the following pathogenic genera: *Aeromonas, Citrobacter, Edwardsiella, Flavobacterium (Flexibacter), Mycobacterium, Pseudomonas*, and *Vibrio.* Bacterial diseases caused by pathogenic vibrios are responsible for larval mortalities and economic losses in finfish and shellfish hatchery rearing systems. Among the Gram-positive bacteria, *Streptococcus* has been shown to cause disease in fishes. So far very few bacterial diseases have been reported from cultured marine finfish in Southeast Asia, and from India there is no report on the maricultured species. Commonly encountered bacterial diseases in fish are:

Aeromonas hydrophila: Gram negative bacteria, **c**auses Bacterial Hemorrhagic Septicemia. The most common symptom is hemorrhage in skin, fins, oral cavity and muscles with superficial ulceration of the epidermis.

Pseudomonas fluorescens: Lesions similar to *A. hydrophila* with hemorrhagic septicemia. *P. anquilliseptica* causes a serious mortalities in Japanese eels with a septicemia resulting in petechial hemorrhage on fins and tail and ulceration of the skin.

Vibrios: **G**ram negative rods, mainly marine. Cause hemorrhage in the skin of the tail and fins, ulceration of the skin, muscles and serosal surfaces. Deep skin ulcers and necrotizing myositis also seen. *V. alginolyticus* / *V. anquillarum* / *V. salmonicida* are the commony encountered species.

Edwardsiella tarda *(Edwardsiella* septicemia) affects primarily channel catfish but also observed in many other species. Is the most serious pathogen involving the eel culture in Asia. Lesions are similar to that of *A. hydrophila* with small cutaneous ulcers and hemorrhage in the skin and muscle.

Edwardsiella ictaluri (Enteric septicemia of catfish): Affects primarily fingerlings and yearling catfish. The most characteristic external lesion is the presence of raised or open ulcers on the frontal bone of the skull between the eyes (Hole in the head disease).

Aeromonas salmonicida (Furunculosis, Ulcerative disease of goldfish): septicemia with hemorrhage in the muscles and other sites. Subcutaneous swelling that often causes an ulcerative dermatitis observed.

Yersinia ruckeri (Enteric red mouth): Generally affects salmonids and rainbow trout are the most susceptible. Symptoms include septicemia with exophthalmus, ascites, and hemorrhage and ulceration of the jaw, palate, gills and operculum, musculature and serosal surfaces of the intestines.

Streptococcus iniae: Disease of tilapia, hybrid striped bass and rainbow trout. *S. iniae* develops either as an acute fulminating septicemia with hemorrhage of the fins, skin, and serosal surfaces and ulceration. The chronic form is limited primarily to the central nervous system. *S. iniae* is a problem primarily of closed recirculating culture system, probably associated with overcrowding and poor water quality.

Flexibacter columnaris (Columnaris disease or Saddleback disease): a highly communicable and serious disease of young salmonids, catfish and many other fish. Lesions usually first appear as small white spots on the caudal fin and progresses towards the head. The caudal and anal fins may become severely eroded. As the disease progresses, the skin is often involved with numerous gray white ulcers. Gills are a common site of damage and may be the only affected area. The disease is frequently associated with stress conditions.

Bacterial Gill Disease: Is caused by a variety of bacteria. *Flexibacter columnaris, Flexibacter psychrophilus* and various species of *Flavobacterium* are the primary bacteria involved in this disease. Fry are most susceptible to the disease.

Renibacterium salmoninarum (Bacterial Kidney Disease) A serious disease of salmonids caused by Gram positive bacillus. The disease follows a slow course with clinical signs not present until the fish is well grown. The fish may exhibit exophthalmus, skin darkening, and hemorrhage at the base of the fins. Cutaneous vesicles and ulcers may also develop.

Mycobacterium species (Tuberculosis): Gram positive, acid fast rods. *M. marinum, M. chelonei* and *M. fortuitum* are the most common Mycobacterium species involved. Infects almost all species of fish. Clinical signs of tuberculosis are quite variable, the most common signs being anorexia, emaciation, vertebral deformities, exophthalmus, and loss of normal coloration.

***Nocardia* sp. :** Gram positive filamentous rods. Chronic disease characterized by raised granulomatous masses in the mouth, jaw, gills and skin

Fungal Diseases

Fungal infections as such have not been considered to be an important factor in marine and brackish water fish culture, but there have been reports implicating various fungal pathogens in fish mortalities. They are opportunistic pathogens/secondary invaders which typically colonize exposed damaged tissue. Fungal infections are of 2 types, superficial and deep seated or systemic. The superficial ones, generally referred to as Saprolegniasis are by far the most commonly observed in fish. These are primarily freshwater fungi, however isolates have been reported from estuarine fishes also. Systemic fungal infections are considered to be more dangerous and difficult to cure. Ichthyophoniasis, a systemic fungal disease caused by *Ichthyophonus* sp. is capable of producing mortalities of epizootic proportions in wild marine fish populations. Epizootic Ulcerative Syndrome (EUS) is a pathogenic, invasive, fungal infection caused by the fungus, *Aphanomyces invadans* in Asian freshwater and estuarine fishes. It causes skin ulceration and death in over 30 species of commercially important cultured and wild fishes both in freshwater and estuarine habitats.

Parasitic Diseases

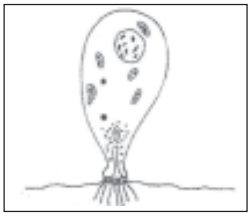
Parasites are generally opportunistic pathogenic organisms, causing diseases under congenial conditions. A variety of parasites are found infecting marine finfishes. Most of the parasites are

normally non pathogenic and do not cause diseases. When large numbers are present in the fish host, they can cause diseases and/or can become a major contributing factor to disease development. They are generally divided into two groups: ectoparasites, which live on the outside of the host (including the gills, mouth, skin and fin surfaces), and endoparasites, which live in the tissues, blood and/or organs (including the gastrointestinal tract). Based on their organization they are also classified into protozoans (single celled) and metazoans (multicellular).

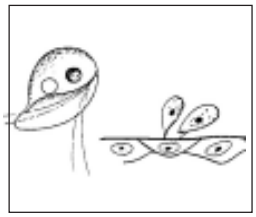
Protozoan parasites are a large heterogeneous group of organisms capable of causing severe damage to any marine fish in intensive culture systems. Protozoans have been reported to be pathogenic to grouper, sea bass and snapper fry and fingerlings at the nursery phase or grow-out phase during the first week after stocking in the cages. The ciliated protozoans, *Cryptocaryon irritans* and *Trichodina* spp., are highly pathogenic to newly introduced fish fry and juveniles in the cage environment. Various species of monogenean parasites are also known to cause serious production losses and mortalities in finfish culture. Other metazoan parasites like digenetic trematodes, nematodes, acanthocephalans and crustaceans are also of importance in finfish culture. Parasitic isopods, can under favourable conditions cause serious damages in cage cultured fin fishes. The pathogenicity of each parasite differs for each species of fish, as well as for each stage of the growth cycle and also the culture site. Some are highly host specific while many others are non-specific thus making their control of outbreaks very difficult.

Amoebiasis: Different species of amoebas have been incriminated in amoebic diseases in fish, of which *Neoparamoeba* spp. Is an important one. In heavy infections, the parasites elicit epithelial hyperplasia, resulting in complete fusion of secondary lamellae and subsequent gill disfunction. The disease is common in salmon.

Amyloodiniosis: known as “velvet disease”, the causative agent is *Amyloodinium ocelatum*, an ectoparasite on the skin and gills of fish. The parasite is least host-specific and affects almost all species of fish. Apart from the velvet appearance, clinical signs consist of anorexia and scratching. Massive infections are frequently associated with mortalities, both in mariculture and sea aquaria, mainly at high temperatures.



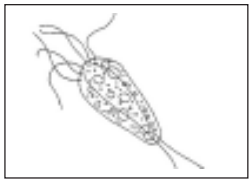
Ichthyobodosis or Costiasis: *Ichthyobodo* spp. (formerly known as *Costia*) are the agents of this disease infecting the gills and skin. *I. necator* is the species parasitizing salmonids in freshwater, but a different species is considered to be present in marine fish. Affected fish appear thin and lethargic, and may show a grey-whitish pellicle on skin, epidermic erosion or even haemorhages or ulcers, as well as gill hyperplasia and edema. Coastiasis is prevalent in different fish species, mainly in larval and juvenile stages.



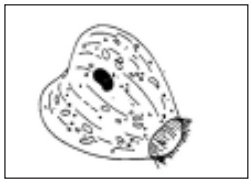
Cryptobiasis: *Cryptobia* spp., have a direct life cycle. Marine ectozoic species include *C. branchialis* and *C. eilatica.* In heavy infections, the parasites produce gill hyperplasia and epithelial destruction, with subsequent respiratory impairment. External signs are anorexia and skin darkness.

Flagellates: Some species of these genera parasitize internal organs of fish. *Cryptobia iubilans* is the only pathogenic intestinal species, common in cichlids. *Trypanoplasma* spp. and *Trypanosoma* spp. include parasites of the bloodstream and of tissues, with indirect life cycles (leeches are the

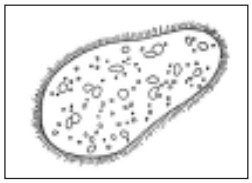
main vectors). The best known is *Trypanoplasma salmositica* (frequently referred as *Cryptobia samositica*) producing cryptobiasis of salmonids. Clinical signs consist of exophthalmia, splenomegaly, hepatomegaly, abdominal distension with ascites, anemia and anorexia. Mortality is dependent on fish stocks and species, but may be high in juveniles. The disease has severe impact in salmonid cultures in North America.



Hexamitiasis: *Hexamita* spp. are parasites of the intestine and gall bladder of freshwater fish, mainly salmonids but also cyprinids and ornamental fish. Hexamitiasis, typical of weak fish, is frequent as a secondary infection. Affected fish can show nervous behaviour, and internally the intestine may appear pale. Mortalities can occur in fry and juveniles.



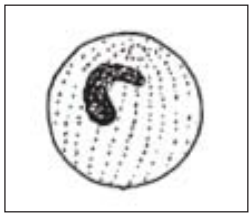
Chilodonellosis: Most *Chilodonella* spp. are free living, but some of them are serious pathogens of freshwater fish, causing heavy loses in aquaria and in cultures. Under favourable conditions they proliferate in the gills and skin of affected fish. The gills suffer hyperplasia, degeneration and necrosis, and respiration is drastically impaired. On the skin they may virtually cover the body surface.



Tetrahymena: Commonly called “Guppy Killer Disease.” Caused by a pear-shaped, ciliated, free-living/parasitic protozoan, common in crowded conditions and in water containing excessive organic debris. Severe infections can lead to mortalities.

Cryptocaryosis: *Cryptocaryon irritans*, a parasite of gills and skin, is the causative agent of this disease. External signs consist of white spots and excess mucus or ulcers on the skin and impairment of respiratory function. Gill histopathology consist of inflammation, haemorrhages, hyperplasia and lamellar destruction. This ciliate is a typical marine fish parasite affecting commercial and ornamental fish and producing high mortality in culture conditions. Outbreaks appears mainly at high temperatures.

Trichodiniasis: Fish trichodinids include mainly *Trichodina* spp., *Trichodinella* spp. and *Tripartiella* spp. These peritrichid ciliates are more commensals than genuine ectoparasites, but can produce different damages in massive infections. The fish show a grey-blue turbid layer on the skin. Respiratory function can be impaired in gill infections. Trichodinids parasitize a lot of freshwater and marine fish species.

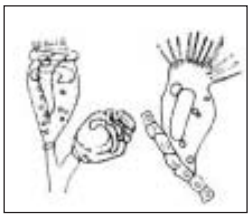


White spot disease: *Ichthyophtirius multifiliis* produces the well known white spot disease or ich. The most characteristic external sign is the presence of white spots on the skin and gills, due to parasite trophonts located under the upper layer of the skin. Affected fish can rub or flash and show breathing problems. In heavy infections the typical white spots are visible with the naked eye. The disease is widely distributed in many freshwater fish species, mainly in aquaria and culture conditions, in which it can produce epizootics. Mortality is mostly dependent on fish size and infection intensity.

Scuticociliatida: Several species of the genera *Uronema*, *Phylasterides* and *Miamiensis* have been recorded as facultative parasites of different fish. External signs include skin lesions or ulcers and

pigmentation changes, but the parasite frequently invade the body muscle and the internal organs, which become destroyed by this histophagous parasite. Nervous system can also be colonised, which can be accompanied by erratic swimming, equilibrium loss or lethargy. The disease cause severe infections and outbreaks in some cultured fish and mortalities can reach 100% of some affected stocks.

Surface fouling Diseases: Caused by a variety of ectocommensal peritrich ciliates. Usually these diseases are never considered serious, but in exceptional cases, along with other pathogens, they can cause considerable damage. In any healthy fish, peritrich ciliates will be present in small numbers, but does not usually cause any harm to their hosts and behave like typical ectocommensals. They attach to the surface of the fish, sometimes giving rise to heavy mat-like outgrowths. In its efforts to overcome the irritation/suffocation the animals rub/scratch their bodies resulting in bruises or open wounds on the skin, which again attract secondary invaders. *Epistylis, Ambiphrya, Capriana* etc are examples of ectocommensal ciliates infestibg fish.The most common ectocommensal found infesting shrimp is *Zoothamnium* sp.



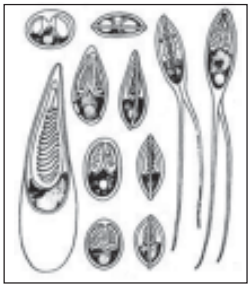
Endoparasitic Protozoans

Coccidiosis: Many species of coccidia infect freshwater and marine fishes with variable pathologies. The genera *Eimeria, Goussia* and *Cryptosporidium* include the species more frequently reported from cultured fish. In freshwater fish, *G. carpelli* parasitizes different cyprinids and *E. anguillae* is typical of eels. In marine fish, *E. sparis* and *G. sparis* have been reported.

Cryptosporidiasis: Fish *Cryptosporidium* spp. affect mainly larvae and juveniles, with deleterious effects not always very evident, but resulting in poor condition. *C. molnari* is more frequent in seabream than in seabass.

Microsporidiosis: Microsporea are represented in fish by different genera, mainly *Enterocytozoon*, *Glugea*, *Loma*, *Pleistophora* and *Tetramicra*. In freshwater fish, *Pleistophora* and *Loma* are relatively frequent. Among cultured marine fish, there have been several reports of *Plesitophora senegalensis* in gilthead seabream, whereas *Glugea* sp. and *Tetramicra brevifillum* have been found in turbot. Pathological concern of microsporidiosis in fish is dependent on location and infection intensity.

Myxosporea (myxosporidiosis) : Myxosporea include numerous genera and species, most of them parasites of fish. Some species are well know pathogens for freshwater fish and marine fish. The most pathogenic species belong to the genera *Ceratomyxa*, *Myxobolus, Myxidium, Spaherospora*, *Enteromyxum, Kudoa, Tetracapsuloides* and *Sphaerospora*. In freshwater fish the most significant diseases are whirling disease, PKD, sphaerosporosis and ceratomyxosis. Myxosporea reported from cultured marine fish include species of the genera *Ceratomyxa, Enteromyxum, Kudoa, Lepthoteca, Sphaerospora and Sinuolinea*.



Whirling disease is caused by *Myxobolus cerebralis* (*Myxosoma cerebralis*): Clinical signs include dark coloration of the posterior part of the body and abnormal swimming in spiral, followed

by skull deformation and spinal curvature. Almost all salmonid species can be infected, but susceptibility is very variable according to the species.

Proliferative kidney disease (PKD): The causative agent, formerly known as PKX, has been recently identified as *Tetracapsuloides bryosalmonae* (syn. *Tetracapsula bryosalmonae*, *T. renicola*). The parasite is highly pathogenic and can produce a severe disease in rainbow trout, with 30-50% mortality. External clinical signs are abdominal swelling, darkening and exophthalmos. Internally, a kidney enlargement is observed, accompanied by ascites in advanced cases.

Spaherospora renicola: Is widely distributed in intensive cultures of cyprinids, mainly *Cyprinus carpio*. Spores and sporogonic stages are located in the renal tubules, but proliferative stages appear in the blood and can reach the swimmbladder, causing inflammation. In the kidney tubules it produces dilatation, atrophy and necrosis of the epithelium, with subsequent impairment of renal function, making it a serious pathogen.

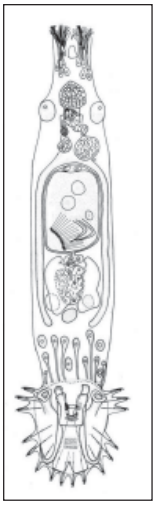
Ceratomyxa : The genus *Ceratomyxa* include a lot of marine species, though they have been rarely associated with significant pathological problems. The main species recorded in cultured marine fish are *C. diplodae, C. labracis* and *C. sparusaurati*. *C. diplodae* and *C. labracis* are quite frequent in wild and cultured seabass. They are not usually associated with clinical disease, but they can induce several histopathological lesions in the gall bladder and neighboring tissues.

Ceratomyxa shasta: Is an important pathogen, causing serious loses in cultured and wild populations of salmonids on the west coast of North America. Intestine is the target organ and parasites can be observed in the epithelium, eliciting lymphocytic infiltration, hyperplasia and necrosis. In advanced stages of the infection, parasites spread to other organs and fish become anorexic, lethargic, and show abdominal swelling, ascites and exophthalmia. Significant mortalities can occur, depending on fish species, as susceptibility is variable.

***Enteromyxum* spp. :** Two species of this genus have special pathological concern for marine fish of high commercial value, and both parasitize the digestive tract of infected fish. *Enteromyxum leei* previously known as *Myxidium leei*, produces enteromyxosis in sparids. The parasite invades the intestinal tract causing severe chronic enteritis, frequently followed by emaciation and death. *E. scophtahlmi* is an important pathogen of turbot cultures, as mortalities can reach 100 % of the affected tanks or stocks, with subsequent economical impact.

***Kudoa* spp:** These marine myxosporeans infect the muscle of many marine fish forming plasmodial cysts. Heavy infections can cause unsightly white cysts or soft texture in filets. with subsequent lowering of market value. In aquaculture, *Kudoa* infections have been described in salmonids and *Seriola*.

Sphaerospora: *Sphaerospora dicentrarchi* is a histozoic parasite with an affinity for the connective tissue of gall bladder and intestine. It is usually a chronic infection, without external clinical signs, though massive infections have been associated with extensive mortalities in juvenile fish. *S. testicularis* infects the testicular tissues. Heavy infections can result in parasitic castration of valuable broodstock fish. In very heavy infections, the parasite invades the serosa and other organs, producing abdominal swelling and ascitis.



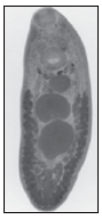
Monogenea : Members of Monogenea, mostly ectoparasites of gills and skin, can cause different degrees of damage in parasitized fish. Mortalities may appear in moderate or heavy infections, mainly in juvenile fish, increasing with water temperature. Clinical signs include lethargy, anoxia, loss of appetite and scratching. Excess mucus, opacity, and even ulcers or haemorrhages may appear. Gill histopathological signs include focal hyperplasia, lamellar fusion, haemorrhages and inflammatory infiltration. Monogeneans are usually very host specific, though in certain culture conditions some species can be found in unusual hosts. Monogenea include 2 main groups, *Monopisthocotylea* (with a simple adhesive disc) and *Polyopisthocotylea* (with a complex adhesive disc including clamps and hooks). Among *Monopisthocotylea* the most significant species for cultured fish are *Gyrodactylus* spp., *Dactylogyrus* spp., *Diplectanum* spp. and *Furnestinia* spp. *Polyopisthocotylea* include several species of pathological concern for fish cultures, most of them belonging to the family Microcotylidae, and some to Heteraxinidae.

***Gyrodactylus* spp.:** causing gyrodactylosis, are mainly typical from freshwater fish. Some species are very pathogenic for salmonid fish, specially *G. salaris*, considered the most pathogenic species.

***Dactylogyrus* spp.:** This genus include numerous species, mostly parasites of freshwater fish. Cyprinids are the main hosts of these monogeneans, but fish of many other families are also affected. The pathologic significance is very dependent on the species and intensity of infection.

***Diplectanum* spp.:** The best known species of this genus are *D. aequans* and *D. laubieri*, parasites of *D labrax*. Their dispersion is very wide in the Mediterranean and Atlantic areas, mostly coinciding with seabass distribution. *D. aequans* is considered more pathogenic, mainly for juveniles and brood stocks.

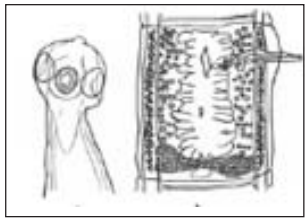
Microcotylosis: In the last years, microcotylosis has affected severely the sparid cultures, causing important loses. Besides the gill dysfunction usually associated with monogenosis, microcotylids produce increased damage due to their haematophagus condition, causing anemia and poor fish condition.



Trematodes: *Diplostomum* spp.: The metacercarial phase of these digeneans parasitizes the eye of fish, though it can be occasionally found in other organs, including the brain. Numerous fish species are susceptible to metacercaria of Diplostomatidae. Clinical signs consist of cloudiness of eye lens, leading to crystalline opacity and blindness. Dark body coloration can be also observed. Acute mortality is very rare, but poor condition is frequent, probably due to the problems with locating food.

Sanguinicolosis: Members of the family Sanguinicolidae are parasites of the circulatory system of fish. Adults are located in the circulatory system. Eggs can accumulate in blood vessels, and are quite frequent in the gills, producing vascular obstruction. Inflammatory response are common in moderate or severe infections. Found in freshwater and marine fishes.

Cestodes: These platyhelminthes may parasitize fishes in larval or adult stages, sometimes causing diseases in cultured fish with variable economic impact. Most species causing disease in fish of economic importance fall within three orders: Caryophyllidea (*Caryophyllaeus* and *Khawia*),

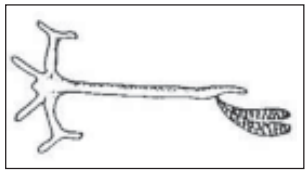


Pseudophyllidea (*Bothriocephalus, Diphillobothrium, Ligula* and *Triaenophorus*) and Proteocephalidea (*Proteocephalus*). Cestodes in fish usually do not cause mortality, though poor condition is frequently observed, mainly in heavy infections. Adult cestodes of the genera *Caryophyllaeus* and *Khawia* parasitize the digestive tract of cyprinids and salmonids, producing different degrees of damage and economic impact in the cultures. In heavy infections, abdominal swelling and poor condition can be observed, mainly in small fish. *Bothriocephalus* spp. and *Eubothrium* spp. are parasites of freshwater and marine fish. *Eubothrium spp.* are frequent in salmonids. *Triaenophorus spp.* can cause severe pathology in fish. Fish are usually the final hosts, but some small fish may act as reservoirs of certain species. Larval stages (plerocercoids) of *Diphyllobothrium* spp. parasitize the muscle and visceral organs of different freshwater fish. Mammals are the final hosts of these tapewormes, which can also infect humans.

Nematodes: Nematodes are common intestinal parasites of fish and can be found abundant in wild species. In some cases the fishes can act as intermediate hosts and may harbour larval nematodes, encysted beneath the skin, musculature or coelomic cavity.

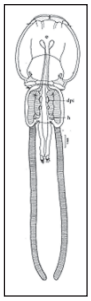
Acanthocephalans: Acanthocephalans are a group of endoparasitic helminths commonly found in both marine and freshwater fishes and are known to cause pathological conditions in many fin fishes. Total loss/degeneration of the intestinal villi and formation of granular tissues and capsule formation affect the digestive and absorptive efficiency of the animal and in heavy infections they can cause occlusion of the gut. Heavy infection with *Tenuiproboscis* sp. have been observed in the red snapper, *Lutjanus argentimaculatus*. Similarly *Pomphorhynchus* sp. have been known to infect many fish species in European countries.

Crustaceans: The main parasitic crustaceans of commercial fish belong to the groups Copepoda and Isopoda. Among Copepoda, species of the genera *Argulus*, *Caligus, Ergasilus, Lernanthropus, Lernaea, Lerneaocera* and *Lepeophtheirus* (sea lice) parasitize different freshwater and marine fish. They are located on the gills, bucal cavity and skin and produce different degrees of damage, even mortality, depending on the fish species and degree of invasion. Clinical signs include occasional rubbing, decrease of condition and gill damage leading to respiration problems. Inflammed wounds, ulcers and mucous excess can be produced as a consequence of crustacean bites, even affecting muscle. Salmons affected by sea lice may show small white-grey spots. In addition, crustaceans can carry or facilitate other infections.

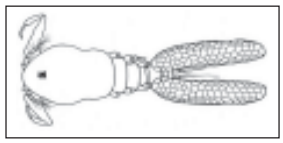


Lernaea : Known commonly as “Anchor worm,” is a common copepod parasite which infects many species of ornamentals. They get their name from the attachment organ which is a highly modified structure resembling the anchor on a ship which is buried in the host’s musculature. A raised ulcer usually develops at the point of attachment, creating an opportunity for secondary infection with pathogenic bacteria.

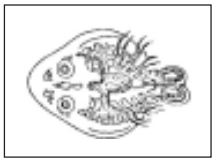
Sea lice*:* are marine ectoparasites that feed on the mucus, epidermal tissue, and blood of host marine fish. *Lepeophtheirus salmonis* is more host specific and is considered as the most important



sea lice species in farmed and wild Atlantic salmon. Sea lice cause physical and enzymatic damage at their sites of attachment and feeding which results in abrasion-like lesions that vary in their nature and severity depending upon a number of factors like host species, age and general health of the fish. Sea lice infection itself causes a generalized chronic stress response in fish since feeding and attachment cause changes in the mucus consistency and damage the epithelium resulting in loss of blood and fluids, electrolyte changes, and cortisol release. This can decrease salmon immune responses and make them susceptible to other diseases and reduces growth and performance.



Ergasilus: Also known as 'gill maggot' is small in size and attacks the gills and sometimes skin of fish, appears as whitish-green threads hanging out of the fish's gills. Heavy infestations result in severe gill damage, emaciation, anemia and death usually due to secondary bacterial infection.



Argulus: commonly known as "Fish Louse", have a flat, distinctive shape and appearance, are found attached to the skin and fins by means of its suckers. Feed on the body fluids and are especially harmful to small fish. Reddish lesions occur at the site of attachment, and this opens the up the skin to secondary bacterial and fungal infections.

Isopods: Different Isopoda have been reported, mainly in sea fish, including *Nerocyla orbygnyi*, *Anilocra physodes*, *Gnathia* and *Paragnathia,* parasitizing seabass, seabream and other sparids, *Seriola* spp., or mugilids. Clinical signs include lethargy, anorexia and respiratory difficulties, as a consequence of gill damage and necrosis. *Cirolana fluviatilis* has been observed causing high mortalities in juveniles of sea bass reared in cages. The picture is frequently complicated by secondary bacterial infections. Mortality can appear mainly in juvenile fish.

Diseases of Molluscs: Compared to the European countries, every little is known about the diseases of molluscs from the Indian subcontinent. The following are the OIE listed molluscan diseases (OIE, 2006).

- *Bonamia ostreae*
- *Bonamia exitiosa*
- *Marteilia refringens*
- *Perkinsus marinus*
- *Perkinsus olseni*
- *Xenohaliotis californiensis*
- Abalone viral mortality

Perkinsosis: Except *Perkinsus olseni* none of the above OIE listed pathogens/parasites of molluscs have been reported from India. *P. olseni,* has been reported from various host species including the pearl oyster, *Pinctada fucata* and *Crassostrea madrasensis* from the Indian sub continent.

Non-infectious diseases: Non-infectious diseases can be broadly categorized as environmental, nutritional, or genetic. Environmental diseases are the most important and may be caused by low dissolved oxygen, high ammonia, high nitrite or natural or man-made toxins in the aquatic environment etc. Managing proper water quality will enable us to prevent most of these. Nutritional diseases can

be very difficult to diagnose. Deficiency of various essential micro nutrients including vitamins can cause a variety of nutritional diseases. The condition seems to disappear when the deficient feed is discarded and a new feed provided. Genetic abnormalities include conformational oddities such as lack of a tail or presence of an extra tail. Most of these are of minimal significance; however, it is important to bring in unrelated fish for use as brood stock every few years to minimize inbreeding.

Prevention, Treatment and Control: The cornerstone of disease prevention is the creation and maintenance of optimum rearing conditions. Good sanitation practices in combination with Quarantine & Biosecurity measures can help a great deal in reducing the incidence of diseases. Treatment of animals in aquaculture is not a viable proposition and preventive approaches is the only option left.

Conclusion

When compared to the past decades, the threat faced by aquaculture is from an array of new/ emerging diseases, and the risk is on the rise. The alarming rate of emergence of new diseases has been driven primarily by anthropogenic influences, the most important of which have been associated with the global expansion of aquaculture. Farming involves displacement from their natural habitat to an environment that is new and often stressful, the use of feeds that are sometimes live and often unnatural, coupled with and high stocking densities futher stresses the animals. This provides opportunities for the existing and emerging pathogens to establish themselves in the hosts leading to diseases. The growth in aquaculture and increasing international trade has resulted in the rapid movement of aquatic animals and their products, with associated risks of the trans-boundary movement of pathogens. Due to the immense export potential, more and more entrepreneurs are venturing into the field of aquaculture and with the intensive nature of culture practices and frequent trans-boundary introductions of many exotic species, new diseases and pathogens are bound to affect this industry. Since chemotherapy/treatment options will definitely reduce the economic viability of the ventures and affect environmental health, it is always better and safe to adopt a proactive approach through better health management practices for the prevention/control of diseases.

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An introduction to fish health management

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Introduction

Achieving food safety in terms of valuable protein for the ever growing population of Indian subcontinent is a major challenge in the 21st century. In this endeavour 'Aquafarming' is to play a major role, as agriculture and animal husbandry has been slowing down in growth. The strength of Indian aquaculture lies in (a) large water bodies suitable for aquaculture, (b) tropical Climate, (c) species diversity and (d) availability of cheap labour. While the weakness include (a) unregulated development, (b) disease problems and (c) lack of scientific approaches. It is estimated that about 5 million tones of aquatic animal products can be produced annually through aquaculture, in India.

As aquaculture production expands, diversifies, and becomes more intensive, the risk and effects associated with pathogen introduction, transfer, disease outbreaks, and pathogen spread are enhanced. The growth, economic viability and sustainability of aquaculture primarily depend on the successful prevention or control of disease outbreaks. Unlike the land based farming, disease problems in aqua farming are complicated due to the three-dimensional nature of culture system where the dynamic interaction of biotic fauna comprising the host and opportunistic pathogens and the environmental factors exists. Disease prevention in aquaculture is not merely a case of dealing with the pathogen and its elimination, but it has to be dealt with a broader perspective, which is now popularly termed as FISH HEALTH MANAGEMENT.

Mariculture – Indian scenario

Shrimp farming dominates Indian aquaculture scene. Importance and need of finfish and shellfish mariculture is coming into lime light, mainly due to the viral disease problem and related crop losses prevailing in the shrimp farming areas of coastal India. The accumulated losses due to white spot syndrome virus (WSSV) alone in India, during the past decade is about Rs. 3000 crores. The importance of finfish and shellfish mariculture is growing as an alternative for the diseases gripped inshore shrimp farming. The research efforts by CMFRI in the development of maricuture technologies of the candidate species such as bivalves, swimming crabs, sand lobsters, finfishes and marine ornamentals have shown initial success. Farming of the green mussel and edible oyster has become a popular livelihood activity by the self-help groups along the costal belt of Kerala, and the farming area is growing every year. CMFRI has succeeded in the hatchery rearing of swimming crabs, sand lobsters and marine ornamentals.

When the rearing activities are in the experimental and demonstrative level, which are at a lower level of intensity, the problem of microbial diseases may not be significant. The absence of clinical or obvious disease problems does not indicate that there are no disease causing pathogens or possibilities of epizootics. Along with domestication and the intense rearing comes an increase in the incidence of infection and potential for disease. Intensive systems lead to higher stocking densities and increasing stress. When animals become stressed, disease outbreaks often occur. Creation of Intensive rearing systems aiming for more and more production and profits, without proper planning and management, invite problems of infection and disease.

One of the most important factors in dealing with the disease is INFORMATION. Knowledge is required on the potential disease causing pathogens, role of environmental factors, health status of the host, diagnostics and therapeutic options.The four K's essential for scientific aquaculture health management are

- Knowledge about the disease process
- Knowledge about the pathogen
- Knowledge about the host and
- Knowledge about the environment.

These information forms the key elements in deciding upon the best means of dealing with a disease or formulating a health management strategy. Hence the best approach in collecting the information should be proactive, rather than waiting for a disease outbreak. One single piece of information, that the disease is caused by a viral pathogen and there is no cure, would desist the farmer from spending large amount of money for 'bogus' cure, and also from additional losses due to delay in harvesting

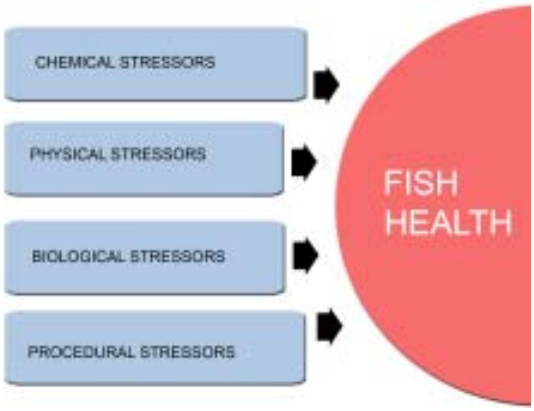
Diseases in aquatic animals can be caused by pathogens as well as by other factors. Common disease causing pathogens include parasites (both protozoan and metazoan), bacteria, fungi and viruses. Other agents causing diseases include toxins, chemicals/pollutants, nutritional imbalance etc. Disease development process is often complicated and involves host-pathogen-environment interactions.

The Genesis of Disease in Aquaculture/Mariculture

Disease is an abnormal condition characterized by a gradual degeneration of fish's/shellfish's ability to maintain normal physiological state due to various factors adversely affecting its well-being. Any impairment that interferes with the performance of normal functions of an animal can be termed a disease. Diseases can be caused by a variety of factors, the most important being pathogens. Other factors contributing towards the development of disease conditions include stress, environmental/water quality, physical agents, nutritional imbalance, toxins etc or a combination of these. Disease condition what we see is thus a complex situation resulting from the interaction/ modification of the primary disease condition by these biotic and abiotic factors. In culture conditions the health status of the animal can become weak due to different stress factors such as chemical stressors, biological stressors, physical stressors and procedural stressors.

Role of Stress in Disease Development

The role of stress in predisposing the fish/shrimp to infections is widely recognized and many of the routine aquaculture practices are known to induce stress. Stress is a non-specific response and



it involves series of changes in the animal in trying to adapt to the changed situation. The adaptive responses of the animal are extended beyond the normal range, which disturbs the normal functions, and the series of changes termed "stress response" tries to help the animal restore the normal homeostasis. This process has both advantages and disadvantages. During stress, hypothalamus-pituitary-interrenal axis (HPI axis) gets stimulated and increases the output of stress hormones called corticosteroids. These stress hormones help to mobilize additional energy during the response to regain the internal homeostasis. At the same time, these stress hormones are basically immuno-suppressive in nature. This can reduce the efficiency of both non-specific and specific immune system of fish significantly and can render the animal more susceptible to disease.

Common husbandry practices like handling, netting, transportation and the normal features of an intensive culture system like suspended solids, low oxygen, high organic matter, overcrowding, high ammonia, etc. can elevate the level of corticosteroids in the blood. Similarly, many of the pollutants at very low levels can also stress the fish and make them relatively more susceptible to infection. Most of the stressors encountered in intensive culture systems are of chronic nature and can keep the level of corticosteroids above basal levels for longer duration.

In such situations, opportunistic pathogens such as parasites, bacteria, fungi, and virus surrounding the animal invades the animal body, resulting in an infection. All infections need not result in disease manifestation. Only when the pathogen build-up disrupts the threshold of animal resistance, the animal succumbs to disease. The situation is a complex one where different factors such as the environment, the animal and the pathogen interacts continuously, making the health management a difficult proposition. This can be further complicated with the involvement of more than one pathogen resulting in a mixed infection which can lead to faulty diagnosis. This entails the need for an integrated management approach to tackle the disease problems with respect to the animal, environment and pathogen using diagnostics as a functional tool.

Disease process

A pathogen can cause a clinical disease only when it can establish on or in the host, proliferate, overcome the non-specific and/or specific defence barriers of the host, produce the pathogenic factors, cause cellular and tissue damage, produce significant pathological changes, impair the function of the target tissue and cause mortality. All infections need not result in disease manifestation. Only when the pathogen build up disrupts the threshold of animal resistance, the animal succumbs to disease condition. The sequence of events in an acute infection is as follows.

- Contact with the pathogen
- Infiltration into the body
- Development / proliferation - incubation (usually short in fishes)
- Spreads throughout the body

- Symptoms appear
- Pathogen restricts itself to specific target organ (mortality)

In the case of chronic infections the pattern of development is

- Slow
- May or may not show pathology / symptoms
- Remain in the body and serve as reservoir / carrier

The sequence of disease development will to a large extent depend on the nature of the pathogen (parasite, bacteria, fungi, virus), environmental factors, size of the host, pathogen load or intensity per unit area or unit weight of the host. The situation is a complex one making the health management a difficult proposition. This can be further complicated with the involvement of more than one pathogen resulting in a mixed infection. A mixed infection can lead to faulty diagnosis. This spells the need for an integrated management approach to tackle the disease problems with respect to the animal, environment and pathogen using diagnostics a functional tool.

Bacterial Diseases: Fish are susceptible to a wide variety of bacterial pathogens. Many of these bacteria become pathogens when fishes are physiologically unbalanced, nutritionally deficient, or there are other stressors, which allow opportunistic bacterial infections to proceed. Bacteria are known to cause infections / diseases in shellfish farming also.

Viral Diseases: Viruses are obligatory intracellular parasites requiring a living cell to replicate. Outcome of diseases due to virus infection is complex and depends on several factors including the immune status of individuals and infectious dose of virus. Mortality need not always be 100%. In some cases, virus remains at a low level of infection establishing a delicate balance with the host. In addition, there are carriers, which are survivors of a mass scale infection and mortality. Usually it is difficult to detect virus in carrier or latent infection stage. Viral diseases are the major cause of disease outbreak and economic loss and known to cause havoc in shrimp culture.

Specific drugs for viral disease treatment are not available or difficult to develop since virus is host cell dependent for all its metabolic machinery. Vaccines in general are not found to be effective in fish viral disease management. Protection from vaccines against viral disease in fish is found to be for short periods with variable results. Poor immune system of the fish and young age at infection are some of the responsible factors for susceptibility to disease. In the absence of successful drug or vaccine, avoidance of the virus in culture system, preventive approaches using quarantine & biosecurity, adoption of crop holiday etc are the best strategies to prevent viral disease in aquaculture, and is already practised in fish health management in the developed countries eg. salmonid culture to prevent IPN disease.

Fungal Diseases: Generally fungal diseases can be external or systemic and are difficult to cure. Except a few, they are generally considered less important pathogens of fishes.

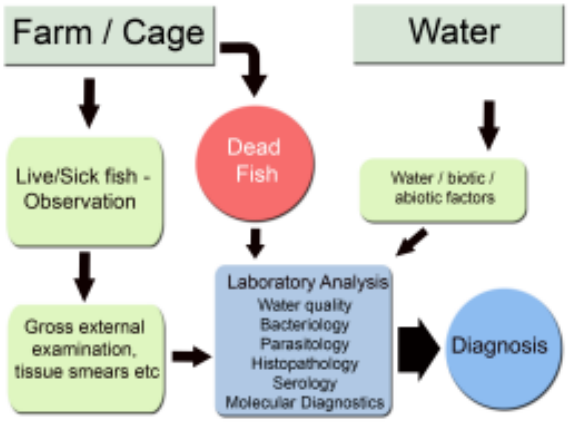
Parasitic Diseases: Parasitic diseases in fishes range from extremely pathogenic ones to those, which are practically harmless. Many of the protozoan parasites are important pathogens of fishes while metazoan parasites are generally less pathogenic in fishes.

Non-infectious Diseases: Feed-derived wastes also affect the culture environment through direct pollution, which in turn affects the culture organisms. Uneaten feeds, faeces and metabolic wastes

contribute to nutrient and particulate loading of the water and substrate which in turn induce stress, depress the growth of cultured organisms and increase their vulnerability to diseases. Improper diets can negatively influence the health of a fish by inducing nutrient deficiencies, imbalances or toxicoses. An impaired nutritional status contributes to defective host resistance. Malnourished fish may harbor latent infections, and certain physiological conditions and environmental stress may predispose them to infection.

Disease Diagnosis in Aquaculture

Diagnosis forms the first step in any disease control programme, which determines the ultimate success or failure of the programme.



Diagnostic Procedure: Once an infection or disease is suspected, the next step is to draw a diagnostic procedure, to fix the root cause of the problem. The diagnostic procedure may include a single diagnostic test or a combination of tests. In the case of routine pathogen watch or health monitoring, a set of selected diagnostic tests are performed to cover the potential pathogens. The approach generally followed is location specific and problem specific, where the first consideration is the availability of the diagnostic facility and expertise. There is no hard and fast method, which can be applied for all cases.

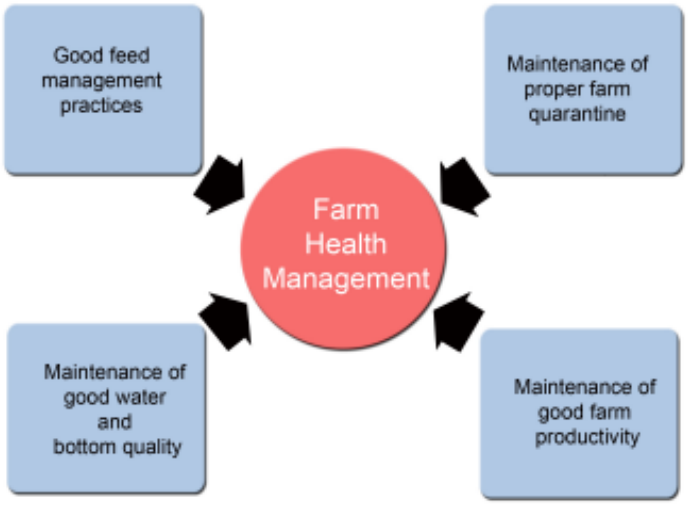
Methods available for disease diagnosis and pathogen detection

History	History of disease at facility or region , facility design, source of seed, type of feed used, environmental conditions etc.
Gross, clinical signs	Lesions visible, behavior, abnormal growth, feeding or food conversion efficiency, etc.
Direct microscopy	Bright-field, phase contrast, or dark field examination of stained or unstained tissue smears, whole-mounts, etc. of diseased or abnormal specimens
Histopathology	Routine histological or histochemical analysis of tissue sections
Electron microscopy	Ultrastructural examination of tissue sections, negatively stained virus preparations, or sample surfaces
Culture and bio chemical studies	Routine culture and isolation of bacterial isolates and identification using biochemical reactions
Enhancement	Rearing samples of the appropriate life stages under controlled conditions to enhance expression of latent or low level infections
Bioassay	Exposure to potential pathogens
Serological methods	Use of specific antibodies as diagnostic reagents in immunoblot, agglutination, ELISA, IFAT, or other tests.
Tissue culture	In vitro culture of pathogens in cell lines
DNA based Diagnostics	PCR, nested PCR, Multiplex, real time PCR

Diagnosis: Once the right diagnostic picture along with the water and soil parameters are available, control measures with respect to the causative factor(s) can be initiated. However, the diagnosis often gets complicated in the cases of mixed infections, with the involvement of primary, secondary and even tertiary pathogens.

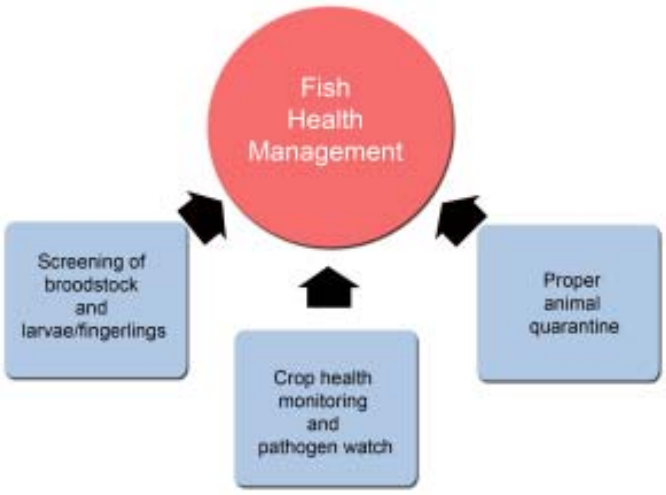
Treatment: Treatment or therapy is intended to restore the normal health of the diseased or infected animal. Drugs can be given oral, intramuscular, intraperitoneal, intravenous or topically as baths or dips. Selection of the proper route depends on the environmental situation, the species and condition of the animal, and the drug being delivered. Unlike the land-based animal rearing systems, where the diseased animals can be identified and treated individually, the scope for disease control in aquaculture through detection and treatment is only of limited value, mainly due to the co-existence of the pathogen and host in the aquatic rearing system. In the case of mariculture, where the extent of the water bodies are without boundaries, the scope of control over the host, pathogen and environment is all the more difficult than in the case of inshore/inland aquaculture. Chemotherapy is not advised in culture systems and should be used only as a last resort since the use of antibiotics can lead to residues in tissues as well as development of antibiotic resistant microbes in the environment, which in turn can create other public health issues. The fish is constantly bathed in potential pathogens, viz., parasites, bacteria, fungi and viruses. Separating the infected or diseased animals from the population and subjecting them to a treatment regime is impractical. Hence, disease treatment becomes a difficult proposition in aquaculture, and disease prevention remains the only natural choice and chemotherapy, if at all required, should be practised judiciously and restricted to broodstock alone.

Aquaculture Health Management: The management practices that are designed to prevent the occurrence of disease in a growout system is termed as the AQUACULTURE HEALTH MANAGEMENT. It is a holistic approach where the focus is given to the health of the animal rather than treatment. Therefore different components viz., animal quarantine, screening of broodstock and larvae/fingerlings, Specific Pathogen Free (SPF) animals, pond and water quality management etc. are involved. Fish health management primarily constitutes two aspects, the **farm health management** and the **fish health management**. Successful integration of these two aspects only can deliver a disease free environment.



Farm health management: It constitutes the maintenance of (a) good soil quality (b) good water quality (c) good farm productivity (d) feed management and (e) maintenance of proper farm quarantine to prevent horizontal transmission of disease causing pathogens.

Fish health management: It constitutes (a) proper animal quarantine b) screening of Broodstock and larvae/ fingerlings and (c) crop health monitoring and pathogen watch. Effective implementation of all the above three aspects of fish health management depends entirely on the early and accurate diagnosis of the disease causing agents. The failure of which can lead to faulty treatment resulting in multiple problems like indiscriminate use of chemicals and drugs, drug resistance, large-scale mortality causing crop failure and economic loss. Timely and early use of proper diagnostics can be used as an effective tool for health care management.



Effective implementation of fish health management depends entirely on the early and accurate diagnosis of the disease causing agents. The failure of accurate diagnosis of pathogens can lead to faulty treatment resulting in multiple problems like indiscriminate use of chemicals and drugs, drug resistance, large-scale mortality causing crop failure and economic loss. Timely and early use of proper diagnostics can be used as an effective tool for health care management. Emerging disease problems, particularly in developing countries, are often slow to be recognized. Thus pathogens become widely spread, often globally, before the seriousness of their nature is recognized and reliable methods of detection, treatment and prevention are developed. Methods for detecting, reporting and responding much more quickly to such emerging diseases should be developed. The recent epizootic of koi herpes virus (KHV) affecting koi and common carps (*Cyprinus carpio*) in Indonesia is a typical example. The design and implementation of effective disease surveillance programs, early warning and reporting systems and contingency plans for dealing with serious disease outbreaks will help in reducing the social, economic and biological impacts of disease.

The integrated approach using diagnostics with farm management, can avoid the introduction of the pathogens into the system. The success of this approach mostly depends on the right choice and use of diagnostics along with other farm management measures, to keep both the animal and its environment in a healthy condition.

The key elements of an ideal health management system can be summarized as:

- Control over the fish/animal stocks at hatchery / farm levels
- Identify excludable disease/pathogens of concern
- Vaccination
- Diagnostics for the detection of pathogens of concern
- Adequate environmental control to prevent the introduction of pathogens of concern (specific pathogen free stock)

- Routine management/husbandry practices to ensure pathogen exclusion (sterilization of influent water, pathogen free feed, prevention of pathogen transfer through men, material and vectors)
- Disinfection, treatment and pathogen eradication methods to contain and eradicate disease outbreaks due to pathogens of concern

In mariculture, development of species specific and location specific health management models with broader management approach for the control of farm/cage level environmental deterioration, pathogen (Virus, bacteria, parasites and fungi) introduction and disease outbreaks is imperative to ensure the sustainability and economic viability of the enterprise.

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Biosecurity in fisheries and aquaculture in the Indian context

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Introduction

Aquaculture is one of the fastest growing industries with an annual growth rate of more than 11% for the past 10 years, producing about 16% of the world supply of animal protein, primarily for human consumption. Driven by population growth, rising demand for seafood and a levelling of production from capture fisheries, the practice of farming aquatic animals has expanded rapidly to become a major global industry. By an estimated production of 47.8 million tonnes in 2005, global aquaculture production in comparison has overtaken the global production of meat from bovine, ovine, porcine and poultry; where the production over the last few decades has not been increased to meet the population growth. In the Indian context, the impressive growth of the sector has raised it to the status of an industry. The strength of Indian aquaculture lies in (a) large water bodies suitable for aquaculture, (b) tropical Climate, (c) species diversity and (d) availability of cheap labour. While the weakness include (a) unregulated development, (b) disease problems and (c) lack of scientific approaches and policies

Shrimp and Carp farming has been the face of Aquaculture in India. By 1993, diseases, especially those of viral etiology have emerged as the major constraint to the sustainability and growth of shrimp aquaculture. Till date, more than 15 viruses have been identified to cause diseases in shrimp and no treatment option is available for their control. The increasing concerns about food safety and the potential negative impacts on the environment makes the control of diseases through chemotherapy/antibiotics a least preferred one. Hence, prevention is the only available option for containing viral disease outbreaks.

Aquaculture has become integral to the economies of many countries especially the developing ones. It provides employment and been a major driver of socio-economic development in poor rural and coastal communities, at the same time relieving pressure on the sustainability of the natural harvest from our rivers, lakes and oceans. Aquatic animals have been displaced from their natural environment, cultured in high density, exposed to environmental stress, provided artificial or unnatural feeds, and a prolific global trade has developed in both live aquatic animals and their products. As aquaculture production expands, diversifies, and becomes more intensive, the risks and effects associated with pathogen introduction, transfer, disease outbreaks, and pathogen spread are bound to be enhanced. Mortality due to diseases or decreased growth rates and/or decreased feed efficiency due to infections are major factors responsible for economic losses in aquaculture. Recent examples

of major losses suffered by aquatic animals from disease outbreaks include the carp mortalities in Java, infectious salmon anaemia outbreaks in Norway, United Kingdom and North America, whitespot syndrome virus and Taura syndrome virus epidemics in prawn aquaculture, the spread of epizootic ulcerative syndrome (EUS) in Asia and Akoya disease in Japanese pearl oysters. Preventing a disease introduction is more cost-effective and easier than control and elimination of an introduced pathogen at a later stage. The situation warranted the sector to look for novel strategies to prevent the crop losses caused by diseases and in this context, the development and implementation of biosecure production practices can play an important role in saving this industry. This has already led to the development and implementation of biosecure production practices for fish rearing industry. Hence, Adoption of BIOSECURITY systems, incorporating 'scientific health management' at hatchery and farm, remains the only strategy for controlling the pathogens, especially those of viral etiology.

What is biosecurity?

Biosecurity is a term used in animal farming industry to describe the preventive measures taken against any infectious disease outbreaks. It can be defined as "the protocols, physical systems and health management procedures that protect fish rearing systems from infectious diseases" or "a set of standard scientific measures adopted to exclude pathogens (prevent the establishment and spread of pathogens) from the host and the rearing environment". Though biosecurity does not have a single definition, a working definition for practical purposes could be "The sum of all procedures in place to protect organisms from contracting, carrying or spreading disease". Thus, biosecurity in aquaculture is the protection of finfish or shellfish from infectious agents.

The key elements of biosecurity are a reliable source of stocks, adequate detection and diagnostic methods for excludable diseases, disinfection and pathogen eradication methods, best management practices (BMPs), and practical and acceptable legislation. A biosecurity program is thus comprised of a variety of practices, policies and procedures used on a farm in order to reduce the risk of pathogen entry into the facility; reduce the risk of spreading and reduce conditions that are stressful to the fish, which can enhance susceptibility to disease. The program should be tailored to the needs of the specific site, business needs of the operation, the fish/shellfish species and life stages grown and the disease profile of the surrounding region. Overall, a biosecurity program would include, but not be limited to, practices and procedures involving: 1) surveillance for the presence of disease organisms; 2) vaccination; 3) quarantine and restricted access; 3) appropriate practices of fish husbandry; 4) disinfection; and 5) disease treatment (including eradication).

Biosecurity can mean different things to different stakeholders. Seafood consumers want to have an assurance that the product is safe to eat. Retailers have a responsibility to provide high quality seafood, and processors should follow Hazard Analysis and Critical Control Point (HACCP) guidelines to ensure that their products are safe for human consumption. At the farm site, workers need to know what practices decrease or increase the risk of a disease outbreak occurring. Investors seek to protect their investments from preventable losses. General security precautions need to be established in each facility to help support the activities of both disease prevention and disease control. A manual of standard operating procedures (SOP) should be assembled to provide a set of standard rules for biosecurity measures and disease monitoring. This should include such things as facility design, facility flow for both personnel and stock, rules for limited or restricted access to

facility, required visitor log book, disinfection procedures for personnel and equipment, a waste management plan, pest control guidelines, and general husbandry and management procedures.

Thus biosecurity is a team effort, a shared responsibility, and an on-going process to be followed at all times. From the breeder to the hatchery, to grow out operators, biosecurity measures and good aquaculture practices have to be observed to contribute to the success of the industry.

Economic Losses

Water is a continuous medium connecting all parts of planet Earth. In terrestrial situation, the discontinuity of land masses generally facilitate the control of trans-boundary movement of pathogens, while in aquatic system, continuity of water could allow the passage of biota along with the micro and macroflora, with potential pathogenic characteristics and related possibility of disease implications. Huge economic losses from aquatic animal diseases have been documented over the last decade, largely due to the lack of proper biosecurity plans/policies.

Very often, disease problems are the major limiting factors in determining the economic viability in any rearing system including agriculture, animal husbandry and aquaculture. The largest economic losses from aquatic animal diseases have been reported from the shrimp farming regions from both eastern and western hemispheres, though authentic information from many parts of the world is hard to obtain. However the figures given in Table 1, provide an indication of the economic losses. The total collapse of the Shrimp farming Industries in Taiwan in 1987, China in 1992, and India in 1995 was due to infectious viral diseases, causing billions of dollars in lost revenue for the industry. Between 1995 and 1996, disease accounted for 71% of the total losses to trout farming in the U.S., part of a continuing trend of a $ 3.02 billion loss to aquaculture from disease worldwide. It was estimated that loss from diseases accounted for 30% of the operating costs in aquaculture.

Table 1. Estimated losses from aquatic animal diseases. Most losses are from the introduction and spread of crustacean diseases

Area	Year	Estimated losses
Thailand	1983-93	US$ 100 million
China	1993	US$ 400 million
India	1994	US$ 17.6 million
Thailand	1996	US$ 600 million
Ecuador	1999	US$ 280 million
Global	1997	US$ 300 million

Aeromonas salmonicida in Europe in the 19th century, to *Aphanomyces invadans* in Asia and North America in the 21st century, caused disastrous epizootics. Furunculosis caused by *Aeromonas salmonicida* is the oldest of the known fish pandemics, believed to be transferred from US to Europe through rainbow trout (*Oncorhynchus mykiss*). Crayfish Plague caused by the fungus *Aphanomyces astacus* was introduced to Europe from USA. Epizootoic Ulcerative Syndrome (EUS) epidemic caused by *Aphanomyces invadans* has spread to whole Asia, Australia and further. It has caused considerable losses to many wild and farmed species in the Philippines, Indonesia, Thailand, Burma, Bangladesh and India, and even US. All the above three disease conditions have been

introduced by fish movements to new ecosystems, where vulnerable host species were present in the wild stocks.

Biosecurity in shrimp farming

By 1993, diseases, especially of viral etiology have emerged as the major constraint to the sustainability and growth of shrimp aquaculture across the world. The past 2 decades witnessed the emergence of more than 15 shrimp viruses and devastating effects caused by them across the world (Table 2). The most important diseases of cultured penaeid shrimp, in terms of economic impact, in Asia, the Indo-Pacific, and the Americas have infectious etiologies. The pattern of spread and trans-boundary movements of many diseases may also pose another important threat. Grossly healthy arthropods tend to carry cryptic viruses (possibly unknown) that can jump to endemic arthropod species and cause massive mortalities. This phenomenon has resulted in 3 major shrimp epizootics that have caused economic losses in the order of several billion US$ since the early 1990's. WSSV which was initially confined to the cultured shrimp has now spread to the marine environment and the brood stock collected from the wild cannot be assumed to be disease free. The threat of many other crustaceans acting as carriers for this pathogen also exists.

Table 2: Viruses of penaeid shrimp (as of July 2001) Adapted from Lightner, 2005.

Acronym	Full name
Parvoviruses (Parvoviridae)	
*IHHNV	Infectious hypodermal and hematopoietic necrosis virus
HPV	Hepatopancreatic parvo virus
*SMV	Spawner-isolated mortality virus
LPV	Lymphoidal parvo-like virus
Baculoviruses and Baculo-like Viruses	
*BP-type	*Baculovirus penaei*-type viruses (PvSNPV type sp.):BP strains from the Gulf of Mexico, Hawaii and Eastern Pacific
*MBV-type	*Penaeus monodon*-type baculoviruses (PmSNPV type sp.): MBV strains from East and SE Asia, Australia, and Indo-Pacific
*BMN-type	Baculoviral midgut agland necrosis type viruses
TCBV	Type C baculovirus of *P. monodon*
PHRV	Hemocyte-infecting non-occluded baculo-like virus
White Spot Syndrome Viruses (Whispoviridae)	
*WSSV	White spot syndrome virus
Iridovirus	
IRIDO	Shrimp iridovirus
RNA Viruses	
Picornavirus (Picornaviridae)	
*TSV	Taura syndrome virus

Reoviruses	
REO-III and IV	Reo like virus types III and IV
LOVV	TOGA-like virus : Lymphoid organ vacuolization virus
RPS	Rhabdovirus: Rhabdovirus of penaeid shrimp
Yellow Head Virus Group	
*YHV/"YBV"	Yellow head virus of *P. monodon*
GAV	Gill associated virus of *P. monodon*
LOV	Lymphoid organ virus of *P. monodon*

*OIE modifiable and listed penaeid shrimp diseases (OIE, 2006)

Among the infectious diseases of cultured shrimp, certain virus-caused diseases stand out as most significant. The pandemics due to the penaeid viruses, WSSV (White spot), TSV (Taura Syndrome) and YHV (Yellow Head), have cost the penaeid shrimp industry billions of dollars in lost crops, jobs, and export revenue (Table 3).

Table 3. Estimated economic losses since the emergence of WSSV, TSV, YHV and IHHNV.

Virus	Year of emergence	Production loss
WSSV	Asia 1992	$ 4-6 billion
WSSV	Americas 1999	$ > 1 billion
TSV	1991-92, Americas & South East Asia	$ 1-2 billion
YHV	1991, South East Asia	$ 0.1-0.5 billion
IHHNV	1981, South East Asia	$ 0.5 1.0 billion

Biosecurity in finfish culture

The international fish disease commission has listed more than 17 diseases of concern among the farmed finfishes (OIE, 2006)

OIE Listed diseases in Fishes

- Epizootic haematopoietic necrosis
- Infectious haematopoietic necrosis
- *Oncorhynchus masou* virus disease
- Spring viraemia of carp
- Viral haemorrhagic septicaemia
- Channel catfish virus disease
- Viral encephalopathy and retinopathy
- Infectious pancreatic necrosis
- Infectious salmon anaemia
- Epizootic ulcerative syndrome
- Bacterial kidney disease (*Renibacterium salmoninarum*)
- Enteric septicaemia of catfish (*Edwardsiella ictaluri*)
- Piscirickettsiosis (*Piscirickettsia salmonis*)

- Gyrodactylosis (*Gyrodactylus salaris*)
- Red sea bream iridoviral disease
- White sturgeon iridoviral disease
- Koi herpesvirus disease

Viral diseases have not been considered to be a significant threat in marine and brackish water finfish culture in India. Other than the Epizootic Ulcerative Syndrome (EUS) and some reports on nodavirus, no other OIE notifiable diseases have been reported from India.

Epizootic Ulcerative Syndrome (EUS) is a pathogenic, invasive, fungal infection caused by the fungus, *Aphanomyces invadans* in Asian freshwater and estuarine fishes. It causes skin ulceration and death in over 30 species of commercially important cultured and wild fishes both in freshwater and estuarine habitats. EUS with its high epizootic potential and mortality rates has already appeared in freshwater systems in many North eastern and some Southern states. In India, losses due to EUS has been estimated at 42.5 million US $ during the period 1992-95. An EUS epizootic on a massive scale can therefore cause devastating losses to the tune of millions for Indian aquaculture.

The recent outbreaks of Koi herpes virus in the neighboring South-East Asian countries is a cause of worry to the country. Koi herpes virus (KHV) is a highly contagious viral disease, capable of causing significant morbidity and mortality in common carp, *Cyprinus carpio*. *C. carpio* is raised as a food fish in many countries and has been selectively bred for the ornamental fish industry, where it is known as Koi. The disease may cause 80–100% mortality in affected populations and affects fish of various ages. Outbreaks of KHV have been confirmed in the United States, Europe and Asia. There is no known treatment for KHV and the virus is believed to remain in the infected fish for life, thus exposed or recovered fish should be considered as potential carriers of the virus. As large numbers of ornamental fishes, especially koi are being imported to India, there is a high risk of KHV entering in the country.

World over, scarcity of water resources suitable for aquaculture purposes and environmental concerns about the wastewater discharges has forced many developed countries to employ “recirculating” technologies - systems that employ an intensive, closed-system approach to fish culture. In such systems, preventing and controlling disease is important because the disease organisms may recycle with the water and, since there is no dilution of the pathogens as in flow-through systems, the resulting rates of infection are greater. In addition, it is extremely difficult to eradicate a disease once a pathogen has become established in a recirculation rearing system.

Biosecurity measures in finfish culture include both physical and biological aspects. The physical aspects start with cleaning and disinfecting measures in hatchery and production facilities. UV treatment of water will control most of the fish viruses. Carefully regulating water temperatures to between 15 °C and 18°C has been shown to be effective at reducing the infectivity of Japanese flounder rhabdovirus (HIRRV)). Dedicated equipment, nets, brushes, etc., are disinfected with ozonated or electrolyzed seawater.

In terms of the biological aspects of disease control, ensure that the broodstock are pathogen-free, and the health of the fry is routinely monitored. Larvae that are cultured in disinfected water should have their normal intestinal flora restored. Immunizing stocks, using commercially available vaccines, is the most effective method for controlling salmonid diseases that cannot be excluded. The “eggs only” policy eliminates the introduction of many pathogens that require a live salmonid

fish host. Any eggs that are imported into the area must have originated from certified, specific disease-free sources, to ensure that diseases are not transmitted vertically.

A pilot scale biosecure production system of seabass (*Dicentrarchus labrax*) in France prevented vertical and horizontal transmission of nodavirus disease in broodstock to market size fish and avoided the use of antibiotics and anti-parasitic treatments, at a final production cost that was similar to traditional system. The strategy combined the use of diagnostic tests for early detection and removal of nodavirus carriers to maintain healthy broodstock and control of specific bacterial populations in the recirculating system. Recycling systems greatly reduce the risk of meteorological and ecological events and mastering of the risks associated with rearing the fish is made easy. Thus by implementing biosecurity, the risk of pathological events can be greatly reduced.

Key To Fish Biosecurity

- Control the fish stocks in hatchery level and farmed level
- Identify excludable disease/pathogen of concern
- Vaccination
- Diagnostics for the detection of pathogens of concern
- Adequate environmental control to prevent the introduction of pathogens of concern (specific pathogen free stock)
- Routine management/husbandry practices to ensure pathogen exclusion (sterilization of influent water, pathogen free feed, prevention of pathogen transfer through men, material and vectors)
- Disinfection, treatment and pathogen eradication methods to contain and eradicate disease outbreaks due to pathogens of concern

Biosecurity in mollusc culture

In the Indian scenario, in terms of volume of production, molluscan culture comes nowhere near shrimp/fish farming and comparatively little importance is given for molluscan farming.

In the case of molluscan culture, for controlling the endemic diseases, the health management procedures include assessing and understanding the state of health of individual and populations of cultured shellfish, early diagnosis of abnormal or pathological conditions and preventing and correcting pathological conditions that may arise. Sanitation procedures are aimed at identifying and monitoring culture systems for contamination sources and management procedures to reduce or eliminate contamination. In intensive hatcheries and nurseries, pathogen-free algal stocks undergo surface sanitation in expanded culture and treated water is used with disease-free broodstock. Health assessments are critical at metamorphosis of larval mollusc cultures, and sanitation and health management hold the keys to ensure production of healthy juveniles. Non-endemic infectious diseases are excluded from mollusc culture operations usually through regulations set by authorities. Regulations to restrict the imported shellfish from being released into the culture waters and strict quarantine measures are to be made mandatory.

The following are the OIE listed molluscan diseases (OIE, 2006)

- *Bonamia ostreae*
- *Bonamia exitiosa*

- *Marteilia refringens*
- *Perkinsus marinus*
- *Perkinsus olseni*
- *Xenohaliotis californiensis*
- Abalone viral mortality

Among these pathogens, only *Perkinsus olseni, has been reported from the pearl oyster and edible oyster from India. N*one of the other pathogens/parasites of molluscs have been ever reported from India. But, this does not rule out their presence in Indian waters. Moreover, we do not have a database of molluscan pathogens in Indian waters since very little studies have been done on this aspect. If our country is free from these parasites/pathogens presently, utmost care should be taken to prevent the entry of these pathogens which may turn out to be a major threat in future. Thus the implementation of biosecurity measures holds great importance for the future of molluscan culture in India.

Lessons from Indian Aquaculture

Indian aqua farming is dominated by the culture of carps in freshwater and shrimp in brackish and higher saline waters. Among the carps, along with the common carp *Cyprinus carpio,* many other species of carps are cultured while shrimp farming is synonymous with the farming of *P. monodon.* In India, viral diseases have emerged as the major constraint to the sustainability and growth of shrimp aquaculture. In fishes, except for the occurrence of Epizootic Ulcerative Syndrome (EUS) and some reports on noda virus, no other OIE notifiable diseases have been reported. No comprehensive information is available regarding the status of the viral diseases infecting cultured fin fishes in India.

Presently, unauthorised/clandestine introductions pose a serious threat to the aquatic systems. Besides competing with the native species for food/other requirements, they bring with them a variety of pathogens (sub-clinical infections/carriers) which may pose serious threats to the native populations. Though blocking the introduction of exotic species may seem to be an attractive option in controlling the introduction of new pathogens into the system, in the present global scenario, production and economic aspects compels us from implementing a total ban on introductions. We cannot turn away from introducing new varieties of ornamental fishes and cultivable species with very high growth/production rates for economic reasons. There should be a balanced approach while recommending introductions.

Viral pathogens of primary concern in India

White spot syndrome virus (WSSV)

Monodon baculovirus (MBV)

Yellow head virus complex (YHV-complex)

Infectious hypodermeal and haematopoietic necrosis virus (IHHNV)

Hepatopancreatic parvovirus (HPV)

Taura syndrome virus (TSV)

Among the OIE listed viral pathogens, only WSSV and MBV have wide spread presence in India, and both are now enzootic in the farming systems. Outbreaks of WSSV were first found in

Penaeus japonicus in China in 1992. The disease first spread geographically within the species *P. japonicus*, and only later, in 1993, spread to other species including *Penaeus chinensis*, which is the major cultured species in China. No one knows how the virus spread throughout Asia after that, but the common practice of moving grossly normal broodstock and PL freely amongst countries was probably the most rapid and effective means of spread. Almost certainly WSSV was spread from Thailand to Malaysia and India in this manner.

WSSV is the most virulent virus known to affect cultured shrimps (Fig 1a, 1b). Considered as the largest known animal virus with a genome size of 305 kb and classified into a new family, *Nimaviridae.* Reported from India since late 1993. Till date, no treatment is known to control the White Spot Disease (WSD). Hence, early diagnosis followed by suitable management practices is the only alternative in tackling this viral disease.

Fig. 1a: Tiger shrimp affected with WSSV fro Indian farms. 1b: Ultrastructure of WSSV virus

The global loss caused by WSSV in 2000 is estimated to be 200,000 metric tons, valued at $ 1 billion. While Indian shrimp farming losses due to WSSV is estimated to be 200-300 crores annually, from 1994, with an accumulated loss of about 3000 crores during the last ten years.

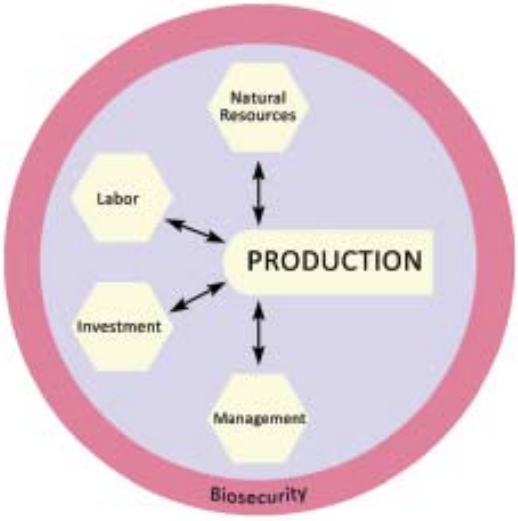

Fig. 2. Relation of production system with other inputs

The viral epizootic such as WSSV results in production losses with a negative impact on different aspects of the production system. Production in any system is closely related to various inputs like natural resources, investment, trade, employment, environment and management costs etc. A common result is that strategies for prevention, control and damage reduction are complementary, and neglect of any of them may lead to unnecessary large social costs (fig.2). Whenever production fails, other than the direct economic losses, these related areas are also affected indirectly. This has resulted in an increased awareness about the shrimp health management concepts including biosecuritiy and hazard analysis and critical control point (HACCP), and their adoption in rearing facilities.

International movement of frozen shrimp products from eastern to western hemisphere for trade and aquaculture has resulted in the transfer of WSSV from Asia to Americas, and TSV from Americas to Asia. Introduction of exotic/dangerous organisms/pathogens through shipping activities (ballast water) also pose a serious risk in this context. Appropriate biosecurity management could have prevented many of the serious losses experienced in aquaculture in recent years.

The main shrimp species cultured in India over the years included *Penaeus monodon* and *Feneropenaeus indicus* until 2003 when *Litopenaeus vannamei* was introduced by some vested farmers. Several farmers have experimented co-culturing of *Macrobrachium rosenbergii* and *P. monodon* in low saline waters. The practice of mixing of species and introduction of exotic species could be one of the reasons for the emergence of new viruses in India. The efforts of Indian shrimp farmers to introduce SPF *L. vannamei* stock and their subsequent experimental introduction to eastern India, in Andhra Pradesh, especially to prevent the specific pathogens such as WSSV and Monodon baculovirus (MBV), and other exotic pathogens brought the focus on the biosecurity facilities in India.

In the wake of the viral pandemics, the shrimp culture industry has sought ways to restore the industry's levels of production to the "previrus" years. Biosecurity risks are increasing every year, as aquaculture develops, new species are cultured and new host-pathogen-environment interactions get tested. Therefore, excluding infectious agents and reducing stress are important in preventing disease outbreaks. The application of biosecurity to shrimp farming is central to those efforts.

The principles of biosecurity is normally considered only for those dangerous pathogens, which are highly virulent, infectious, untreatable, vertically transmitted, have a diverse host range, and threaten the very survival of the industry. From the implementation point, biosecurity can be more easily implemented in small, intensive, and controlled farming systems than in outdoor and large-scale operations. A two-pronged approach is suggested: excluding pathogens from stock (i.e., post larvae and broodstock) through the use of quarantine and specific pathogen-free (SPF) certified stocks and restricting imports of live and frozen shrimp. Excluding vectors and external sources of contamination and preventing internal cross contamination is also important.

Legislations

Several procedures and guidelines developed by different agencies, organisations or nations deal with the components of biosecurity issues and plans. The common objectives include aspects of protecting animal populations, environment, food and the humans itself. Many instruments falling under the terms such as policies, codes, agreements, plans, conventions, regulations and treaties

has been made to achieve the objectives of biosecurity. Examples are given in the Table 3.

Table 3. Examples of International or multinational policy instruments containing elements pertinent to aquaculture biosecurity. Dates are years of initial adoption

Lead Organization	**Title**
World Trade organization (WTO)	Agreement on the application of Sanitary and Phytosanitary Measures (SPS Agreement), 1995Convention on Biological Diversity (CBD), 1992, and its Cartegena Protocol on Biosafety, 2000
Food and Agricultural Organization of the United Nations (FAO)	Organization of the United Nations (FAO)Codex Alimentarius (Codes of Hygienic Practice for the Products of Aquaculture), 1981-1999Code of Conduct for Responsible Fisheries, 1995Code of Conduct for the Import and Release of Exotic Biological Control Agents, 1995International Plant Protection Convention (IPPC), 1997International Council for the Exploration of the Sea (ICES)
International Council for the Explorations of the Sea (ICES)	Code of Practice on Introduction and Transfer of Marine Organisms, 1994
International Maritime Organizations (IMO)	Guidelines for Control and Management of Ships' ballast Water to Minimize the Transfer of Harmful Organisms and Pathogens, 1997
United Nations (UN)	Biological Weapons and Toxins Convention, 1972
International Union for the Conservation of Nature	Guide to Designing Legal and Institutional Frameworks on Alien Invasive Species, 1999

Issues regarding aquatic animal health are usually referred to the *Office International des Epizootices* (OIE) *International Aquatic Animal Health Code* (OIE 2000). Its mission is to inform governments of the occurrence and course of diseases throughout the world and of ways to control these diseases, to co-ordinate studies devoted to the surveillance and control of animal disease, and to harmonize regulations for trade in animals and animal products among its 158 member countries.

The majority of countries possess basic animal health legislation of different levels. In most countries, there is no clear distinction between terrestrial and aquatic animal health legislation. In cases where specific regulations for aquaculture exist, their enforcement is applied mostly as an emergency procedure to deal with a specific problem, and not as the result of an established program for surveillance and monitoring of the health status of cultured organisms. Several countries have specific legislation to regulate the import and export of live aquatic organisms and their products for use in aquaculture, for human consumption, or other purposes. Generally, these laws and regulations are in conformity with the rules of the OIE and WTO-SPS.

Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy provides guidelines

for developing a list of pathogens, diagnosing diseases, implementing effective programs for health certification and quarantine measures for aquatic animals, guidance for creating disease zones and monitoring and reporting new diseases. This manual outlines the development of contingency plans and stresses the importance of risk analysis and need for national strategies and policy frameworks.

National legislation and policy

Legislation may play a useful role to enhance responses to aquatic animal health emergencies. Such legislation should address a number of issues, like disease surveillance, control and eradication; contingency plans; new species introductions; domestic movement of live fish and their products; and fish inspection and quarantine. Besides, the challenge is also to design legislation that enables and guides both public and private sectors involved in fish health related activities. It should clearly redefine the duties of various authorities involved in matters of fish health at the national, provincial and district levels and promote effective coordination, power-sharing and communication between all those involved. As providers of information and reports, fish farmers should be enabled to play a role in decision-making processes relating to the implementation of fish health management programmes. Scientists, enforcement officials and fish farmers need to be motivated to carry out relevant emergency preparedness response measures in aquaculture practices.

Legislation with respect to the Indian Fisheries sector

According to the Indian constitution, the states have the power to make laws and regulations with respect to fisheries and hence regulations and control of exotic organisms and diseases have to be enforced by the respective states. At the central level, the Indian Fisheries Act (1897) which is a century-old is still in existence. The legal mechanisms for control of both legally and illegally introduced aquatic organism and enforcement of the quarantine has to be made strict. Provisions should be made for regulating the movement of the native aquatic organisms within India in the wake of disease outbreaks. A draft legislation on “Live aquatic organisms importation Act 2006” has been proposed. Based on the existing international agreements and codes of practices for the trans-boundary movement of aquatic animals, the recommendations made in various consultations on invasiveness, disease diagnostics, risk analysis, emergency preparedness, capacity building etc., and existing legal provisions adopted by different countries, an act becomes inevitable to strictly implement the provisions needed in safeguarding the existing conservation and management of aquatic animal diseases and biodiversity in Indian fisheries.

Normally all introduced/imported organisms (brood stock/egg/larvae) arrive through the ports, and recently through the airports. There should be a mechanism to check and ensure that every consignment entering the country should be absolutely free of any pathogen. Imports should be allowed through selected ports and facilities should be established in collaboration with scientific organizations at these ports to establish their pathogen-free status.

Biosecurity in Indian aquaculture: Future strategies

The term ‘Biosecurity’ appears to be new to the Indian aquaculture industry. While aquaculture has made rapid advances in the past few years in fish and shellfish diagnostics, disease prevention and disease control measures lag significantly behind. Therefore, it is imperative to develop and enforce Ecologically Sustainable Development (ESD) strategies to meet the needs of the present

without compromising the ability of the future generations to meet their own needs. A biosecurity program is developed through the scientific analysis of information with the aim of adopting procedures to manage risks to an acceptably low level, through a risk assessment approach. The use of sound epidemiological principles and a logical, structured approach will result in a more accurate outcome. Thus designing an effective biosecurity program requires a thorough understanding of the aquaculture operations, general principles of disease transmission, and knowledge of the fish or shellfish maintained in the facility.

Aquaculture biosecurity policies

Aquaculture biosecurity policies vary from farm-level to the international level, and between areas at each of these levels, but several characteristics are essential if aquaculture biosecurity polices are to be successfully implemented. They include:

- Science-based decision making,
- Economical and socio-political rationales,
- Standardized and uniform methods,
- Relative ease of application,
- Wide recognition,
- Vertical and horizontal integration, application, and agreement,
- Consistent enforcement,
- P7rimary focus on prevention, but with contingencies in place for control and management, or eradication.

Successful implementation of biosecurity at various levels throws up lot of challenges. Implementing strict biosecurity measures at the Hatchery can be the choice, while at the farm level, biosecurity can be very expensive and may not be feasible in open farming systems. Biosecurity measures would be adopted at the farm level only if they are shown to effectively prevent the occurrence of the disease and at a cost the farmer can afford. So, in the present Indian aquaculture scenario, 'Biosecurity' could be a choice and not an option.

*A biosecure system could therefore be based on **specific pathogen-free** (**SPF**) stocks.*

- Wild populations are surveyed to select animals free of the important diagnosable pathogens to establish the initial stock.
- These stock are kept under strict quarantine conditions to maintain the pathogen free status
- Breeding & rearing
- The population is qualified for SPF status, when the progeny is considered free of any diagnosable pathogens
- The quarantine, SPF Nucleus and multiplication centre located in different places.

Strategies used for the pathogen exclusion are:

- Identification of pathogen and their entry routes

- Quarantine and screening of hosts introduced in the system
- Disinfection at defined critical control points
- Restricted access
- Identification of risk factors that favor pathogen establishment and spread

Ideal elements for the setting up of a biosecurity facility are:

- Selection of a site away from potential source of pollution/contamination
- Availability of good quality water
- Water treatment facility to exclude the potential pathogens and other hazards
- Measures to prevent the entry of carriers of infectious agents
- Appropriate back-up for water supply and life support systems
- Operation procedure which allow one-way flow
- Prevention against the escape of animals
- Quarantine facility for screening specific pathogens with novel diagnostic facility
- Routine health monitoring progarmme
- Application of HACCP principles as a risk management tool to check the entry of viral pathogens
- Maintenance of complete records of operation
- Adherence to standards prescribed by international disease commission (OIE)

Recommendations to the Industry

- Restrict movement of sick animals (no “emergency harvest” or “dumping”).
- Implement yield verification programmes to assist in analysis of cost versus benefits of husbandry methods, breeding, vaccine use etc.
- Develop an identity as a united industry for effective interactions with regulatory agencies.
- Include vaccination as part of health management or biosecurity plan for aquaculture.
- Find ways that farmers can use biosecurity procedures to increase their profits.
- Include rapid and early detection tests for pathogen and stress.
- Hire media consultants to survey popular media for aquaculture-related news items and to prepare statements for the press.
- Develop a greater awareness of environmentalist/animal ethics opposition philosophy. Identify agendas of such groups and the consequences that necessarily follow from their position. Uncover basic values and philosophical rationale that underpins them. Enlist professional help to obtain a critique of their position.
- Insist on transparency and stakeholder involvement in your government’s responses to OIE and International trade requirements.

At the shrimp farm level, biosecurity refers to producing healthy finfish/shellfish in a well-controlled environment that excludes the introduction or propagation of unwanted organisms and includes the prevention or escape of organisms back into the natural environment. If past experiences can be taken as an indication, implementing these measures at the national level will be herculean task. It is a well known fact that *L. vannamei* has already been cultured in many regions of the country even before the legal clearance was given. Such unauthorized introductions can raise serious challenges to the very concept of biosecurity in the country. If introductions are not done with sufficient care, the pathogens entering our waters may turn out to be a serious problem for years to come.

An integrated plan for maintaining aquatic animal biosecurity and health, where all levels from border to the farm, including the environment need to be developed. The most important aspect is the management of the disease and pest risks associated with the importation of aquatic animals and animal products. Process of import risk analysis (IRA) and application of Quarantine measures are the scientific approaches to be adopted. The Australian Govt has successfully adopted an AQUAPLAN comprising of different modules viz., International linkages; Quarantine and Surveillance; Monitoring and Reporting; Preparedness and Response; Awareness; Research and Development; Legislation, policies and jurisdiction; and Resources and Funding (AFFA 1999). Such a measure can definitely be taken as a model for India.

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Laboratory Approaches in Fish Microbial Disease Diagnosis and Mangement

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Introduction

In recent years, there has taken place an exponential growth of shrimp farming in coastal India because of the lucrative income earned by early entrants. In the same way around 10, 00,000 heactares are under finfish cultivation in India of which around 6, 50, 000 hectares are under fresh water zone. Among the finfish produced 3.22 million metric ton is the share from fresh water fish culture indicating heightened increase in aquaculture. However, the expansion of the culture was not planned and many vital scientific principles in site selection and management were ignored. The defective water intake and drainage systems created a eutrophic condition of many water resources. As a cumulative effect aquaculture ran into increasingly complex problems like partial or mass mortalities due to either diseases or environmental impairment. Ultimately the sudden outbreak of white spot disease in late 1994, threatened even the very existence of the shrimp industry. This article deals with the important microbial diseases observed in the culture systems in India in general and the laboratory approaches to the detection and identification of the pathogens and briefly their management.

Microbial Diseases of shellfishes (Shrimps)

Infectious Diseases

Infectious diseases are the ones with definite aetiologic agent and can be transmitted both vertically and horizontally. Viruses, bacteria, fungi and protozoans cause these diseases.

Bacterial Pathogens

Bacteria are opportunistic pathogens usually causing infections secondarily in penaeids. They are always found in the shrimp body and pond water and cause disease only when the shrimp is exposed to stress. A number of bacterial infections have been found in cultured shrimp from the larval stage to adult. Most of the isolates from the infected shrimp have been *Vibrio* spp. and others include *Aeromonas* and *Pseudomonas* spp.

Shrimp infected with bacteria shows discolouration of the body, necrosis of appendages and shell, aggregation of blood cells, lesions with the vital organs and loss of appetite. Bacterial infections in penaeid shrimp cause three forms of disease conditions like erosions, lesions and septicemia. When the bacteria infect body fluids of shrimp, general septicemia and local lesions like

hepatopancreatitis, opthalamitis and enteritis develop. If shrimp is infected by chitinovorous bacteria which are capable of shell lysis, blackened areas or brown spots form on the exoskeleton. Bacterial infections are common in both hatcheries and grow-out ponds which are grouped as Luminous Bacterial Disease, Vibrosis, Shell Disease**,** Tail Rot, Chronic Tail Rot, Gill Rot, and Bacterial Red Disease. Major fungal diseases are Fusariosis and Larval mycosis.

Nutritional Diseases

Nutritional imbalances result in a variety of diseases, which need further investigation. However, the incidence and mortality because of them is negligible. They are Black Death Syndrome, Chronic Soft-Shell Syndrome, Red Discolouration. Exuvia Entrapment Disease (EED) and Blue Discolouration.

Environmental Diseases

Often, sudden and wide fluctuations in water quality parameters like dissolved oxygen, salinity, temperature and pH result in abnormal conditions in shrimp. The most important of them are Abnormal Gill Colours, Muscle Necrosis, Cramped Tail, Blisters, Gas Bubble Disease, **and** Whitish Discolouration of Shell.

Toxic conditions

Rarely, some species of dinoflagellates, blue green algae, diatoms and certain microbes secrete toxic substances, which are thought to be responsible for toxic conditions in pond-cultured shrimp.

Haemocytic Enteritis

Blooms of *Oscillatoria* spp. causes hemocytic enteritis in the juveniles and sub-adults of *P.monodon*. In the affected shrimp the gut becomes red and mucosal epithelium undergoes necrosis with hemocytic inflammation. Mass mortality is noticed within two days of the onset of the problem. Secondary bacterial infection of *Vibrio* spp. has also been noticed.

Diseases of unknown aetiology

There are certain conditions and abnormalities in cultured penaeid shrimp, the causes of which are unknown and hence termed mysterious. They are Winged Branchiostegite, Depigmented Shrimp, Structural Deformities, Emaciation and Tumors

Finfish Diseases

Viral Diseases

Depending up on the pathology of target organ, the animals exhibit various clinical signs leading to behavioural alterations. Therefore, the identification of a viral pathogen begins with the study of behavioural changes and other clinical manifestations before collecting the samples for virus isolation and identification. They are EHN - Epizootic haematopoietic necrosis, IHNV - Infectious haematopoietic necrosis,SVC - Spring viraemia of carp, VHS - Viral haemorrhagic septicaemia, ISA - Infectious salmon anaemia, RSIV - Red sea bream iridovirus disease, KHV - Koi herpesvirus disease, VER – Viral encephalopathy and retinopathy, LDV – Lymphocystis disease virus, IPN – Infectious pancreatic necrosis, OMV – *Onchorhyncus masou* virus disease and CCV – Channel catfish virus disease.

Bacterial Diseases

Fishes are susceptible to a variety of bacterial pathogens, most of which are saprophytic in nature. These bacteria become pathogenic when fishes are physiologically unbalanced, nutritionally deficient, or there are other stresses, such as poor water quality, overstocking, which allow opportunistic bacterial infections to proceed. Pathogenic bacteria can spread disease throughout the fish's body if they are absorbed through the gills or gut, or gain entry via the skin known as systemic infection. Other bacterial infections cause localized surface disease such as fin rot and ulcers; however, if these are not resolved they can also lead to a systemic infection. In general there are four types of bacterial infections which affect finfishes. They are Fin rot – usually resulting from environmental stress, Bacterial body ulcers – open, shallow to deep, lesions on the fish's body, Bacterial gill disease – in which the gills are the primary target, Systemic bacterial disease – in which bacteria invade the fish's body and damage internal organs.

There are two types of pathogenic bacteria, Primary or obligate pathogens and – Opportunistic pathogens. The first category of pathogens is not part of the normal aquatic flora and is capable of causing disease in healthy individuals, for example Aeromonas *salmonicida.* Opportunistic pathogens are normally free-living, either in water or on fish, but become pathogenic under certain circumstances. Many of these are saprophytes, normally living on dead organic matter such as plant and animal remains or faeces, for example *Aeromonas hydrophilia.* In general, most of the bacterial diseases that affect fin fish are caused by opportunistic bacteria.

Major Bacterial Diseases of fin fishes are Columnaris Disease, Disease caused by *Edwardsiella tarda,* Fish mycobacteriosis, Motile Aeromonad Septicemia (MAS), Disease caused by *Aeromonas salmonicida,* Mouth Fungus disease, Bacterial tail rot and fin rot and Vibriosis

Fungal Diseases

Major fungal diseases are Dermocystidiasis caused by *Dermocystidium*, Cotton wool disease caused by *Saprolegina* spp, *Branchiomyces, Ichthyophonus hoferi,* Epizootic Ulcerative Syndrome (EUS *Aphanomyces inadans, A. pscicida, A. invaderis* and ERA (EUS-related *Aphanomyces*).

Methods of Detection and Identification of Viral Pathogens

Sampling of animals for disease diagnosis

Use moribund specimens or freshly dead specimens. Non-random sampling has to be carried out when the disease is suspected for virological analysis, small fish may be bagged, sealed and transported as a whole on ice/frozen gel-packs. For larger fish, viscera can be aseptically removed, placed in sterile containers and shipped on ice/frozen gel-packs. The bags with fish should be filled with five volumes of Hanks' basal salt solution containing either gentamycin (1,000 mg/ml) or penicillin (800 IU/ml) + dihydrostreptomycin (800 mg/ml). Anti-fungal agents such as mycostatin or fungizone may also be incorporated at a level of 400 IU/ml.

Diagnosis

The recent guidelines of disease diagnosis in fish diseases could be classified into three levels of identification of the infection:

Level I: includes farm/production site observations, record-keeping and health management. This background information helps a great deal in confirmatory diagnosis of infection using level II and level III diagnostics.

Level II includes the specialized techniques like histopathology which generally cannot be conducted at the farm site.

Level III comprises advanced techniques requiring high level of infrastructure investment, trained personnel and significant expenditure for carrying out the tests.

Preservation of tissue samples

Samples required for histology, *in situ* hybridization, PCR or electron microscopy should be fixed on site by chemical preservation to prevent tissue breakdown and decay. For virological examination, the samples should be brought to the laboratory in ice with 24 hours of sampling.

Samples for PCR analysis

Samples for PCR analysis can be transported on ice or should be fixed in either 95% or 70% ethanol. It is best to use cold fixative that has been stored in the freezer or kept on ice, as this helps arrest autolysis and secondary microbial proliferation, as the tissues are preserved.

Methods of diagnosis of viral infection in finfish and shell fishes

Viral infection of fish may be diagnosed using different procedures, either alone or in combination:

1. Clinical examination of affected fish (Include both behavioural changes and physical abnormalities).
2. Electron microscope examination of fish tissue or cell culture samples.
3. Cell culture isolation of virus.
4. Immunological detection of viral antigen in fish tissue or cell culture.
5. Molecular detection of viral genes (PCR, nucleic acid probes)

Behavioural changes in fishes due to viral infections

Wherever possible fish sampled for disease diagnosis should be examined for any behavioural abnormalities differing from that of the normal behaviour. This may include listlessness, swimming near surface some times putting the head out of water, gulping of air, loss of balance, corkscrew swimming, belly up rolling motion, flashing and scraping, lethargic movement, increased respiration, increased feed uptake followed by anorexia and reduced reflexes. While the behavioural changes though do not give unambiguous identification of the aetiological agent, it does give a preliminary idea about the probable cause of the disease. In this regard, the pattern of mortalities is an indicator of infection. Depending on the pattern of mortalities and the observation of dead specimen, a preliminary diagnosis could be made. While uniform mortalities across the system point to adverse environmental conditions, irregular or sporadic mortality may be due to an infectious cause. In case of viral infection, the loss of animals and rate of mortality may be higher compared to other infectious agents.

Physical examination

Examination of fish can be done on sacrificed specimens. Kill the fish just prior to examination by decapitating or anaesthetic over dose. Tricane methane sulphonate (MS – 222) 1: 1000 or Benzocaine 0.1g/4l (Dissolve benzocaine in small quantity of acetone and then dissolve in water) will anaesthetise the fish within 5-10 minutes. Examine the outside of the fish for any external lesions or abnormalities. Specific cases of lesions may indicate characteristic viral infection like lymphocystis disease

Histological examination

Samples for histology must be taken as soon as possible after the fish death and fixed immediately, preferably in neutral buffered Formalin.

The organs sampled depend on the nature of the problem but in most cases all the major organs are sampled e.g. gill, skin including muscle around lateral line, heart, liver, pancreas/ gut, spleen and kidney. The samples should be sufficiently small to allow the fixative to penetrate rapidly through the tissue. Therefore the dimensions of the tissue removed should be less than 7.5 mm. The tissues should be placed in at least 10 times their volume of fixative i.e. one part tissue to ten parts fixative. The tissue can be kept in formalin for prolonged periods but they should be stored in such a way as to avoid any portion of the tissues remaining out of the fixative.

Electron microscopy (EM)

Electron microscopy could be broadly classified into 2 sections 1. Transmission electron microscopy (TEM) and 2. Scanning electron microscopy (SEM). The former is used for observing changes in the tissues by sectioning them using ultra microtome and staining them before observing in the electron microscope. SEM is used for observing the surface level changes in the tissues at higher magnifications using EM.

TEM requires similar processing of tissues as in normal histology although the chemicals involved and the protocols are different. Tissue sections or virus infected cell cultures are used for observing viruses or cellular changes by electron microscopy.

Virus isolation in cell culture and identification (In the case of finfish viruses)

Method

Virus isolation and identification involves 3 stages

1. Obtaining samples from the host tissue
2. Growing virus in a permissive cell culture
3. Identifying the virus by a suitable technique

1. Obtaining samples from the host tissue

Whenever possible the tissue should be collected from fish showing signs of the disease; e.g. moribund, external lesions, abnormal behaviour or appearance. The tissue samples should be collected from live fish. If live fish are not available, freshly dead specimens kept on ice for not more than 48 h should be used. External or internal tissues as appropriate may be taken as single tissue

or as pooled sample. The tissue should be taken with minimum possible contamination and should be preferably processed immediately or may be kept in ice for 48 h. Single or multiple tissue samples of not more than 1- 2 g are sufficient for virus isolation procedures, even for the largest fish.

Materials to be examined

1. Fry (< 4 cm length): Remove and discard heads and tails from just posterior to the vent. Retain remaining portion of the body
2. Fingerlings (4 – 10 cm length): Open the body cavity and remove the viscera, including kidney, by cutting and scarping. Retain these tissues for examination.
3. Larger fish: remove and retain a portion of the kidney, spleen, caecae-pancreas, liver and gill. In certain situations, however, it is sufficient to sample only kidney material.
4. Take no more than 1 g of tissue in total from each fish.

Sample material depends both on the size of animals and the objective of testing, i.e. diagnosis of overt disease or detection of fish that are subclinical pathogen carriers.

Pooling of samples

Tissues from a maximum of 10 fry or fingerlings may be pooled and treated as one sample. When larger fish are sampled, tissues from a maximum of 5 fish may be pooled and treated as one sample.

Transportation / storage of samples

Pooled samples must be held in a clearly labeled leak proof container. These should be packaged in separate sealed plastic bags for each sample. Samples must be transported only on ice without freezing to a laboratory as quickly as possible. The samples should be packaged in a robust container and laboratory notified at dispatch. Samples must reach the laboratory and be processed within 48 hours of collection.

2. Tissue processing

Following steps are involved in the processing of the tissues:

a) Weigh the tissue and transfer to a cooled sterile pestle and mortar.

b) Add a little sterile sand or glass wool and grind the tissue into a smooth paste. Homogenise the tissue in 9 volumes of balanced minimum salt solution supplemented with 2 % serum.

c) Centrifuge the resulting tissue suspension at 1500 g for 15 minutes to remove tissue debris.

d) Dilute the clarified extract 1:5 with BSS + 2 % serum. Filter the diluted extract through a 0.45 m membrane filter to remove any bacterial contamination. The BSS is also supplemented with antibiotics (Penicillin and streptomycin and kanamycin 50 IU/ml, 50mg/ml and 50 mg/ml respectively)

3. Growth of the virus

All viruses require living cells as a substrate for growth and multiplication. Appropriate Fish cell lines have become the standard requirement of the isolation characterization and identification of

fish viruses. Cell lines for the inoculation of the extract is selected based on the suspected identity of the virus and if no specific virus is suspected, one or more cell lines more appropriate for the fish species under examination are selected.

Important cell lines that are required to test most of the fish viral diseases include:

Bluegill fry (BF-2), Chinook salmon embryo (CHSE-214), *Epithelioma papulosum cyprini* (EPC), Rainbow trout gonad (RTG-2), Fathead minnow (FHM), Grunt fin (GF), Salmon head kidney (SHK1), Atlantic salmon kidney (ASK)

For the isolation and growth of the viruses either the method of inoculation of preformed monolayer cultures or simultaneous inoculation and culture of cells could be used.

In the case of shrimp as permanent cell lines are not available primary cell cultures have to be used

Inoculation of preformed monolayer cultures:

Monolayer cultures of selected cell lines in tissue culture flasks are prepared and actively growing sub-confluent (80 – 95% confluent) monolayers and not more than a few days old are selected for the inoculation of the prepared tissue extract. The medium present in the tissue culture flask is removed and the monolayer is overlaid with the tissue extract at a rate of approximately 1 ml/25 cm^2 cell sheet. The inoculum is allowed to adsorb for 30 – 60 min. Control cultures are mock infected at the same time with an equal volume of balanced salt solution used to prepare the tissue extract. Antibiotic supplemented culture maintenance medium is added to 3 – 5 mm depth.

Simultaneous inoculation and culture of cells:

Flask cultures of recently grown, confluent monolayers of the required cell line are selected as the source of cells. Filtered tissue extract is directly added to sufficient freshly trypsinised cells, which once settled would give 70 – 90 % confluence. The tissue culture flasks are then be incubated under appropriate atmospheric and temperature conditions. The incubated flasks are examined daily under an inverted phase contrast microscope for evidence of any cytopathic effect (CPE) compared to uninoculated control culture. After 10 – 21 days, the flask is blind passaged to fresh tissue culture flasks with the same cell lines and examined daily for CPE as before. If there is no CPE even at the end of the blind passage, the sample is discarded as negative.

4. Virus identification

Direct electron microscopic examination of the infected tissues, cell cultures or cell culture supernatant medium also give indication to the identity of the virus but not specific identity. Currently techniques are also available to detect the virus genetic material in the tissue directly by molecular biology techniques, which eliminate the requirement for the isolation of the virus in cell culture. But this method is in the process of development for many viruses.

Observation of the cytopathic effect (CPE)

The CPE can either be specific or non-specific. Specific CPE arise out of the infection due to virus, which would be characteristic to different virus groups. Non- specific cell changes can also appear due to toxicity of the inoculated tissue extract or microbial contamination.

If CPE is observed, the culture fluids are harvested, clarified by low speed centrifugation and diluted in the maintenance medium and passaged to fresh cultures to determine the nature of CPE. If the CPE is specific, it reappears in the fresh culture but the non- specific CPE would not reappear in the fresh cultures due to dilution. The type of CPE is a useful indication to the virus identity though not absolutely diagnostic.

Antibody-based virus detection method

Viruses are generally identified by use of antivirus serum. The methods used include: Serum neutralisation (SN), Fluorescent antibody technique (FA), Immunoperoxidase techniques (IP), Enzyme-linked immunosorbent assay (ELISA), Immune electron microscopy etc.

Detection of virus using immunoperoxidase of infected cell cultures or fish tissues

Viruses replicate within susceptible cultured cells and addition of a mild detergent permeabilises the cells allowing a rabbit antibody to bind to intracellular viral proteins. Virus could be detected by a biotinylated anti-species antibody and a streptavidin-peroxidase conjugate. The addition of a substrate results in 'brick-red' staining in areas labelled with antibodies. In fish tissues the presence of virus particles is detected by using the histological sections and subjecting them to antibody based staining technique as above.

Detection of viruses using antigen-capture ELISA

Antigen-capture ELISA has been validated to detect different viruses in cell cultures and directly in homogenates of fish tissues. ELISA is useful for both diagnosis and certification. Antibodies used for ELISA should have sufficient level of specificity and neutralization indices. Monoclonal antibodies directed against major epitopes of capsid protein would give dependable results. Different modifications of ELISA standardized against different viruses could be used for detection of the specific viruses.

In antigen capture ELISA, the virus particles are captured from the sample by a purified mouse or rabbit antibody that is coated to the plate. The virus is detected by a second antibody and a peroxidase-labelled conjugate using the chromogen such as ABTS (2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulphonic acid). The enzyme is inactivated after 20 minutes and the resulting optical density (OD) is compared with standards.

Immunoelectron microscopy

Gold-labelling of sections containing tissues or cell cultures are used in this test. Conventional electron microscopy generates data on virus structure and morphogenesis. Negative contrast electron microscopy will produce images that can be used to examine the particulate structure of the virus. The use of antibodies and conjugated gold with these preparations permits both ultrastructure and antigenicity to be examined.

Neutralisation test

Cell culture supernatant showing CPE or fish tissue extracts are centrifuged at 2000 g for 15 min at 4 °C and clarified. In the test a positive control of the homologous virus and a negative control of heterologous virus is also taken. Dilute the virus containing medium to 10^{-2} -10^{-4} and virus stocks to about 10^{3} TCID 50. Mix aliquots of about 200 µl of each virus dilution with equal volumes of an

appropriate dilution of neutralising antibody (rabbit polyclonal or mouse monoclonal antibody) against the virus, and similarly treat aliquots of each virus dilution with cell culture medium. (The solution should have a 50% plaque reduction titre of at least 2000).Incubate all the mixtures at 15-25°C (depending on the virus) for 1 hour. Transfer aliquots (50 µl) of each of the above mixtures on to drained cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5-1 hour at 15-25°C. When adsorption is complete, add cell culture medium, supplemented with 2% FCS. Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase-contrast preferable) or after discarding the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 10% buffered formalin. The tested virus is identified based on CPE if prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the specific antibody, whereas CPE is present in the untreated sample.

Indirect fluorescent antibody test

Prepare cell monolayers on glass cover slips or plastic cell culture plates with 80% confluence. Seed six cell monolayers per virus isolate to be tested, plus two for positive and two for negative controls. The FCS content of the cell culture medium can be reduced to 2-4%. Inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks. Dilute the control virus suspension in a similar way, to obtain a virus titre of about 5000-10,000 infectious units/ml. Incubate for 24 hours at appropriate temperature. Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at -20°C) for glass cover-slips or a mixture of 30% acetone and 70% ethanol, also at -20°C, for plastic wells and allow to fix for over 15 minutes. Air-dry cell monolayers for about 30 minutes and process immediately or freeze at -20°C.

Prepare a solution of antibody against the virus in PBS containing 0.05% Tween 80 (PBST) at the appropriate dilution. Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution and decant completely after the last rinsing. Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber. Rinse four times with PBST. Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer. Examine under UV light using a microscope. Positive and negative controls should give the expected results for successful analysis.

Nucleic acid based detection methods

DNA probe

Infect cells with the virus isolate along with positive and negative controls. Extract RNA/DNA from cells as soon as the cells show CPE. A biotinylated probe with complimentary sequence to viral targets is made as antisense probe complementary to a conserved region in the virus. This would react only with the specific virus and should recognise all isolates of the virus. The final concentration of the biotinylated probe will be 0.1 µg/ml in hybridisation solution.

Extraction of mRNA from infected cells

Remove culture medium from infected cells and add extract RNA using Trizol. Partially dry the

RNA pellet following the manufacturer's instructions. Dissolve RNA pellets at 65 °C in deionised, RNAse-free water. Add RNA to blotting device along with virus positive denatured PCR products (DNA). Microwave NC membrane for 60 seconds on high power to attach nucleic acids to the membrane.

Prehybridise membrane for 1 hour at 55°C in the buffer and add the probe solution and allow to react for 1 hour at 55°C. Rinse the membranes with post-hybridisation solution and wash at 55 °C for 15 min. Incubate the membranes in a solution containing streptavidin/alkaline phosphatase in for 30 minutes at room temperature. Rinse the membranes briefly followed by washing twice in buffer with about 7 minutes incubation at room temperature. The membranes are subjected to color development using solution containing nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). In case of DNA viruses the DNA could be extracted and used for analysis.

Polymerase chain reaction

Polymerase chain reaction (PCR) involves enzyme-based amplification reaction in the assay. Several cycles of copying a specified stretch of DNA or target nucleic acids of an infectious agent is done using PCR. Two short nucleotide sequences, termed primers are designed complimentary to the sites that flank the target sequence, bind to the DNA strand to be copied. Using a DNA polymerase, which is active even during high temperatures, copies the target sequence into a new strand using the free nucleotides joined to the primers. By repeating the heat-cycling regime 20-40 times, the amount of copied target DNA gained is enough for further operations, such as detection, cloning or sequencing.

Collect aliquots of culture medium from cell monolayers exhibiting CPE or fish tissue homogenates and centrifuge at 2000 g for 15 minutes at 4°C to remove cell debris. Release RNA/DNA from virus solution by diluting the sample 1/20 in sterile deionised water, heating the tube at 95°C for 2 minutes, then store on ice. This simple procedure is especially suitable for virus grown in cell culture where few PCR-inhibiting substances are present. Alternatively, RNA/DNA can be extracted from a pellet of infected cells or fish tissues using standard methods or by use of other commercially available kits.

DNA extraction

For DNA extraction, ground the preserved tissues to powder. Around 10 volumes of extraction buffer (NaCl [100 mM], ethylene diamine tetra-acetic acid [EDTA, 25 mM], pH 8, sodium dodecyl sulfate [SDS, 0.5%]) are added with proteinase K (100 µg/ml). Following overnight incubation at 50°C, DNA is extracted using a standard phenol/chloroform protocol, and precipitated with ethanol.

Reverse-transcription PCR

RT PCR is used to detect the presence of target RNA by generating a complimentary DNA copy and amplifying it to detectable levels. Prepare a master mix for the number of samples to be analyzed. The master mix for one 50 µl reverse-transcription PCR is prepared as follows: 21.75 µl ribonuclease-free (DEPC-treated) or molecular-biology grade water; 5 µl 10 × buffer; 5 µl 25 mM $MgCl_2$; 5 µl 2 mM dNTP; 2.5 µl (20 pmoles/µl) each of forward and reverse primer; 0.5 µl Taq polymerase (5 U/µl); 0.5 µl AMV reverse transcriptase (9 U/µl); 0.25 µl RNasin (39 U/µl). To each of the tube add 20 µl extracted template RNA.

Nested round PCR protocol

If the first round PCR provides insufficient amplified product, an internally nested set of primers is used for additional DNA amplification. Prepare a master mix for the number of samples to be analysed. Work under a hood and wear gloves. Dispense 48 µl of master mix into each tube and add 2 µl of first round PCR template.

Visualize the specific band amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

Methods of Detection and Identification of Bacterial Pathogens

Bacterial pathogens can be diagnosed under following categories:

1. Gross clinical signs of disease on individual fish
2. Internal abnormalities apparent during post-mortem examination
3. Histopathological examination of diseased tissues
4. Bacteriological examination of tissues.

1. Gross clinical signs of disease

Fish may display many behavioural and physical changes, some of which give valuable clues as to the nature of the diseases. Many of these signs are common to a multitude of diseases. Thus, many external signs of diseases are recognized pertinent to the fish pathogens.

2. Internal abnormalities apparent during postmortem examination

Careful internal observation of diseased fish may reveal the presence of clearly discernible abnormalities.

3. Histopathological examination of diseased tissues

Although many histological procedures may be routinely used, it is important for the bacteriologist that Gram stained sections should be prepared. Thus, the presence of any offending bacterial pathogen will quickly be recognized. Microscopic examination of Gram stained material will enable the determination of basic staining reaction and micromorphology of the pathogen. Possibilities include the presence of Gram positive or Gram negative rods (spore bearing or asporogenous), cocci and mycelium. For Gram positive organisms, the acid-fast stain will help in the recognition of *Mycobacterium*, *Nocardia* and possibly *Rhodococcus*. *Flavobacterium columnare* can be diagnosed by the microscopic scrapings from an eroded area. Classically, small tufts or haystack-like colonies of the organism may be seen.

4. Bacteriological examination of tissues

Selection of specimens

In order to determine the extent of bacterial infections in fish stocks, it is necessary to have a random sample comprising 10 fish for the bacteriological investigation. For this, only live or moribund fish can be used. Dead fish should never be used for bacterial diagnosis, since the dead fish become quickly colonized by numerous bacteria, which would bear no relationship with the disease under

investigation, and which may completely mask the original bacterial flora responsible for the condition. In the bacteriological routine investigation, the detection of bacteria normally occurs from the visceral organs, which seldom contain microbes in healthy fish. The bacteriological investigation can also be extended to abscesses, ulcers, skin, gut, blood, ascitic fluid and so on.

The gills are the poor samples for bacteriological studies, since they are heavily contaminated.

In the case of acute bacterial infections, the pathogens are mostly detected in all the organs, while in the case of a chronic course of the disease, bacteria are usually encountered as foci in the abscesses, ulcers and the organs.

It is necessary, in many cases, to extend the bacteriological investigations to the water or the food to trace the source of infection.

General processing of samples intended for bacteriological examination

Bacteriological studies should be carried out before the fish is opened or cut.

Sacrificing

The fishes have to be euthanized with an anaesthetic overdose (MS-222) or narcotized by a narcotic (e.g. trichlorobutanol).

Scraping and squash preparations

If there is a suspicion of bacterial infection, first observation about the presence of bacteria can be made by microscopic examination (with oil immersion) of unstained or stained smears of body fluids, blood, ascitic fluid and scrapings, teased or squash preparations of organs, skin, gills, liver, kidney, spleen, gut and so on.

Inocula from fish for bacteriological examination

Inocula from fish are taken when there is a suspicion of bacterial infection. For this, material should be removed from the infected foci, in which the typical bacterial pathogens without contaminations could be expected (liver, spleen, kidney, swellings, blood). If inocula from intestines, open ulcers or skin are considered necessary, then several bacteria with no pathological significance may also be expected. The prerequisite for this is that the material should be taken out under aseptic conditions, i.e., without contaminations from the air, water and intestines of fish. As far as possible, the removal of inocula should be carried out in a laminar flow chamber, thus preventing the microbial contamination from the air. The surgical instruments used for the removal of inocula should be sterilized by thoroughly heating and repeated immersions in a high percentage alcohol or other disinfectant.

External surface

For removal of inoculum from the skin, the infected spot on the skin is scraped with a sterile, pointed needle and adhering material inoculated onto a solid medium. In the case of abscesses and ulcers, their surface is disinfected by swabbing with high percentage alcohol or seared with a hot spatula and then from the depth of the abscess or ulcer, some material is removed by means of a sterile platinum loop.

For collecting the mucous samples, pass a sterile swab along the lateral surface of the fish. Streak the sample on to medium.

For collecting the vent samples, place a sterile swab or loop approximately 1/2 to 1 inch into the anal vent and remove. Streak on to the medium.

If the samples are collected from external lesions, plate serial dilutions of the inoculum to decrease the number of interfering bacteria and fungi that are likely to be present in this type of sample.

Internal organs

The removal of inocula from the inner organs is done by opening the body cavity of the fish by an appropriate direction of incision. The fish is placed on its right side with the head to the left. With a large needle, the caudal peduncle is fixed to a board. The surface of the fish is flooded with 70% alcohol or other disinfectant. This having been done, a ventral and a lateral incision respectively are made with a sterile pair of scissors. With a flamed loop, the surface of the liver, spleen, heart and kidney is punctured, and a little of the infectious material is plated onto the appropriate culture medium. Small tissue fragments (2-3 mm^3) can be removed from the exposed organs by either cutting off with a pair of scissors or pulling off with a pair of forceps, and placed in a liquid culture medium.

If a very small fish, whose dissection cannot be done aseptically, is to be bacteriologically investigated, the entire fish is grasped with a pair of forceps, plunged in alcohol, then singed and ventrally dissected with a sharp dissecting needle. The swollen organs can then be crushed with forceps and conveyed to the nutrient substrate.

It is most convenient to remove inocula from the intestines by cutting out a piece of the intestinal part (mostly the terminal intestine) held with forceps or by inserting a platinum wire loop into the intestinal lumen.

If the body cavity of the fish contains fluid as in the case of abdominal dropsy, it is usually not possible to carry out the removal of inocula in the usual manner, since microbes can spread to other places through the ascitic fluid. For this purpose, the fish is suspended from the lower jaw by a hook, and the whole trunk, nearly at the centre of the body cavity, is cut transversely, so that the dorsum, the vertebral column and the walls of the body cavity are completely severed, but the viscera remain intact. The fluid flows down, and the removal of inoculum from the internal organs can be undertaken by inserting a platinum wire loop.

Any instruments used are sterilized by dipping them in alcohol and immediately flaming before use.

Collection of blood

The blood as an inoculum is obtained with a sterile syringe by puncturing the heart or caudal vein or by cutting off the base of the tail.

Isolation of bacteria

The use of suitable culture media, which can afford the maximum conditions of growth for the various kinds of bacteria, is a prerequisite for the isolation, culture and differentiation of fish pathogens from suspected or diseased fish. If there is suspicion about a specific pathogen, nutrient media with

special additives are employed. After incubation of the inoculated culture at room temperature in an incubator, the percentage of positive cultures of potential pathogenic microorganisms becomes recognizable by the turbidity of the liquid medium or the growth of colonies on solid media.

In liquid cultures, there is the risk that fish pathogenic microbes are masked by non-pathogenic bacterial flora or the microbes are impeded in their growth by their metabolic products. Transfer or isolation of pure cultures must therefore be performed quickly. These risks can be avoided by inoculating solid media with inocula from the infected organ or impure liquid or crude culture, since the bacteria can grow in separate colonies into pure cultures.

Nutrient agar is mostly used at present as a standardized, ready-made culture medium. The nutrient agar can be enriched with additives for fastidious microbes and for differentiating bacteria.

Isolation of pure cultures

In order to study and characterize a microorganism, it is first necessary to isolate the microbe from all others and maintain it in pure form throughout the study. Cultures consisting of only one type of microorganism are called pure or axenic cultures.

For obtaining pure or single cultures, only solid media can be employed in the routine investigation. However, liquid media containing several types of bacteria can yield pure cultures by dilution of the original culture and reculturing in test tubes containing liquid nutrient media; thereafter, by several dilution stages, the aliquots can be brought to the stage of containing only one type of microbe. It can then be confirmed by microscopic, cultural and biochemical investigations, as to which tubes containing only bacteria of one species.

In the routine isolation of pure cultures, the suspected material under investigation is inoculated on a solid culture medium using a sterile platinum loop.

If the inoculum contains only one type, there would be no difficulty in the isolation of pure cultures. Material containing several types is diluted, before smearing, by passing a loop of material containing microbes into one or more drops of sterile physiological saline solution or sterile tap water. The loop is freshly sterilized for every passage. If the loop has been flamed to red-hot, care must be taken that the loop is adequately cooled before taking an inoculum, since all fish pathological microorganisms are affected at temperatures above 37^0C.

The isolation of pure cultures on nutrient agar plates is done by streaking directly with material previously diluted or of low germ content. Here, the microbial mixture is transferred to the edge of an agar plate with an inoculating loop or swab and then streaked out over the surface in one of several patterns. As the microorganisms are spread over the surface of the agar, their concentration on the inoculating loop decreases. Eventually, single microbial cell lands on an isolated area of the agar-solidified medium. As the isolated microorganism multiplies, a population develops, forming a clone of cells called colony. If the original specimen possesses several different types of microorganism, each type may form a distinct colony that can be distinguished from the others. The different organisms can then be purified by picking a colony with an inoculating loop and streaking onto fresh medium.

The diverse morphology of colonies of fish pathogenic bacteria on solid culture media can contribute to determine the nature of bacteria. The type of colony is described by the size, the shape of the contour edge or marginal elevation, surface appearance, pigment formation and texture.

If the nutrient agar employed for pure culture is a differential culture medium, then, besides the colony morphology, certain biochemical properties of the microbes in the immediate neighbourhood colony (e.g., the formation of acid from carbohydrates, formation of haemolysin on the blood plate and so on) can indicate differentiation of the types of bacteria.

The pure cultures of bacteria isolated on nutrient agar or by other methods can be maintained as the strain stock on agar slant or in stab cultures.

Characterization of isolated bacteria

Many characteristics are used in classifying and identifying microorganisms.

1. Colony morphology, staining morphology and micromorphology

2. Biochemical tests

- Phenyl alanine deaminase, Indole production, Reduction of nitrate to nitrite and to nitrogen, Methyl red test and Voges Proskauer reaction, Decarboxylation of aminoacids, Aminoacid dihydroxylation, Levan and glucan production, Oxidative and fermentative metabolism, Kovac's oxidase reaction, 3-keto lactose production, 2-keto gluconate production, H_2S production, Sensitivity to 2,4-diamino-6,7-diisopropyl pteridine.

3. Degradation characters

- Gelatin production, Lipase production, Urease production, Starch hydrolysis, Caseinase production, Haemolysis, Chitinoclastic activity, Cellulolytic activity, Lecithinase activity, Aesculin hydrolysis, DNAase activity.

4. Physiological characters

- Varying pH, temperature, NaCl concentration, Presence of heavy metals (growth)
- Growth in the presence of stains, Requirement of ions

5. Utilization of substrates as sole carbon sources

Carbon sources such as ribose, xylose, raffinose, melibiose, rhamnose, trehalose, cellobiose, galactose, mannose, fructose, sucrose, arabinose, inulin, glycogen, sorbitol, sodium pyruvate, calcium lactate, sodium glucuronate, lactose, adonitol, tyrosine, dulcitol, glycine, proline, serine, valine, sodium malonate, m-inositol, leucine, uracil, tryptophan, cystine, sodium formate, sodium acetate, sodium gluconate, mannitol, sodium citrate, glycerol, salicine, sodium-potassium tartarate, succinic acid, citrulline and glutamic acid.

6. Fermentation (production of acid and gases)

Glucose, fructose, sucrose, lactose, maltose, ribose, xylose, raffinose, melibiose, rhamnose, trehalose, cellobiose, galactose, mannose, arabinose, glycerol, mannitol, salicine, adonitol, inositol, sorbitol, starch, dulcitol, glycogen, inulin, butanol, ethanol and dextrin.

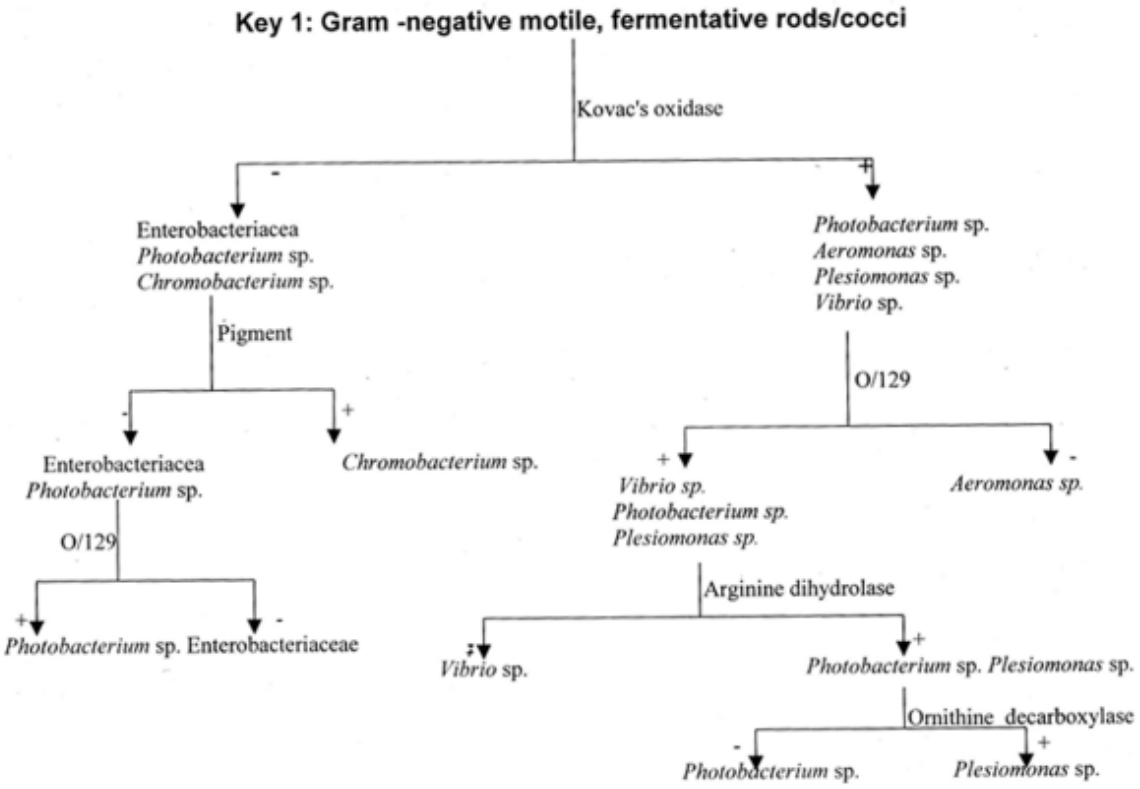
7. Antibiotic sensitivity

Penicillin G, streptomycin, oxytetracycline, ampicillin, tetracycline, gentamycin, polymyxine B, chlortetracycline, neomycin, cefazolin, amoxycillin, nalidixic acid, novobiocin, chloramphenicol,

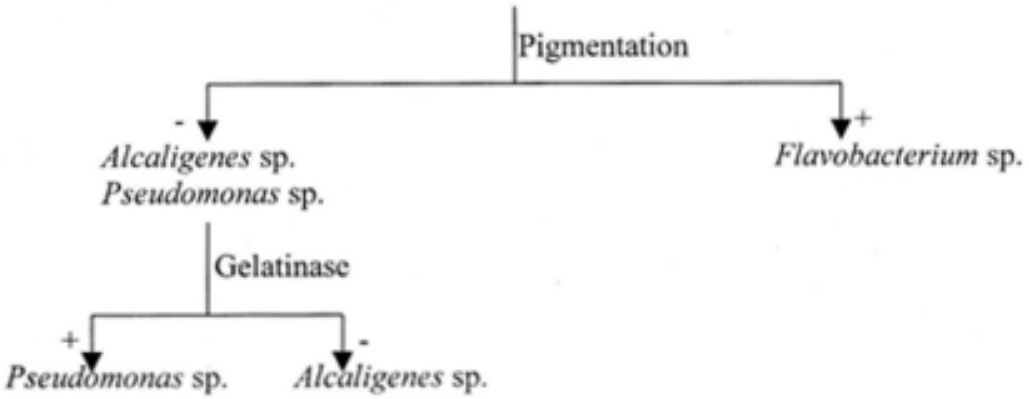
erythromycin, kanamycin, bacitracin, lincomycin and sulphadiazine.

V. Identification

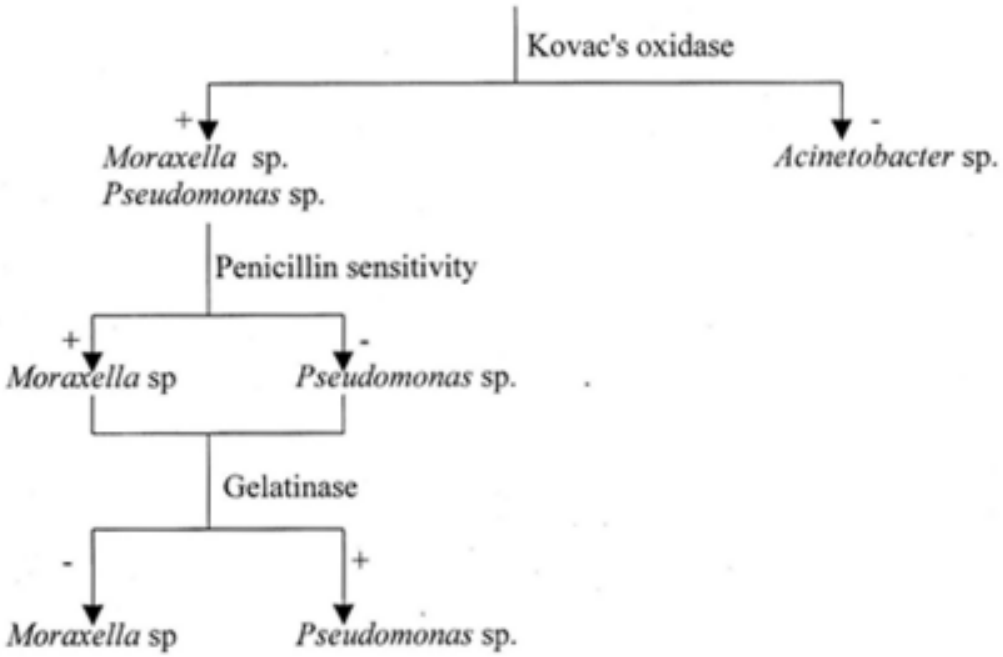
There are several diagnostic keys available for the fish pathogenic bacteria. Once the morphology and Gram staining reaction of bacteria within a lesion have been defined in smears, subsequent rational isolation and identification should be possible. These generally involve biochemical testing for the presence of specific enzymes. A simplified dichotomous key for the identification of bacterial pathogens are shown below;



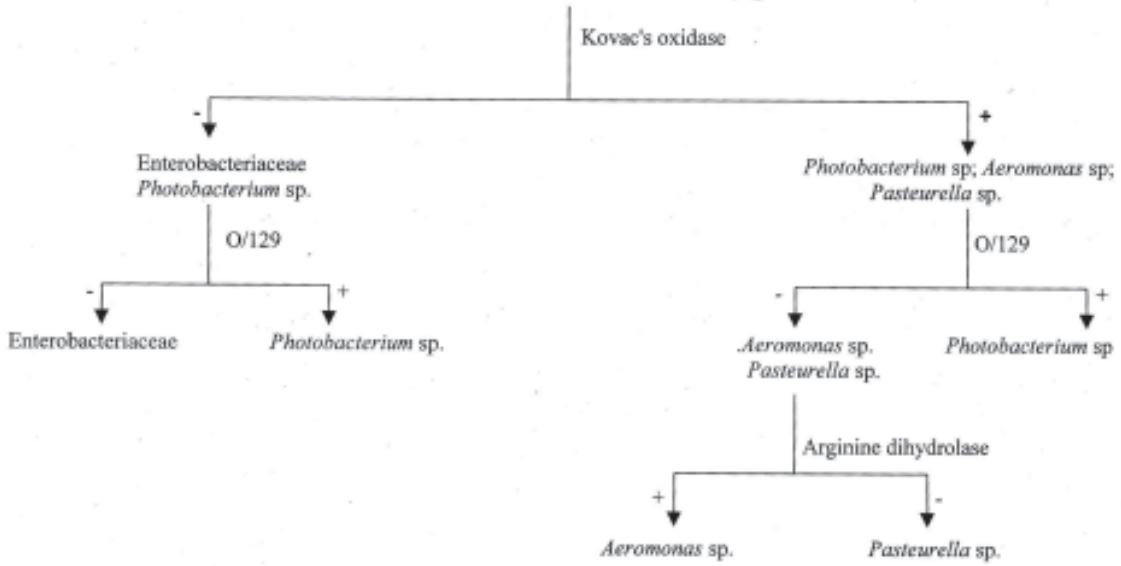
Key 2: Motile, Gram-negative rods with alkaline reaction to dextrose



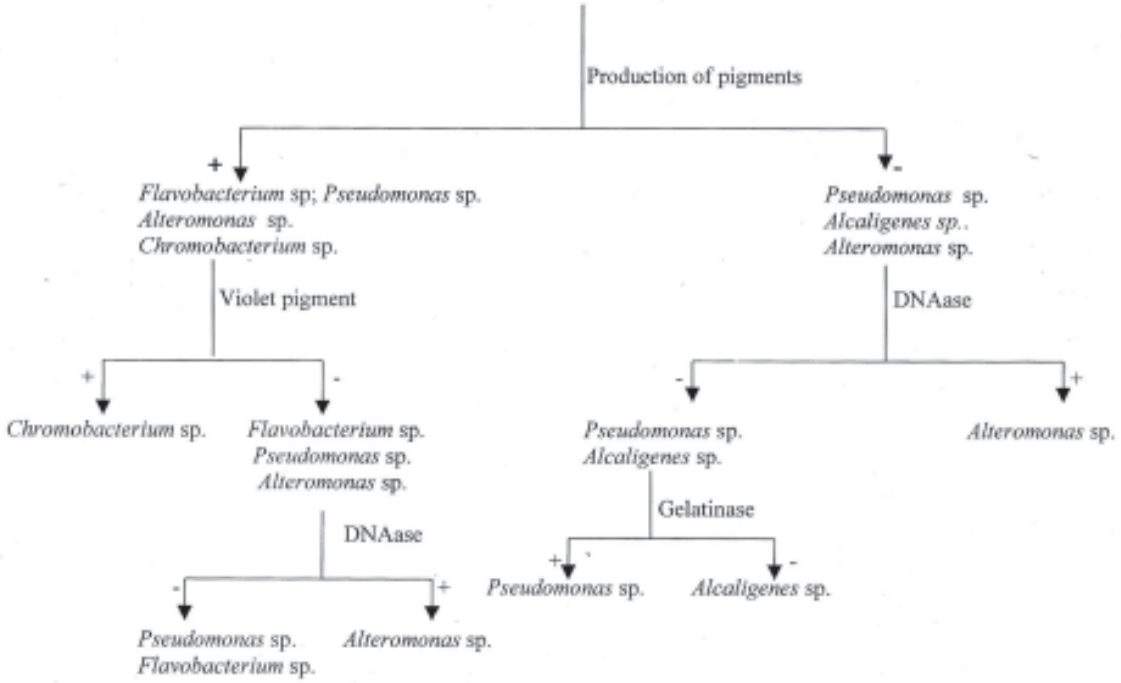
Key 3: Gram-negative, non-motile, non-pigmented, oxidative/alkaline/no reaction to dextrose

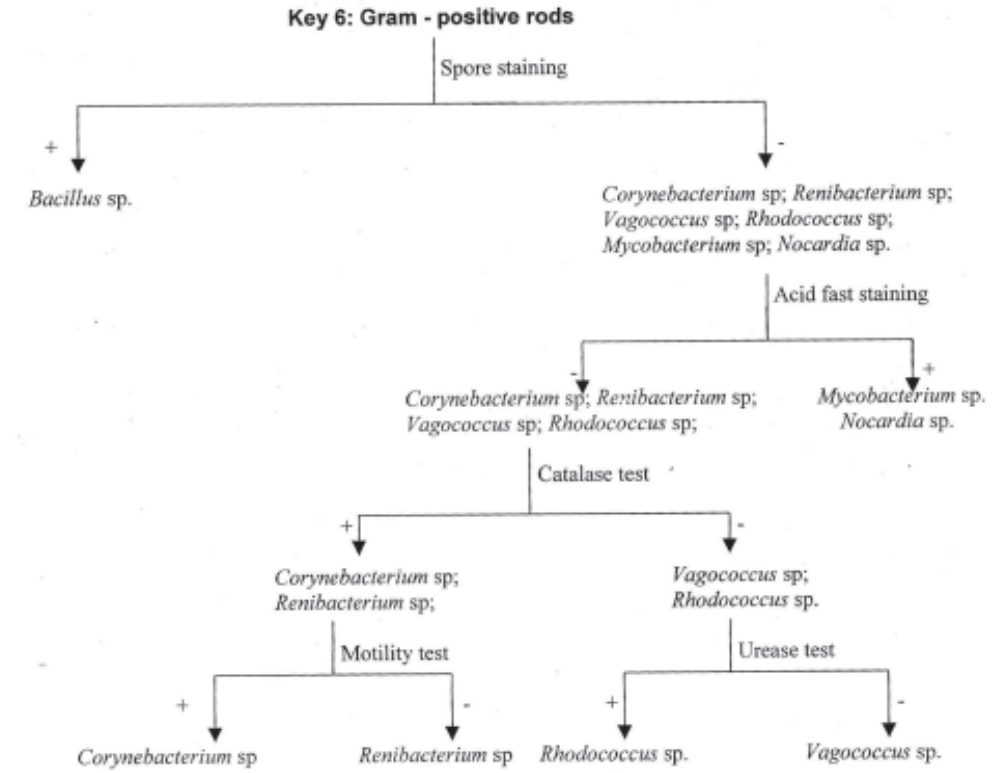


Key 4: Gram-negative, non-motile, fermentative, non-pigmented rods/cocci



Key 5: Gram -negative motile rods/cocci with no acid production/oxidative reaction to dextrose




Key 6: Gram - positive rods
Spore staining
+
-
Bacillus sp.
Corynebacterium sp; Renibacterium sp;
Vagococcus sp; Rhodococcus sp;
Mycobacterium sp; Nocardia sp.
Acid fast staining
-
+
Corynebacterium sp; Renibacterium sp;
Vagococcus sp; Rhodococcus sp;
Mycobacterium sp.
Nocardia sp.
Catalase test
+
-
Corynebacterium sp;
Renibacterium sp;
Vagococcus sp;
Rhodococcus sp.
Motility test
Urease test
+
-
+
-
Corynebacterium sp
Renibacterium sp
Rhodococcus sp.
Vagococcus sp.


Key 7: Gram positive cocci
Utilization of glucose
Oxidative
Fermentative
Micrococcus sp.
Planococcus sp.
Staphylococcus sp; Streptococcus sp;
Enterococcus sp; Lactococcus sp.
Motility and Beta- galactosidase
Catalase
-
+
-
+
Micrococcus sp.
Planococcus sp
Streptococcus sp; Enterococcus sp;
Lactococcus sp.
Staphylococcus sp.
Arginine dihydrolase
+
-
Streptococcus sp.
Enterococcus sp.
Lactococcus sp.
Acid from sorbitol
+
-
Enterococcus sp.
Streptococcus sp.

Methods of Detection and Identification fungal pathogens

Diagnosis of fungal infections:

Identification fungal infections can easily be done by direct **microscopic examination of tissue materials.** One of the methods is to put drops of 10% KOH on clean grease free glass slide and to place the sample of tissue (Scales, Scraping from skin or exoskeleton or pieces of soft tissue) and pass the slide through a flame. When the tissue is softened, a clean glass cover slip is placed on the sample and pressed. The slides are kept with mounts for a variable duration from 5minutes to 30 minutes depending upon the thickness of the scales, and examined every 5minutes. Examine each slide thoroughly for the presence o filamentous, septate, branched hyphae crossing margins of the cells. Another method is to keep the sample on slide with 10% KOH and 40%DMSO and examine the slide after 5minutes for fungal hyphae.

Lacto- phenol Cotton Blue Slide Mounts:

This is another method under wet mount preparation which is widely used for staining and observing fungi and is simple to prepare. The preparation has three components such as phenol, which kill live fungi; lactic acid which preserve fungal structures and cotton blue which stains the chitin in the fungal cell walls.

Demonstration of fungal growth in tissues by special stains:

a) Periodic Acid Schiff Stains (PAS) - These stains demonstrate the Polysaccharides in fungal cell wall and thus make the morphological features visible.
b) Gridley Method (Lillie Modification)- Here chromic acid oxidation of carbohydrate and subsequent Schiff reaction is used
c) Fluorescent method- Fluorescent dye like acridine orange is used.
d) Grocott method of silver impregnation to demonstrate structure of hypha and mycelium.

Isolation and identification fungi by cultural methods

Bacteria tend to grow in a neutral or slightly alkaline medium , where as fungi prefer acid medium, typically pH 5.0-6.0 and fungi require high carbohydrate content (usually 2-4% glucose). The acidity of the medium and high carbohydrate content in medium discourages bacteria. In addition, the efficiency of fungal media is enhanced by incorporation of antibacterial compounds like antibiotics.

The commonest couture media used in a diagnostic laboratory for the isolation and cultivation of infectious fungi is Saboraud's medium (Saboraud's glucose agar, pH 5.4) having a concentration of 2-4% glucose/ carbohydrate. There are a number of mycological media available and it is not possible to describe all of them in this note. Czapek Dox medium and Cornmeal agar are other media used in clinical labs. Cycloheximide is incorporated in many media to prevent contaminant moulds. Chloamphenicol, penicillin, oxolinic acid and Gentamycin etc. are added to inhibit bacterial contamination.

Biochemical and serological tests, which form the main tools of bacterial identification, have little use in mycology. The morphological features and growth characters are used for identification of fungi. The rate of growth, colour morphology of the colony and the pigmentation are valuable

observations. The morphology of hyphae, spores and other structures is studied. Hyphal diameter, presence or absence of septa and special structures found during examination are valuable in diagnosis. Special hyphal structures frequently observed are spring- like helical coils (spiral hyphae). Localised swelling formed by tightly twisted hyphae resembling tennis racquet (raquet hyphae) and numerous short branches appearing at the ends of hyphae (favic chandelier) are important features in identification. The morphology of asexual spores or conidia is of diagnostic importance. They may be small, single celled 'microconidia' or large, single or multicelled 'macroconidia'. The type of spore formation is distinctive for different fungi *Blastospores* are formed by budding (yeasts). *Arthrospores* are formed along mycelium by segmentation and condensation of hyphae. *Chlamydospores* are thick walled resting spores formed by rounding up and thickening of hyphal segments.

One of the methods in fungal identification is the examination of slide mounts. The preparation of slide mounts causes disruption of structures leading to difficulty in visualising all morphological features intact. To overcome this methods of visualising cultures *isitu* without disturbing the fungal growth are available. These are *slide cultures* and *cellophane culture*.

Slide Mount

A sterile glass slide is placed on a bent glass rode kept in a sterile petridish. The bottom of petrish dish has a moist pad over which the glass rode is placed (the glass rode and moist pad are sterile). A block of 1 centimetre square is cut from solid fungal growth media and placed over the slide in centre under aseptic condition. Inoculate the edges of the square with the fungus. Put a sterile cover glass over the square of culture media. Close the petridish and incubate. When sufficient growth is attained remove the cover glass; put a drop of alcohol on cover slip to remove air from fungi. The cover slip is stained with lactophenol cotton blue. The cover slip is inverted over a glass slide and examined. A second preparation is also made by removing the remaining agar gel block from glass slide and then treating it with alcohol; the block is stained and mounted on a fresh glass slide with cover glass.

Cellophane culture

Small pieces sterile cellophane, about 1.5 centimetres are placed on the surface of a solid growth medium and are inoculated with the fungus to be studied. The fungus grows well on the surface of cellophane drawing the nutrient through the membrane by diffusion. When sufficient growth is obtained, the cellophane squares are removed and placed on microscopic slides. Then add alcohol and lactophenol cotton blue over the cellophane squares. Put a cover slip over it and examine.

Disease Management

Viral Diseases

Avoidance

Avoidance is the prime option in the management of viral pathogens and diseases. This must be through precise and rapid detection of the pathogen using either immunological or molecular tools and by resorting to quarantine.

Vaccination

Vaccination of shrimp either orally or by intra muscular injection using WSSV subunit vaccines has remained a popular strategy for immunization against WSSV. Most studies have focused on the use of vp28 as recombinant plasmid constructs injected as such or as oral vaccines when expressed in yeast or bacterial hosts and mixed in feed. There are reports of successful use of antibodies against envelope proteins of WSSV raised in egg yolk. Other envelope proteins used in vaccination include vp19, vp281, vp26, VP15, VP35. Recombinant viral proteins rVP19 and rVP466 have been reported to afford protection when used as recombinant protein vaccines. Formalin-inactivated WSSV administered orally to shrimp has also been reported to protect shrimp against WSSV infection. However, the feasibility and efficacy of all these vaccination strategies in a large-scale field trial is not yet determined.

RNA interference (RNAi)

Of late the application of RNA interference as an antiviral therapeutic in aquaculture has garnered much interest owing to the success stories reported in human and veterinary medical sciences. RNA interference (RNAi) is described as the post transcriptional silencing of gene expression that occurs when double-stranded RNA (dsRNA) is introduced into a cell (Fire and Mello, 1998). Long dsRNAs in the cytoplasm produced by viral infections, transposons are processed into 21 to 23 nucleotide (nt) RNA duplexes by an RNase III nuclease called Dicer. These short guide RNA sequences are incorporated into an RNA-induced silencing complex (RISC) which then identifies the homologous mRNA in the cytoplasm and cleaves the target mRNA resulting in silencing of gene expression.

Ever since it was first demonstrated that dsRNA induces antiviral immunity in the shrimp *Litopenaeus vannamei*, there have been many reports on the efficacy of dsRNA and siRNA against shrimp viruses like WSSV, Taura Syndrome Virus (TSV) and Yellow Head Virus (YHV). Several recent papers have reported that siRNAs (Westenberg et al. 2005; Xu et al. 2007) or dsRNA synthesized by *in vitro* methods (Kim et al. 2006; Robalino et al. 2005; Robalino et al. 2004) serve as potential therapeutic agents for treating white spot syndrome disease. Experiments conducted at CIBA have demonstrated the efficacy of dsRNAs synthesized against vp28 and ribonucleotide reductase 1 in controlling WSSV infection. Thus RNAi promises to be a potential means of treating brooders to ensure the production of WSSV-free seeds.

Although several treatment protocols are in the pipeline they can be used effectively only after the demonstration of their success in extensive field trials. At this time the most effective method for the management of WSSV infection is the adoption and implementation of better management practices. The application of scientific principles to farm practices will go a long way in the control of the white spot syndrome and other shrimp viral pathogens. With the recent advances in research in molecular therapeutics, the day is not far when there will be a viable therapy for the viral diseases.

Bacterial Diseases

All species of fish are susceptible to bacterial pathogens, which may, under certain conditions, give rise to outbreak of disease. Poor water quality and temperature changes are the most commonly encountered factors, which predispose pathogenic bacterial disease. In most instances, the infection

is widely disseminated to all the fishes sharing the same environment. Many of the disease agents give rise to similar clinical signs and lesions and a bacteriological examination is often required to establish the identity of the casual organism. Diagnosis is thus based on the results of an examination of a sample of the affected population. Rapid diagnostic methods such as examination of wet mounts or stained smears from selected tissues can be useful, particularly when prompt treatment or prophylactic measures are required, but specific identification demands the isolation of the organisms on an appropriate culture medium and the application of a range of physical and biochemical tests.

In food fishes antibiotics are not permitted and hence the issue is not dealt with here. However, in the case ornamental fishes when target bacterial pathogens are detected, antibiotic treatment can be executed after sensitivity testing so that the specific pathogen shall be eliminated.

As the bacterial pathogens are opportunistic prime concern should be to establish optimal environmental conditions besides the application of probiotics, immunostimulants and vaccines.

Fungal Diseases

Fish fungal infections are difficult to treat and mould cannot ever be eliminated from any fish keeping systems. Currently, the most effective strategy for controlling and preventing *Saprolegnia* infections is a combination of good fish management and husbandry techniques, combined with chemical treatment, especially during the 2 to 4 day period after handling. The reduction of stress appears to be the single greatest factor to help fish resist saprolegniasis.

Several treatments for fungal infections are suggested in the literature.

Eggs

Fungal infections of eggs can be minimized by the reduction of organic material in the water and dips in antifungal medications. Formalin and malachite green, particularly when combined, are universally considered to be the most effective treatment.

Zero water exchange and recirculating aquaculture systems

Zero water exchange shrimp culture system

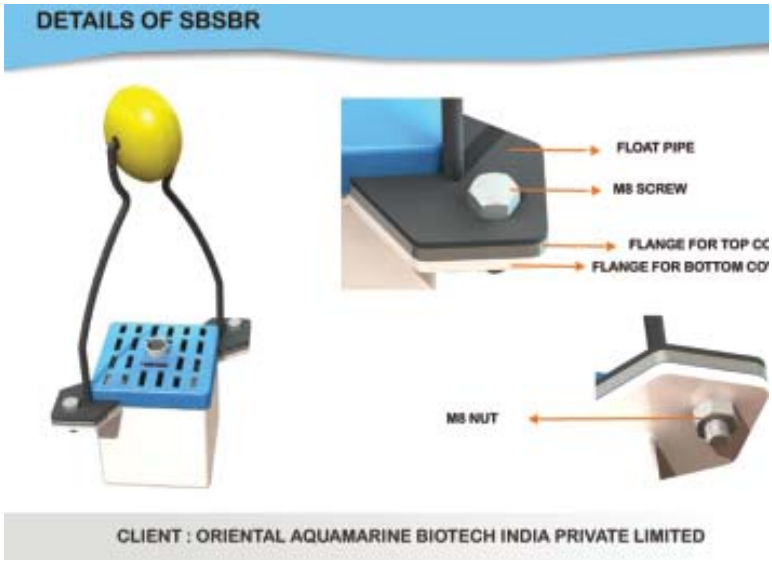
(Integrated Disease Management)

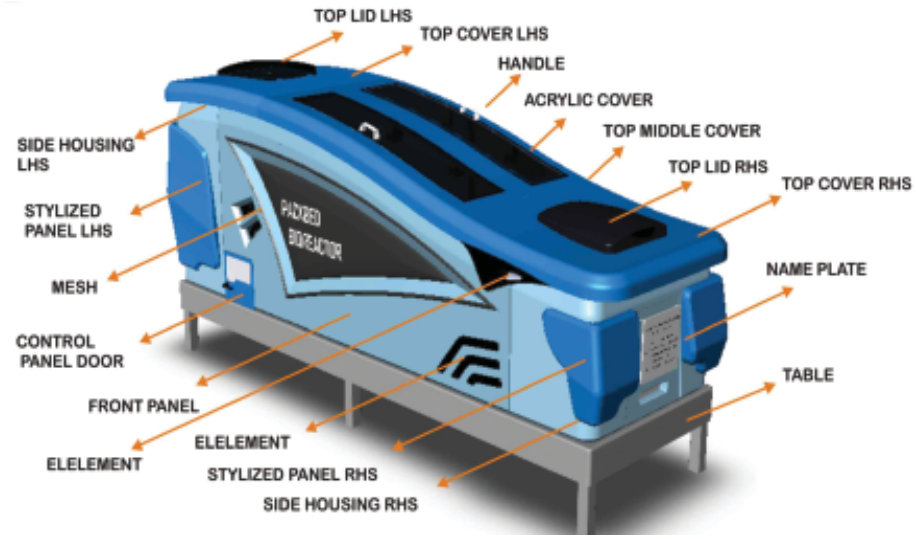
Apart from viral pathogens most of bacterial fungal and protozoan pathogens are opportunistic which require conditions unfavorable for shrimps to cause infection. There fore an appropriate disease management strategy should be evolved to implement at field conditions integrated with the disease management practice. This include selection of disease free (pathogen free) seed from disease free broodstock. Development and maintenance of pond conditions having the parameters within the range, implementation of bioremediation for active removal of detritus from pond bottom, unionized ammonia from the water column and lessening the formation of hydrogen sulfide from the sediment and bioremadiation of the same. Prominent environmental quality parameters to be managed are dissolved oxygen (>3.0 ppm.) alkalinity (50-80 ppm.) unionised ammonia (<0.01 ppm.) nitrite (<0.1ppm.) H_2S (< 0.03ppm.), pH 7-8.5 soil E*h* (> -100mv) and sufficient phytoplankton bloom to have sechi disc reading of about 30 cms. Apart from this, application of an appropriate gut probiotics antagonistic to vibrios, vitamin c, prior to molting, digestive enzymes after molting, feeding calcium and magnesium prior to harvest are the major management measures to be adopted for preventing

the occurrence of diseases in grow out system. The inevitable component of the management strategy is the routine health assessment of the stock.

Recirculating Aquaculture Systems integrated with nitrifying bioreactors for brood stock development and seed production

Nitrifying bioreactors have been developed for the establishment of recirculating aquaculture system for finfish and shell fishes. The patented technology has been commercialized through M/S Oriental Aquamarine Biotech India Pvt Ltd, Coimbatore. There are two categories of reactors named as Stringed Bed Suspended Bioreactor (SBSBR) suitable for water holding capacities ranging from 0.5 to 1.0 tonne capacity and Packed Bed Bioreactor (PBBR) having a capacity to nitrify 60 tonnes water per day. Activated reactors are supplied for fresh, brackish and marine aquaculture systems.





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In vitro Culture of Finfish Cells - Principle and its Applications

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Introduction

Vertebrate cell cultures are in vitro models. The term in vitro refers to keeping entities of an organism outside the living body in an artificial environment, in contrast to in vivo, i.e. in the organism. Vertebrate cell lines arise from primary cultures. A primary culture starts from cells, tissues or organs taken directly from organisms. If a primary culture can be divided into new culture vessels and successfully propagated, it becomes a cell line. A cell line may be propagated a limited number of times, in which case it is finite, or indefinitely, in which case it becomes an immortal or continuous (or permanent) cell line.

Although animal cell culture was first successfully undertaken by Ross Harrison in 1907, it was not until the late 1940's to early 1950's that several developments occurred that made cell culture widely available as a tool for scientists. First, there was the development of antibiotics that made it easier to avoid many of the contamination problems that plagued earlier cell culture attempts. Second was the development of the techniques, such as the use of trypsin to remove cells from culture vessels, necessary to obtain continuously growing cell lines (such as HeLa cells). Third, scientists were able to develop standardized, chemically defined culture media that made it far easier to grow cells. These three areas combined to allow many more scientists to use cell, tissue and organ culture in their research. During the 1960's and 1970's, commercialization of this technology had further impact on cell culture that continues to this day. The overall result of these and other continuing technological developments has been a widespread increase in the number of laboratories and industries using cell culture today.

Fish cell line : Fish cell lines being of poikilothermic origin, grow well at room temperatures without the need of thermo regulated incubators, furthermore, an amino acid-rich nutrient medium such as Leibovitz- 15 that does not require CO_2 buffering has been successfully used with fish cell lines, thus CO_2 incubators are not necessary and cells can be grown conveniently in any undisturbed areas. Additionally, because of lower metabolic rates than eurythermic cells, fish cells can be maintained with little care for long periods of time.

In 1962, the first teleost cell line was reported in the literature. The first continuous fish cell culture (RTG-2) was originated over 20 year ago from rainbow trout gonad tissue. Subsequently, many other cell lines of poikilothermic origin have been developed. Most fish cell lines have been

established and utilized for isolating, identifying, and studying viruses that cause economically important diseases. Consequently, the majority of these cell lines originate from species that are artificially propagated to some extent. Moreover, most such fish cell lines have been developed in North America or Europe. Nowadays, more fish cell lines are available from fishes indigenous to Asia, since aquaculture and fish farming are pursued on a large scale in this part of the world.

Wolf & Mann (1962) enumerated 61 cell lines originating from 36 fish species, representing 17 families. These cell lines were chiefly used for viral diagnostic purposes, and many had not been well characterized or previously reported. Fryer and Lannan (1993) have compiled a new listing of the fish cell lines reported in the literature that have been at least partially characterized. Recently Lakra et al (2010) made a comprehensive review on the characterized fish cell lines of both freshwater and salt water that have been developed after 1993.

Most fish cell lines originate from normal tissues, and embryos or normal fins are most frequently listed as the source of the tissue used in the primary culture. However, few cell lines were initiated from fish tumors, and in some cases, these cells remained tumorigenic *in vivo* following repeated in vitro passage. Traditionally, the chief uses of these cell lines were for detection and study of fish viruses and for diagnosis of the diseases caused by these agents. Today, fish cell cultures are increasingly utilized in research unrelated to disease, and with the recent identification of rickettsial fish pathogens, the diagnostic role of cultured fish cells has also expanded. Along with the multiplying uses of fish cell culture is a concomitant increase in the need for guidelines for the health and maintenance of fish cell lines.

Fish Cell Culture characteristics : The physiology and the blood plasma constituents of teleost fishes are very much like those of terrestrial vertebrates; therefore the methodology for culture of cells and tissues is also similar. Most fish cell lines are readily propagated in vitro using unmodified media and techniques developed for mammalian cells, with appropriate adjustment of incubation temperatures to reflect the temperature range normal for the donor fish species. Also, osmolarity of the media must be adjusted upward for fishes of marine origin. Most important, fish tissue culture often requires less time for preparation and maintenance. Mammalian cell culture techniques need only be modified to reflect the lower incubation temperature requirements and the slower replication rates of the poikilothermic cells.

Appropriate incubation temperatures for cultured fish cells correspond to the normal temperature range of the fish species from which the cell line is derived. For lines from coldwater fishes, incubation temperatures range from 4-24°C with an optimal range of 15-21°C. For lines from warm water fishes, incubation temperatures range from 15-37°C, and the range of optimal incubation temperatures is 25-35°C.

Cell Culture Systems : Once in culture, cells exhibit a wide range of behaviors, characteristics and shapes. Some of the more common ones are described below. Two basic culture systems are used for growing cells. These are based primarily upon the ability of the cells to either grow attached to a glass or treated plastic substrate (Monolayer Culture Systems) or floating free in the culture medium (Suspension Culture Systems).

Types of Cells : Cultured cells are usually described based on their morphology (shape and appearance) or their functional characteristics.

There are three basic morphologies:

Epithelial-like: cells that are attached to a substrate and appear flattened and polygonal in shape.

Lymphoblast-like: cells that do not attach normally to a substrate but remain in suspension with a spherical shape.

Fibroblast-like: cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures.

Endothelial cells: Endothelial cells are very flat, have a central nucleus, are about 1-2 µm thick and some 10-20 µm in diameter.

Other types: Macrophages, neuronal cells, melanocytes, etc.

It is important to remember that the culture conditions play an important role in determining shape and that many cell cultures are capable of exhibiting multiple morphologies.

Development of cell line

Primary Culture : There are several ways with which monolayer cultures of fish cells may be initiated. This is a quick method that employs multiple explants of tissues of either fresh water or marine fish as the simplest way to produce monolayer cultures. When cells are surgically removed from an organism and placed into a suitable culture environment, they will attach, divide and grow. This is called a Primary culture. There are two basic methods for doing this. First, for Explant Cultures, small pieces of tissue are attached to a glass or treated plastic culture vessel and bathed in culture medium. After a few days, individual cells will move from the tissue explant out onto the culture vessel surface or substrate where they will begin to divide and grow.

The second, more widely used method, speeds up this process by adding digesting (proteolytic) enzymes, such as trypsin or collagenase, to the tissue fragments to dissolve the cement holding the cells together. This creates a suspension of single cells that are then placed into culture vessels containing culture medium and allowed to grow and divide. This trypsinization method describes warm (1-2 hrs at 37^0 C) and cold (4 ~ to 6~ overnight (16 hr) trypsinization of fish tissues which yields cultivable cells and small aggregates of cells for monolayer cultures. The disaggregated cells obtained by this procedure generally yield more uniform monolayer more quickly than do cultures initiated with minced tissues alone. This method is called Enzymatic dissociation.

Before starting the preparation of primary cultutre, food should be withheld from donor fish for a day or more before use. Healthy specimens free of external lesions are preferred; otherwise there is risk of encountering systemically disseminated bacteria. Cells and tissues should be cultured at a temperature similar to the environmental temperature preferred by the donor species. Extended exposure of tissues from cold-water fishes such as salmon and trout to 30°C can be lethal. In contrast, many and perhaps most fish tissues remain viable even if held for a day or two on ice or at 4°C to 6°C. Internal tissues may be safely removed after thorough topical disinfection; this is conveniently done by total immersion of the fish for several minutes. A solution of liquid household bleach having 500 ppm available chlorine, or a 1:1000 dilution of a quartenary ammonium compound may be used. Excess disinfectant should be rinsed off with chlorinated tap water or sterile water and the surgical site sponged with 70% isopropanol or ethanol. External tissues such as those of fins,

gills, corneas and barbels are severely damaged by such disinfection. Consequently, such tissues should be excised first and decontaminated separately. Immersion for 1 hr in a solution containing 500 IU polymyxin B, 500 µg neomycin and 40 IU bacitracin is suggested, for these are bactericidal antibiotics.

Subculturing : When the cells in the primary culture vessel have grown and filled up all of the available culture substrate (called monolayer) they must be subcultured to give them room for continued growth. This is usually done by removing them as gently as possible from the substrate with enzymes. These are similar to the enzymes used in obtaining the primary culture and are used to break the protein bonds attaching the cells to the substrate. Some cell lines can be harvested by gently scraping the cells off the bottom of the culture vessel. Once released, the cell suspension can then be subdivided and placed into new culture vessels. Once a surplus of cells is available, they can be treated with suitable cryoprotective agents, such as dimethylsulfoxide (DMSO) or glycerol, carefully frozen and then stored at cryogenic temperatures (below -130°C) until they are needed.

Development of continuous cell lines : Some cell lines may give rise to continuous cell lines. The ability of a cell line to grow continuously probably reflects its capacity for genetic variation, allowing subsequent selection. Genetic variation often involves the deletion or mutation of the p53 gene, which would normally arrest cell cycle progression, if DNA were to become mutated, and over expression of the telomerase gene. Possibly the condition that predisposes most to the development of a continuous cell line is inherent genetic variation, so it is not surprising to find genetic instability perpetuated in continuous cell lines. The alteration in a culture that give rise to a continuous cell line is communally called *in vitro* transformation and may occur spontaneously or be chemically or virally induced. Immortalization means the acquisition of an infinite life span and transformation implies an alteration in growth characteristics (anchorage independence, loss of contact inhibition and density limitation of growth) that will often, but not necessarily correlate with tumorigenicity

Many (if not most) normal cells do not give rise to continuous cell lines. Normal human fibroblasts remain euploid throughout their life span and at crisis will stop dividing (around 50 generations), although may viable for 18 months. Human glia cells and chick fibroblasts behave similarly. Epidermal cells, on the other hand, have shown gradually increasing life span with improvements in culture techniques and may yet be shown capable of giving rise to continuous growth. Continuous cell line of lymphoblastoid cells is also possible by transformation with Epstein-Barr virus.

Stem cell cultures

Stem cells have the remarkable potential to develop into many different cell types in the body during early life and growth. Stem cells are distinguished from other cell types by two important characteristics. First, they are unspecialized cells capable of renewing themselves through cell division, sometimes after long periods of inactivity. Second, under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions. In some organs, such as the gut and bone marrow, stem cells regularly divide to repair and replace worn out or damaged tissues. In other organs, however, such as the pancreas and the heart, stem cells only divide under special conditions.

Until recently, scientists primarily worked with two kinds of stem cells from animals and humans: embryonic stem cells and non-embryonic “somatic” or “adult” stem cells. Scientists discovered ways

to derive embryonic stem cells from early mouse embryos nearly 30 years ago, in 1981. In 2006, researchers made another breakthrough by identifying conditions that would allow some specialized adult cells to be "reprogrammed" genetically to assume a stem cell-like state. This new type of stem cell, called induced pluripotent stem cells (IPSCs).

Stem cells are important for living organisms for many reasons. In the 3- to 5-day-old embryo, called a **blastocyst**, the inner cells give rise to the entire body of the organism, including all of the many specialized cell types and organs such as the heart, lung, skin, sperm, eggs and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

Given their unique regenerative abilities, stem cells offer new potentials for treating diseases such as diabetes and heart disease. Laboratory studies of stem cells enable scientists to learn about the cells' essential properties and what makes them different from specialized cell types. Scientists are already using stem cells in the laboratory to screen new drugs and to develop model systems to study normal growth and identify the causes of birth defects.

Stem cell research is one of the most fascinating areas of contemporary biology, but, as with many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

Characterization of Cell Lines : In contrast to mammalian cells, are easier to maintain and manipulate, and unlike primary cultures, produce highly reproducible results. This ease of handling and simpler growth requirements makes cross-contamination of cell lines a more likely possibility, Proper characterization and identification of the cell lines are hence critical for scientific usage.

Authentication of a cell line is the sum of the process by which a line's identity is verified and shown to be free of contamination from other cell lines and microbes. Tests used to authenticate cell cultures include iso-enzyme analysis, antigenic markers, karyotyping/ cytogenetic analysis and more recently molecular techniques of DNA profiling. Whilst most of the techniques above are generalized tests and are applicable to all cell lines additional specific tests may also be required to confirm the presence of a product or antigen of interest.

Cell line contamination : Cell line contamination is a major drawback of main cell banks of the world and it has cost of losing important biological products or valuable research. The causative agents are different chemicals, invertebrates, bacteria, fungi, parasites, viral species and even other cell lines. Bacteria, fungi, parasite, viruses, invertebrates and mycoplasmas are main causative agents of cell line contamination.

The bacterial and fungal (including molds and yeast) contamination of cell lines (except mycoplasmas) can be readily detected, as these organisms cause increased turbidity, shift in media pH (change in medium color) and cell destruction. Some reports have indicated that putative pathogens such as nanobacteria also will not be detected by this method.

In the case of mycoplasmas their cell line contamination is always undetected for many passages. They can proliferate within the cell, tolerate antibiotics and their growth always does not have any obvious microbial evidence like turbidity and pH changes or cytopathic effect. Their contamination also spreads quickly to the other cell lines

Sources of contamination : Another approach to cell culture contamination is sources of contamination. The sources of microbial culture contamination are different and may be grouped under four subjects.

- Contaminated cells, which are used as the primary starting material for cell culture.
- Glassware or apparatus, including storage bottles and pipettes
- Culture media (serum, basal cultural media containing heat-sensitive essential amino acids and vitamins, enzymes like trypsin, pronase and collagenase, and basic salt solutions).
- Airborne modes which can occur anytime the culture vessel is opened or contact is made with culture fluid through a defective culture vessel, stopper, or poor technique

Cross-Contamination and Misidentification : The problem of intraspecies and inter-species cross-contamination among cell lines has been recognized for half a century. For those scientists working on cell lines derived themselves or received from a colleague, basic authentication tests such as STR profiling, isoenzyme analysis, and contamination tests are readily available and should be routinely used. Transferring cell lines to colleagues should be avoided, or when it does occur, accompanied with comprehensive documentation verifying the integrity of the material or tests need to be repeated. Although cross-contamination of fish cells with other cell types has not been widely reported, conveyed the identification of a cell line dubbed Clone 1A believed to be derived from rainbow trout as being CHSE-214, a cell line derived from Chinook salmon embryos. Accordingly, awareness of good laboratory practices and careful vigilance with fish cell cultures as detailed by Lannan should be followed to avoid confusion of cell lines.

Applications of cell culture

Fish cell lines have been useful in many areas of research. Originally developed to support the growth of fish viruses for studies in aquatic animal viral diseases, fish cell lines have grown tremendously in number covering a wide variety of species and tissues of origin and an array of applications. Fish immunology, physiology, genetics and development, toxicology, ecotoxicology, endocrinology, biomedical research, disease control, biotechnology and aquaculture are some of the areas in which fish cell lines have made significant contributions

Toxicity Testing : Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver- and kidney-derived cell cultures.

Cancer Research : Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. Since, the normal cultured cells could be induced into cancer cells, the mechanisms that cause the change can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

Virology : One of the earliest and major uses of cell culture is the replication of viruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as basic research into how they grow and infect organisms**.**

Cell-Based Manufacturing : Cultured cells can be used to produce many important products, like viral vaccines, genetically engineered protein of medicinal and commercial value and replacement of tissues and organs.

Genetic Counseling : Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

Genetic Engineering : The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biologists wishing to study the cellular effects of the expression of theses genes (new proteins).

Gene Therapy : The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then "infect'' the patient with the virus in the hope that the missing gene will then be expressed in the patient's cells.

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Microbial Safety of Fish and Fishery Products

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Fish is a highly perishable commodity. Spoilage of fish begins as soon as the fish dies. In tropical conditions, fish spoils quite rapidly, within a few hours of landing, if not preserved properly. The spoilage rate of fish can be reduced by good handling practices and effective temperature control from the very beginning. In raw fish, spoilage takes place mainly due to three reasons viz., (1) enzymatic action, (2) chemical spoilage, and (3) microbial spoilage. Among these three the microbial spoilage is very important and described in detail.

Microbial spoilage

The major cause of fish spoilage is bacteria, particularly in the case of marine fishes. The body fluids and flesh of freshly caught fish are free from bacteria except when the fish has bacterial disease. The bacteria present on skin, adhering slime, gills and intestine is normally saprolytic. Once the fish is dead, the bacteria invade the fish tissues causing spoilage and production of undesirable compounds. The type of bacteria on fish is very much dependent on the microbial flora of the environment.

Two types of microorganisms are of concern

1. Saprophytic or spoilage bacteria
2. Pathogenic bacteria

Spoilage bacteria

The important classes of spoilage organisms found in tropical species are *Pseudomonas, Flavobacteria, Acinetobacter, Aeromonas* and *Moraxella.* The spoilage bacteria are characterized by their ability to produce hydrogen sulphide (H_2S), reduce trimethyl amine oxide (TMAO) to trimethyl amine (TMA) and convert urea to ammonia. Many volatile compounds are also produced by *Pseudomonas* group of bacteria. The quantitative measurement of these compounds indicates the degree of spoilage. In the freshly landed fish total bacterial counts are in the range of 10^3-10^6 cfu/g and when the counts rise above 10^7 cfu/g, fish flesh starts visibly to spoil.

Pathogenic bacteria

Pathogenic bacteria associated with seafood are of two types

a. Indigenous bacteria
b. Non-indigenous bacteria

Indigenous bacteria

They are widely distributed in the aquatic environment. These pathogens occur in minimal numbers, and are not a serious problem in fresh fish. However, their growth and multiplication in seafood is a serious problem and cause illness. The important examples of this class are *Clostridium botulinum Vibrio* and *Aeromonas.*

Non-indigenous bacteria

Non-indigenous bacteria enter in seafood through external contaminants. The main sources include polluted aquatic environment, sewage, excreta from animals, birds, human beings, workers handling the material as well as the surface and environment where the seafood is processed. The examples of this class of bacteria are *Salmonella, Shigella, Escherichia coli,* and *Staphylococcus aureus.*

Factors influencing growth of different classes of bacteria

Bacteria are single-celled (unicellular) microorganisms widely distributed in nature belonging to the kingdom Prokaryote. The most outstanding characteristic of the organisms belonging to this kingdom is that the genetic material is not bound by a membrane. Bacterial growth is markedly influenced by many factors such as temperature, pH, salinity, oxygen content etc.

(1) Temperature

Each bacterial species has temperature range in which it has optimum growth. Also, there is a minimum temperature below which the bacterial growth does not takes place and a maximum temperature above which bacteria cannot grow. The optimum temperature is the most favourable temperature for growth. Depending on their temperature preference bacteria are broadly divided into three groups.

Psychrophilic bacteria : They are cold loving bacteria. They grow usually between temperature ranges of 0-20^{o}C, the optimum being 15^{o}C. This group includes most of the bacteria causing spoilage of refrigerated or iced food items. The examples of this class are *Pseudomonas, Alteromonas,* and *Moraxella*

In actual practice, truly psychrophilic bacteria are not usually encountered. These cold loving bacteria usually have a growth temperature range of 0-35^{o}C and hence also called as psychrotrophic bacteria.

Mesophilic bacteria : Majority of the bacterial species belong to this group. They grow within the temperature range of 20-45^{o}C with an optimum of 30-37^{o}C. Most of the pathogens belong to this group. The examples of this class are *Salmonella, Vibrio,* and *Streptococcus.*

Thermophilic bacteria : Bacteria which grow best at higher temperatures come under this group. Their growth temperature range is 45-70^{o}C, the optimum being 55^{o}C. Bacteria belonging to this group are quite rare. Such bacteria are found in natural hot springs. Certain bacteria causing spoilage of canned foods belong to this group. The examples of this class are *Bacillus coagulans, B. stearothermophillu* etc.

(2) Oxygen / air

Depending upon the requirement of oxygen or air for growth, bacteria are divided into 4 groups viz., *aerobic bacteria (*Bacteria requiring the presence of free oxygen or air for growth are called

aerobes. e.g. *S. aureus),* anaerobic bacteria (bacteria which can grow only in the absence of oxygen or air. e.g. *Clostridium* sp), facultative anaerobic bacteria (bacteria growing in the presence and absence of free oxygen), and microaerophilic bacteria (bacteria grows in the presence of very little free oxygen, e.g. *Lactobacillus, Streptococcus*).

(3) Salinity and pH

Salinity and pH are the primary physicochemical parameters controlling bacterial growth and have a marked effect on the maximal proliferation of bacteria.

Majority of the bacterial flora of marine fishes are gram negative, non-spore forming rods/cocci whereas gram positive in freshwater fishes. Brackish water fishes have both gram negatives and gram positives bacteria. Bacterial count from skin, gills and intestine of fish varies with the geographical origin of fish. The body fluids and flesh of freshly caught fish are mostly free from bacteria but saprophytic bacteria are usually present on the skin, gills and intestine. Soon after death these bacteria invade the fish tissue and leads to the spoilage of fish. There are three main routes for this attack.

1. From the gills into the flesh through the vascular (circulatory system)
2. Through the skin by penetration
3. Through the peritoneal lining, from the intestinal lining

Invasion of bacteria through the first and second routes is faster. Entry through the peritoneal lining can take place only after perforation of stomach and intestinal walls, which normally takes longer time. When left in ambient temperature, which is usually $28\pm4^{o}C$ tropical fishes get spoiled within 6-12 hours, depending on their size. In order to prevent such spoilage, many methods are in practice. Drying, icing, freezing, canning and smoking are some of the preservation methods followed in fish processing industry.

(A) Drying : A minimum level of water should be present in the medium/ substrate allowing the bacteria to grow. Such available water in foods or other substances is described by the term water activity (aw). Below a minimum level of water activity, microorganisms cannot grow. Most of the fish spoilage bacteria do not grow below aw of 0.91. During the drying of fish, water is removed from the fish muscle to an aw of 0.9 or below so that bacterial action is completely prevented.

(B) Icing : Icing is the most prevalent method of preserving fish. Ideal icing involves packing crushed ice and fish in layers in insulated boxes, in the fish to ice ratio of 1:1 (w/w). By this, the temperature of the fish is lowered to near $0.1^{o}C$ in about 2-3 hours. Reduction in temperature arrest most of the enzymatic changes and kills 50-60% of mesophilic bacteria. It also reduce the activities and growth of all other bacteria, those are cold loving and cold tolerant. As a combined effect of all these 3 factors discussed earlier, the spoilage of fish will be delayed to a considerable duration in ice. During iced storage of fish, there is an initial drop of bacterial count due to the death of the bacteria; however, some group of bacteria may get adapted and survive at low temperature. Consequently, there will be gradual increase in bacterial count, which may takes 6-8 days to reach one million/gm or above and the fish may reach to the incipient spoilage stage. In tropical fishes, psychrotophs are the actual spoilers during iced storage than psychrophiles. These psychrotophs, whose population is very low in the fresh tropical fish, easily adapt to grow at the low temperature in ice and may

flourish very rapidly, leads to the spoilage of fish. Further, psychrotrophs have a shorter generation time compared to psychrophiles.

(C) Freezing : Freezing of fish is done at -40°C, and the frozen fish is stored at -18 °C to -20 °C. During freezing, 80-90% of the Gram negative bacteria die out, and the residual bacterial can not grow in the frozen storage. But, before cooking, the frozen fish has to be thawed. During the thawing process the residual bacteria, which are predominantly Gram positive, can cause spoilage of the thawed fish. Hence, frozen fish will have to be thawed within the shortest possible time.

(D) Salt curing and fermentation : There are essentially two types of products where the preservative action of salt is the predominant process, dry salted and wet salted or pickled fish products. Dry salting is used only for non-fatty fish. There are two types of spoilage of this product. One is growth of the extremely halophilic bacteria which causes a condition known as “pink”. These pink halophilic bacteria (*Halococcus, Halobacterium*) are strongly proteolytic and produce off-odours and flavours in the product. The other type of spoilage is moulding by a highly osmophilic type of fungus known as “dun” (*Sporendonema and Oospora*). Wet salting is used for fatty fish species such as herring and anchovy. The fishes are mixed with salt and kept in a closed container. Three types of spoilage are known for these products characterized by the presence of sour, sour/sweet and putrified off-odours and off-flavours. This type of spoilage is caused by growth of Gram negative, halophilic, obligate anaerobic rods. The second type of spoilage is characterized by the development of fruity off-odours and is caused by growth of osmotolerant yeast species. Third type of spoilage is caused by a Gram negative, halophilic, aerobic, non-motile, rod-shaped *Moraxella* like bacteria.

(E) Canning : The canning process is a sterilization technique that kills microorganisms already present on the fish, prevents further microbial contamination, and inactivates degradative enzymes. In this process fish are hermetically sealed in containers and then heated to high temperatures for a given amount of time. Canned fish can be stored for several years. However, sterilization does not kill all microorganisms, and bacterial growth and gas production may occur if the products are stored at very high temperatures. Fish is a low acid food and can be processed safely only at temperatures reached in a steam pressure canner. Failure to heat process fish at 240° F or higher may allow spores of the dangerous heat-resistant bacteria, *Clostridiurn botulinum,* to survive, germinate, and grow. The poison produced by botulinum bacteria cause botulism, a deadly food poisoning.

E) Smoking : Smoking or smoke curing of fish is a method of preservation effected by a combination of drying and deposition of smoke constituents. When fish is smoked it is subjected to four basic treatments viz., brining, drying, smoking and heat treatment. Formaldehyde, acids and phenols are the important constituents of smoke involved in smoke curing of fish. Among these, phenolic constituents are supposed to be the most effective in preserving fish.

Quality control and its maintenance are the most important criteria for all the processed and preserved seafood. Quality control in seafood means all the steps taken between harvesting and retail trade to protect the quality of the final product. Many advanced and developing countries have already adopted the Hazard Analysis Critical Control Point (HACCP) concept for the seafood processing. With a view to harmonizing the various quality management systems the International Standards Organisation has developed a set of standards called ISO 9000 series. The HACCP

concept can become a part of these standards. The HACCP concept proposed by the US FDA has been taken as a standard process control system for assuring food safety by international bodies. It has been identified as the global unified quality assurance system for producing safe and better quality fish products at a global level.

(A) The main elements of the HACCP system are:

1) Identify potential hazards AND TO Assess the risk of occurrence.
2) Determine the Critical Control Points (CCPs)
3) Establish criteria to be met to ensure that each CCP is under control.
4) Establish a monitoring system.
5) Establish corrective action when CCP is not under control.
6) Establish procedures for verification.
7) Establish documentation and record-keeping

(B) Application of HACCP system in fishery harbor

HACCP concept is elaborated to the fishery harbors and landing centers for ensuring seafood safety. The main elements of it are:

1) Landed fish should not be exposed to the sun and should be iced.
2) Inspect fish for appearance and odour and reject fish of unacceptable quality.
3) Periodically perform bacteriological tests on representative samples.
4) Follow a cleaning schedule for all work areas and surfaces, using water containing 5 to 10 ppm of free chlorine.
5) Remove all fish slime and blood by hosing down with chlorinated water. At the end of the day, rinse all surfaces with clean water having 5 ppm of chlorine.
6) Apply personal hygiene rules strictly to prevent contamination of fish. Smoking and spitting in work areas should not be permitted. Hands must be washed with bactericidal soap prior to handling fish and after a visit to the toilet.
7) Check that water supply and treatment systems are in order. Water and ice samples should be analysed as per testing schedule by ISO certified laboratories for levels of chemical and bacteriological contamination and potability certificates obtained.
8) The harbour should be free from litter and other wastes.
9) Check to ensure that all drainage systems are in good working order**.**
10) The harbour should be free of animals, rodents and pests.
11) Ensure that there are no bird nests in the fish handling area.
12) Check that wastes are being disposed of sanitarily.
13) Check cold storage equipment to ensure that the right temperature is being maintained.
14) Ensure that all precaution and warning signs are readable

C) The ISO-9000 series certification of the International Standards Organization

For seafood processing establishments, the most relevant standards of the ISO 9000 series are the ISO 9001 and 9002. The former is a quality system standard that lays down requirements for product development, production, delivery and after sales functions. The latter concerns only production and delivery. The ISO 9003 deals with quality system requirements for final inspection and testing.

Microbiological criteria (MC) and testing of quality of fish and fishery products

Traditionally, control of microorganisms in food was demonstrated by microbiological testing of samples at various stages of production and the final product. Results were compared with criteria developed to give some degree of assurance that the food was safe and of good quality. It is now fully recognized that this type of activity can never give an absolute assurance of product quality and safety. A much higher degree of assurance can be provided by a preventative approach based on the application of the Hazard Analysis Critical Control Point (HACCP) principles at all steps in the food supply and processing system. Three types of MC are generally recognized according to their use:

1) Standards
2) Guidelines
3) Specifications

These terms have been defined and redefined a number of times, but it is generally recognized that the term “standard” is a MC contained in a law or regulation with mandatory compliance. A microbiological “guideline” is a MC applied at any stage in food processing and aids in identifying situations requiring actions for food safety or quality reasons. A “specification” is a MC used for contractual purposes by food business as part of their own safety management system and should not be confused with legal requirements.

Microbiological standards to be met

Sampling plan and recommended microbiological limits for seafood (ICMSF 1986)

Product	Test	Case	Plan Class	no. of samples	no. of positive results	Limit per gram or per cm^2	
						Per g	Per cm^2
Fresh and frozen fish	APC	1	3	5	3	5×10^5	10^7
	E. Coli	4	3	5	3	11	500
Precooked breaded fish	APC	2	3	5	2	5×10^5	10^7
	E. Coli	5	3	5	2	11	500
Frozen raw crustaceans	APC	1	3	5	3	10^6	10^7
	E. Coli	4	3	5	3	11	500
Frozen cooked crustaceans	APC	2	3	5	2	5×10^5	10^7
	E. Coli	5	3	5	2	11	500
	S. aureus	8	2	5	0	10^3	-
Cooked, chilled, and frozen crabmeat	APC	2	3	5	2	10^5	10^6
	E. Coli	6	3	5	1	11	500
	S. aureus	9	2	5	0	10^3	-
Fresh and frozen bivalve molluscs	APC	3	2	5	0	5×10^5	-
	E. Coli	6	2	5	0	16	-

Standards and criteria of fish and fishery products under the food sanitation law, Japan.

Classification	Standards and criteria	Remarks
Fish paste products (fish sausage and ham)	-Coliform organism: negative/g	There are also processing & preservation standards
Boiled octopus	- Viable bacteria count: 1.0 x 10^5/g or less- Coliform organism: negative/0.01 g	Only frozen octopus. There are also processing & preservation standards*
Raw oyster for uncooked	- Viable bacteria count: 5.0 x 10^4/g or less- *E. coli* MPN/100 g: 230 or less	-

US FDA Regulatory requirements for pathogens

Parameters/indicators of microbial safety	Bacterial counts
Total viable count	Not to exceed 100,000 per gram
Salmonella	Not to be detected in 25g of meat
Vibrio cholerae	Not to be detected in 25g of meat
Listeria cooked only	Not to be detected in 25g of meat
E. Coli	Less than 10 per gram
S. aureus	Less than 1000 per gram
Faecal coliforms	None

As a relatively “high-risk” perishable food, fish and fishery products are subjected to a range of food safety requirements related to general hygiene and specific microbiological and chemical contaminants. Thus maintenance of microbial quality and ensuring the safety of fish and fishery products at all the levels of production to final consumption is the prime challenge for the fishery industry. Food production and processing sector can start on the journey towards world class quality by building a foundation using the quality tools: ISO 9000, HACCP and Good Manufacturing Practices (GMP).

Suggested Reading

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Laboratory approaches towards disease diagnosis: Histopathology & Parasitology

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Successful diagnosis is the most important step in any disease control programme, the outcome of which determines the ultimate success or failure of the programme. Once an infection or disease is suspected, the next step is to draw a diagnostic procedure, to fix the root cause of the problem.

When and where to start an investigation?

In farm conditions,

- Any mortality greater than 0.3% and less than 0.5% per day should be suspected and investigated.
- Mortality greater than 0.5% but less than 1.5% indicates that a fish health problem/disease is present.
- Mortality reaching/exceeding 1.5% per day should be treated as an epizootic
- The sick animals should be isolated as much as possible to prevent transmission of the disease to other lots and control measures initiated.

Sampling procedures

In clinical cases of disease (0.5% mortality/day) 10 moribund fish or shellfish are generally a sufficient sample size to make a diagnosis. In situations where no excessive mortality or clinical disease is apparent, a larger sample size of 60 animals may be necessary. However, depending upon individual circumstances, sample sizes may vary between 10 and 60.

The diagnostic procedure may include a single diagnostic test or a combination of tests. In the case of routine pathogen watch or health monitoring, a set of selected diagnostic tests are performed to cover the potential pathogens. The approach generally followed is location specific and problem specific, where the first consideration is the availability of the diagnostic facility and expertise. There is no hard and fast method, which can be applied for all cases.

Records/History on	Water source and Pond, Water parametersStocking, feeding, growth performance, handling, Recent pathologies and treatments EpizootiologyThe more precise and consistent the farm records are the more likely it is for the pathologist to reach a sound diagnosis
On site observation of	Water conditions / soil / benthos and plankton samples. Live / sick fish for shoaling behaviour, reactions to stimuli and feeding. Presence of dead

	and moribund fish. External lesions (ulcers, exophthalmus, reddening, fin erosion)
Examination	Gross external examination for parasitesPreparation of smears
Sampling for	Laboratory examination (whole fish, organs) for Bacteriological, fungal examinations Blood tests / Rapid diagnostic testsElectron Microscopy
On site necropsy	Skin, fins, gills, internal organs Sampling for histopathology / virology
Laboratory analysis	Bacteriology, Parasitolgy, Histopatholgy, Serology, PCR / DNA Probes

Importance of documentation/case sheet

Majority of disease problems in fish are linked intimately to water quality and management issues, factors that do not always translate into directly observable pathology. Interpreting the cause of disease based solely on biopsies or necropsy may be misleading. History of disease at the facility or in the region, farm design, source of seed stock, type of feed used, environmental conditions etc. should be recorded. This systematic record keeping will help to arrive at a primary diagnosis but need not be conclusive. Behavioural aspects like movement pattern, feeding pattern, morbidity etc. along with gross clinical signs such as lesions, haemorrhage, colour changes, fouling etc should also be recorded.

Transportation of samples to the lab

As far as possible live fish are preferred and samples should be immediately send after removal from water. If dead, they should be held on ice and processed within 1-2 hrs. Bacteriological samples should be taken first to reduce chances of contamination and virology samples should be processed within 48 hrs.

Laboratory work sheet: A laboratory case/work sheet has to be kept with all the details of the investigation including the case history (See appendix I & II)

Standard Necropsy Procedures for Finfish (adapted from Theodore R. Meyers)

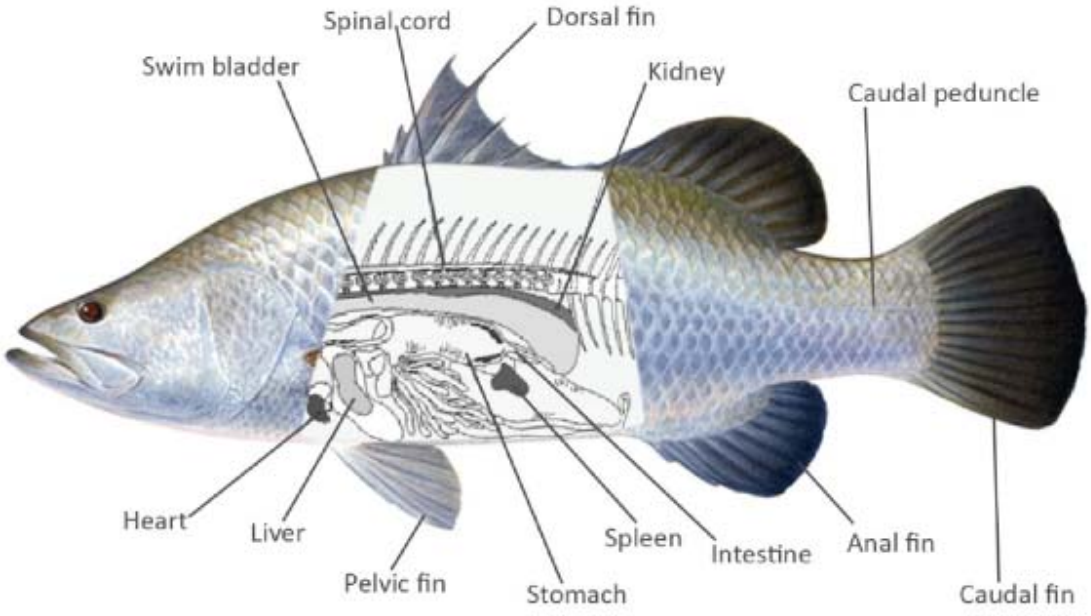
As a first step the sample is given an accession number and the case data information received with the sample should be used to fill out the laboratory worksheet. Live fish should be examined for behavioral abnormalities (spiral swimming, flashing, flared gill opercula, prostration, etc.) then anesthetized to avoid tissue artifacts caused by alternate methods of euthanasia such as pithing or a blow to the head. Some external abnormalities (whitened or eroded fin tips, cloudy cornea, body discoloration, excessive mucus) are best observed while the fish is submerged in water. In many cases postmortem changes in fish received dead will prevent this latter opportunity.

General Necropsy Procedure

- The fish should first be examined for external abnormalities or lesions that could include: poor body condition; exophthalmia; cloudy cornea or lens opacity; hemorrhaging within the anterior chamber of the eyes, fins, body surface or body orifices (anus, nares, mouth, gill chamber), frayed or missing fins; gas bubbles within the fin rays or connective tissues of the eyes; ulcerations, abscesses, abrasions; body discolorations; excessive mucus; trailing fecal casts or rectal prolapse; external foreign bodies such as fungus, metazoan or protozoal parasites,

cysts or tissue growths; potbelly or other protrusion or body malformations (spinal deformities, cranial swelling, shortened opercula, pugheadedness, microeye).

- External lesions such as ulcerations or abrasions should be struck onto TSA (Tryptone Soy Agar) for bacteriological studies. Use of TSA with 1% NaCl may be necessary depending upon case information and whether fish are in saltwater and a halophilic bacterial pathogen is suspected.
- A peripheral blood smear should be made by excising the caudal peduncle (for small fish) and allowing a drop of blood to be deposited near the frosted end of a clean glass slide. The blood is smeared before clotting with a second glass slide by touching the drop with the slide at a 45^{o} angle to the first slide and pushing the angled slide to the end of the first slide. Capillarity draws the smear across the first slide and the smaller the angle the thicker the smear. Stain the smears using Giemsa and observe under the microscope at 1000X. Larger fish may be bled by caudal vein puncture.



- Fish should be placed on their right sides for performance of the remaining necropsy procedures. Skin scrapes of normal and lesion areas mounted with a drop of PBS (Phosphate Buffered Saline) and coverslip on a glass slide should be made by using either the edge of the coverslip as the scraping instrument, or a scalpel. Bacteria or fungus from lesion areas or protozoal parasites such as *Ichthyobodo* and *Trichodina* are common subjects to look for beginning at 40x and then at 200X on a compound microscope.
- Wet mounts of gill filaments are made by using a small pair of surgical scissors to remove a portion of one gill arch. Gill filaments should be slightly teased apart for good viewing of filament and lamellar profiles and mounted in PBS with or without a coverslip. These should be examined immediately since branchial epithelium rapidly deteriorates causing postmortem artifact. Look

for gas bubbles in the capillaries, telangiectasia, hyperplasia, external parasites (bacterial, protozoal, fungal, metazoan), or other foreign bodies.

- Disinfect the outer surface of the fish by flooding with 70% ethanol. Disinfect a pair of scissors, forceps and scalpel by immersion in 100% ethanol and passing the instruments through a Bunsen flame allowing the alcohol to ignite and burn off. Repeat one or two more times. Wipe instruments clean of any organic matter beforehand for effective disinfection.
- The abdominal cavity is entered by pulling the pectoral fin with sterile forceps while cutting into the abdominal wall at the base of the pectoral fin with a pair of small sterile scissors. The cut is continued dorsally to just below the lateral line where resistance is encountered. Start again at the base of the pectoral fin and continue the incision towards the posterior of the fish along the ventral abdominal wall to the vent. Stay slightly above the intestinal tract when making the incision so that it is not punctured, thereby contaminating the tissues. At the vent continue dorsally to just below the lateral line and continue cutting anteriorly to connect with the first incision. Remove the flap of abdominal tissue, thus exposing the internal viscera and cavity. When done correctly on a moribund specimen the air bladder should remain inflated and the GI tract completely intact. Instruments may need wiping of organic material and flaming repeatedly during this procedure.
- Visually examine viscera (heart, liver and gall bladder, kidney, pancreas, adipose tissue, spleen, air bladder, pyloric caeca and entire GI tract) for abnormalities such as: discoloration or mottled appearance; enlargement (hypertrophy); hemorrhage or erythema; abscesses or cysts; fluid in the abdominal cavity (ascites causing potbelly); foreign bodies such as fungus, metazoan parasites or tissue growths, etc.
- If bacteriologic samples are to be taken they should be struck onto TSA from the kidney and/or from visceral lesions before other samples are taken to avoid bacterial contamination.
- Tissues to be taken for viral assay of larger fish (kidney/spleen pool) should also be placed into sterile tissue culture fluid for refrigeration and homogenization at a later time. Fry are generally processed whole for virology
- If the spleen has not been completely removed for virus assay, a spleen squash can be made by placing a cut section of the tissue with a drop of PBS on a glass slide and covering with a coverslip. Whole spleen squashes will be necessary when small fish are examined. Look for the presence of motile or non-motile bacterial rods and fungal hyphae. The coverslip may be removed and the squash Gram stained for confirmation of bacteria as described for gill tissues.
- A squash of lesion material from a visceral organ or organs may be warranted if present and if its cause is not readily discernible. Gram stain and/or Geimsa stain of this material may also be warranted.
- If the cause of mortality or morbidity is in question as to whether or not the above procedures will provide an answer, histology samples should be taken as a backup measure, but only if moribund fish are available. Fish that have been dead for several hours or longer are generally not suitable for histology due to postmortem tissue autolysis. If fry are involved, whole fish may be dropped into Bouin's fixative or 10% buffered formalin. Fingerlings should have the abdomens opened with scissors for better fixative penetration.

- If clinical signs suggest a central nervous system disorder the top of the cranial cavity should be opened and the brain included in bacteriologic sampling using TSA. Heads from additional affected fish should be severed behind the gill opercula and placed into fixative for later histological sectioning of the brain.
- This necropsy procedure should include at least 5-10 moribund or otherwise affected fish. Control or healthy fish should be requested for comparison of whether abnormalities perceived are real or not. The number of control fish processed will depend upon the particular case and may range from 10 to none.

Histopathology procedures for finfishes: Due to the rapid rate of autolysis of fish tissues compared to that of homeotherms, they must be handled rapidly to prevent degenerative changes within the specimen, making ultimate diagnosis either unreliable or impossible. For satisfactory histological preparations, only freshly killed or moribund fish should be considered. Proper fixation is fundamental to all satisfactory histological preparations. The primary objective of fixation is to preserve the morphology of the tissues in a condition as near as possible to that existing during life. The most widely used fixatives are formaldehyde and Bouin's fixative. Small fish/fry can be directly dropped into the fixative while in the case of larger samples, incisions should made into the abdomen so that the fixative reaches the internal organs/viscera.

Histology of Bivalves: Bivalves less than 6 cm in length (shucked) can be fixed whole by dropping into preservative. Animals must be shucked cleanly from the shell by severing adductor muscles prior to fixation. For good fixation, larger bivalves require 3 incisions (anterior, mid, posterior) made across the surface of the animal about mid-way through the tissues. Do not cut completely through the animal so that individual specimens remain intact and tissues do not become mixed. Fixatives used for bivalves are Helly's fixative, Bouin's fixative or Davidson's fixative. After fixation bivalve tissues are firm enough for further processing. Each animal body is cut through the anterior, middle and posterior areas resulting in 4 separate pieces of tissue. One section, about 2 mm in thickness, is shaved with a razor blade from each of the faces of the tissues representing the 3 major body areas. The sections are placed within 1-3 tissue cassettes depending upon their size for dehydration and embedment. Small bivalves can either be embedded whole or cut longitudinally on the median axis and both tissue halves placed face down within a cassette.

Bivalve Larvae: Fix in a test tube of Helly's fixative, then centrifuge @ 1,500 rpm for 10 minutes. Discard supernatant and embed the larvae in an agar plug. Remove the plug from the test tube for dehydration and embedding in wax in the usual manner (trim if necessary).

Histology of Shrimp: The chitinous exoskeleton of shrimp prevents adequate penetration of any fixative by simple immersion. Consequently, the fixative must be injected into strategic internal areas of each animal prior to dropping the whole shrimp into the fixative. Inject fixative into the shrimp using a 10-ml syringe and appropriately sized needle, depending upon the size of the animal (small shrimp; i.e., small-gauge needle). Immediately after injection, slit the cuticle of the animal from the last (6th) abdominal segment to the base of the rostrum. The incision in the cephalothoracic region should be just lateral to the dorsal midline and that in the abdominal region should be mid-lateral. Do not cut too deeply into the underlying tissue. The objective is to break the cuticle to allow fixative penetration. After injection and body incisions, the animal may be dropped into the fixative. Transfer

shrimp to 70% ethyl alcohol after 48 hrs. Commonly used fixatives for shellfish: Davidson's fixative, Buffered formalin & Helly's Fixative.

Always ensure that the volume of the fixative is at least ten times that of the tissues. The fixed tissues are washed, dehydrated in ethanol series, cleared, embedded in paraffin and sectioned at 5-7 µm thickness. The sections are then stained with Haematoxylin & eosin and observed under the microscope.

Routine screeing of fish for parasites

Whenever possible examine fresh material. Fish should be freshly killed, without anaesthetic, and kept moist throughout examination, the reasons being

- Parasites are more easily recognised and identified.
- Parasites, especially ectoparasites, may leave the host or die after death.
- Collection of blood parasites is nearly impossible after death.
- Decomposition starts immediately after death and internal parasites may be destroyed by host's enzymes.

Other important points to be noted are:

- Handle fish as little as possible.
- Kill fish by cutting though cranium or though spinal cord immediately behind the head.
- Fish should be kept WET at all times during examination.
- If the fish is already dead, refrigerate, but keep moist. Do not freeze as most small parasite become unrecognisable and only large helminths and crustacea can be recovered. If examination is to be delayed, fix in 10% Formol saline and slit open the body cavity to allow fixative to penetrate internal organs.
- It is essential to examine skin and gills for ectoparasitic protozoa immediately after death as these may die or leave the host within a short time, e.g. flagellates.

Examination procedure

Kill fish quickly by cutting through the spinal cord with a sharp scalpel in the region immediately posterior to the gills.

Blood can be collected at this stage from the heart of major vessels using a Pasteur pipette. Place a few drops on a slide and allow to clot. A smear can also be made, fixed in methanol for 10 minutes and stained later.

1. Examination of skin

i. Take "scrapings" for high power examination (several, if fish is large). Scrape with a sharp scalpel in an 'anterior to posterior' direction and place mucus and epithelial cells on a slide in a drop of water. Avoid scraping scales as these reduce the visibility of small protozoa. *Thin preparations* are essential. Spread scrapings thinly and cover with a coverslip. Examine under high power.

NB. Scrapings should be made along the dorsum in an anterior to posterior direction including head, from the fins and from any discoloured areas or lesions.

ii. Examine the entire fish under low power using a stereo microcope. Be sure to examine under fins as well as other areas. Large metazoan parasites and *Argulus* can be seen in this way.

2. Examination of gills

i. Remove operculum and examine inside.

ii. Remove a whole gill and place on a slide or in a petri dish (add water if necessary and examine under low power on the stereo microscope. Separate the primary lamellae with needles to observe large monogenea and crustacea. Examine any lesions in detail.

iii. Cut off lamellae and remove gill arch. Place lamellae on a slide and spread thinly, chop if necessary and cover with coverslip. Examine under high power.

3. Other organs

i. Make incision along the ventral region. Remove abdominal wall to expose viscera. Examine visceral surfaces, abdominal cavity and pericardial cavity carefully under low power using stereo microscope. Examine any abnormalities or cysts, spots etc. in detail under high power.

ii. Remove alimentary canal and associated organs by cutting across oesophagus and around anus. Divide alimentary canal into stomach, pyloric caeca, fore,-mid and hind-intestine, and rectum. Examine surface and scrape contents onto a slide. Examine contents under high power. Compress sections of alimentary canal between slides and examine under High Power.

iii. Dissect and make squash preparations from heart, liver, gallbladder, spleen, kidney, gonads, urinary bladder and swim bladder.

iv. Dissect out eyes and open nares. Examine under low Power and high power for helminths. Squash lens and examine for eye flukes. Dissect out the separate tissues of the eyes carefully to determine the location as the site is helpful for identification.

v. Remove skin and slice muscle for helminth larvae and protozoan cysts. Squash muscle between slides or glass plates.

vi. Open cranial cavity, examine and make smear of brain tissue.

Fixation of parasites

The most commonly used fixative for preserving and storing parasites include alcohol-formoi-acetic (AFA or Davidson's Fixative). Bouin's, formalin and glycerine alcohol (Humason 1979). Formalin is probably the most commonly used and preferred fixative. Preservation in cold fixatives is not recommended because most parasites will contract and make identifications difficult or impossible.

Prior to fixation, worms should be thoroughly washed in saline and cleansed of mucus.

Protozoa: For Myxosporea: cut out the cyst with enough adjacent tissue and place in 10% formalin. For trophozoites of motile forms, place as many protozoa as possible on a clean microscope slide, add one drop of PVA-AFA (polyvinyl alcohol-acetic acid formalin alcohol) fixative adhesive, mix. spread over slide and allow to dry. Also, protozoans can be transferred to a vial of 10% formalin (keep in mind they will usually shrink).

Monogeneans/Trematodes: Trematodes should be transferred to a small glass petri dish. Remove excess saline or water. Heat 10% formalin or AFA to 85-90°C (begins to steam but not boil) in a fume hood. Add hot fixative to dish containing trematodes. For thicker worms, flatten under a coverslip and flood with warm fixative. For monogenea, drop infected gills into 10% formalin. Larger monogenea can be removed and fixed under light coverslip pressure.

Cestodes: Procedures are similar to that of trematodes; kill in 80'C water or formalin and store in buffered 10% formalin.

Nematodes: Kill in warm (80^0C) glycerine alcohol (1 part glycerine:3 parts 95% ethanol) and transfer to cold glycerine alcohol for storage.

Acanthocephala : For acanthocephalans it is necessary to evert the proboscis prior to fixation. Place worms in distilled water and refrigerate overnight. Transfer to warm 10% formalin or AFA.

Leeches: Fix in warm 10% formalin, or if very thick, flatten between two slides and flood with 10% formalin.

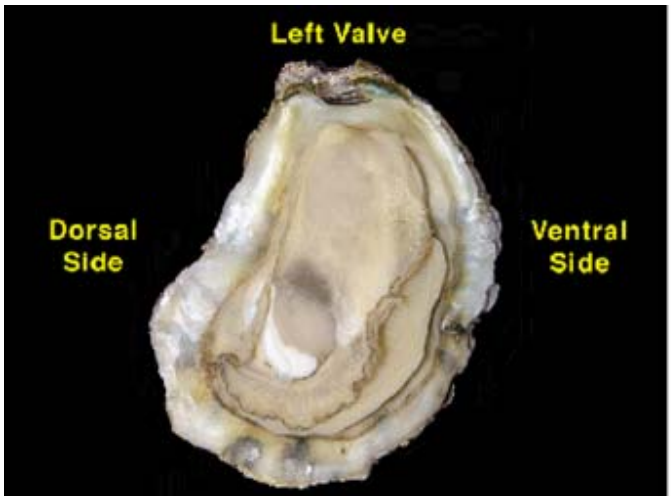
Copepods: Remove copepods. if possible, and drop into glycerin alcohol or 70% ethanol. If not easily detached, cut out a small piece of tissue containing parasite and place m 10% formalin or 70% ethanol.

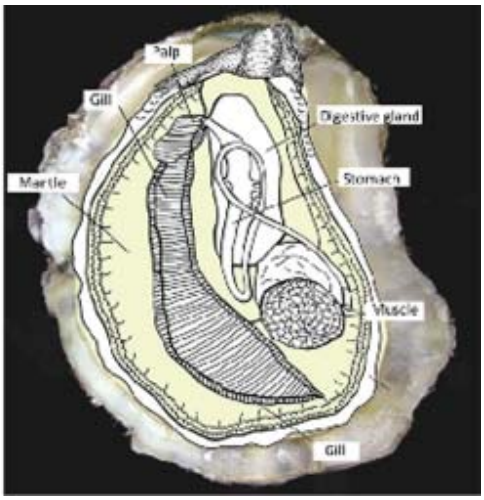
Routine screeing of bivalves

Oysters can be transported live from the collection site to the laboratory by keeping them wet by covering with a wet gunny cloth or cotton. In the lab, detach oyster individually, clean the sediment and debris from the shell by washing under tap water.

Orienting and opening the shells: Shell is composed of two unequal valves; the upper, flatter right valve and the lower, concave left valve which is cemented to the substratum and holds the animal. Orient the oyster pointing the anterior end (hinge region) away from you. Right valve or upper valve is opened by inserting a knife and cutting the adductor muscle. Detach the right valve leaving the soft tissue in the left valve.

Locating the organs: Tissue layer above is the right mantle skirt, the water space enclosed between the two mantles is the mantle cavity. Posterior adductor muscle can be observed in the





mantle skirt. Lifting the right mantle exposes the gills. The pericardial cavity and the heart lie anterior to the adductor muscle. On the ventral side (on our right) gills labial palp mouth etc can be located and on the dorsal side (on our left) rectum and anus. Visceral mass fills the space between hinge and the adductor muscle. In reproductive individuals gonads fill space in the visceral mass between stomach and digestive ceca.

The condition of the oysters are assessed by observing their general appearance, fouling, shell damage, presence of abnormalities, gaping, retraction of mantle, wateriness of the tissues, abnormal coloration, presence of abscess, lesions, pustules, tissue discoloration etc.

Morphometry and condition index (CI): general health and physiological state of the oyster can be assessed by the CI value. Morphometric features like dorso-ventral (length), anterior-posterior (height) and width (thickness) measurements are taken along with the total (shell on) weight, tissue weight and shell weight. Several formulas are in use for assessing the CI value like

CI= (dry soft tissue weight (g)*1000)/internal shell cavity volume (ml)

CI=Tissue weight (g)/(length(mm)*height(mm)*width(mm)*1000)

Tissue condition can be graded according to the table below

Condition index	Code No	Oyster appearance
Very good	1	Animal firm and filling shell cavity; creamy white and evenly textured; usually ready to spawn
Good	2	Not quite as firm or large as above; usually ready to spawn
Good minus	3	Coloration less opaque, often slightly yellow or grey
Fair plus	4	Animal distinctly not filling shell cavity; coloration often mottled, with blood vessels and muscle fibers showing through the more translucent epithelium
Fair	5	Oyster well developed but not opaque or tending towards white; grayish and translucent; flesh flaccid
Fair minus	6	Translucency more pronounced
Poor plus	7	Oyster not well developed, darker grey, often greenish ;pericardial cavity clear ;small portion of shell cavity filled
poor	8	Negative qualities more accentuated
Very poor	9	Animal distinctly atrophied; coloration dark and uneven, very translucent ;seldom more than third of shell cavity occupied; adductor muscle often discolored and transparent even in the normally white sector

Pathological observations

Shell observation: External shell is observed and the presence of foulers and borers are noted. *Polydora* is the major shell borer encountered and, barnacles, ascidians, mussels etc are

frequently observed foulers. Inside shell is viewed for any calcareous growths or discolorations. Heavy *Polydora* infections produce black discoloration, brittleness, foul smell and mud blisters are also visible in certain cases.

Tissue observation: the general appearance and condition of the tissue is noted. Usually a creamy white plumpy appearance observed in case of good condition and reproductive phase. But tissue appear dirty white and watery when it is in spent condition or diseased.

The mantle cavity of the animal is examined for the presence of parasites/ commensals. Mantle fluid is collected using a Pastuer pipette from the mantle cavity and a drop is placed on a clean alcohol wiped slide and observed under microscope after placing a cover slip. A number of protozoan ciliates and other algae etc can be found. Small pieces of mantle, gill and digestive gland tissues are squashed and examined for the presence of parasites.

Collecting hemolymph: After shucking the oyster excess mantle fluid is drained off. Using a sterile syringe (1ml, 27 gauge) 1-2 ml hemolymph is collected either from pericardial cavity or from the adductor muscle sinuses. Hemolymph collected is stored at 4^0c until observation. Hemolymph is mixed with May Grunwald's eosin-methylene blue solution and kept aside to stain the cells and later observed under microscope. There are three major types of hemocytes in oyster namely granulocyte, semigranulocyte and agranulocyte which can be identified after staining.

Ray's Fluid Thioglycollate Medium (RFTM) Culture for detecting Perkinsus infections:

Tissue samples from gill and mantle measuring approximately five to 10 mm are excised and placed in separate 30 ml tubes containing 15 ml fluid thioglycollate medium supplemented with 500 ìg ml^{-1} streptomycin and 500 U ml^{-1} penicillin-G potassium. The tubes were incubated at 24°C for 7 days, in the dark. After incubation, the fragments of tissue from each tube are collected, placed on a glass slide and macerated along with a drop of Lugol's iodine solution. The preparation was covered with a cover-slip, allowed to sit for 10 minutes and examined under the microscope.

Molecular diagnostic studies: Bivalve tissues for molecular diagnostic studies are fixed in 95% ethanol and stored under refrigeration. DNA extraction done using standard phenol/chloroform protocol followed by ethanol precipitation. This will be followed by PCR screening for major pathogens viz *Perkinsus.*

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Electron Microscopy in Disease Diagnosis

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Understanding the pathogen and the pathogenesis at cellular levels are imperative in the studies of disease causing organisms. With its very high resolving and magnifying powers, Electron Microscopy has opened up new vistas in studying the ultra structure and has become an indispensable tool in understanding many of the diseases and their etiological agents. The limitations of Light Microscopes, low magnifying and resolving powers (1000 x magnification and a resolution of 0.2 micrometers) paved the way for the development of electron microscopes. Electron Microscopes are instruments that use a beam of highly energetic electrons to examine objects on a very fine scale and function exactly like their optical counterparts. Present day electron microscopes are capable of giving magnifications up to 1000000 X and 800000 X and a resolving power of 0.1 nm and 0.4 nm in T E M and S E M respectively. Transmission electron microscopy (TEM) can reveal the ultra structural details at cellular levels, whereas Scanning electron microscopy (SEM) can show the morphology of minute structures/organisms in its three-dimensional state. Combining the TEM and SEM, it has become possible to study and classify the viruses and virus like organisms.

Commonly employed methods for disease diagnosis include histology, serology, microbiology, molecular diagnostics and electron microscopy and each method has its own advantages and disadvantages.

Pathogens	**Size**	**Microscopy**
Helminth	mm - cm	Light microscopy
Helminth eggs	50 mm and above	Light microscopy
Fungi	5 mm and above	L M & E M
Protozoa	2 mm and above	L M & E M
Bacteria	0.2 mm and above	L M & E M
Rickettsias	0.3 – 0.6 mm	L M & E M
Virus	0.01 – 0.4 mm (10 – 400 nm)	Electron microscopy

Among these diagnostic techniques, electron microscopy remains the most important tool to establish a viral etiology in the case of disease outbreaks without any previous history, and stands out as the only technique, which can visualize and record viral pathogenesis at cellular levels.

Histology uses light microscopy and is still an invaluable tool in disease diagnosis. It does not require sophisticated instruments and is useful in many disease conditions. However, misleading

observations may make confirmatory diagnosis difficult. In the case of viral infections, one can find lesions or inclusions, which are only suggestive of a specific viral infection through histopathology. TEM provides information about the morphology of pathogens, sub cellular changes / particles / structures etc. Moreover, due to the limited magnification and resolution, ultra structural / sub cellular changes and minute pathogens/stages cannot be observed.

Sero-diagnostic methods play an important role in disease diagnosis, especially in field conditions. Serology still remains the mainstay of viral diagnosis. The tests are normally based on specific antibodies (immunoprobes) and can detect sub clinical / latent / carrier states of infection. However, the draw backs of serological tests are (a) highly variable sensitivity & specificity (b) many viruses often produce clinical disease before the appearance of antibodies (c) Less useful in the case of latent viruses (d) antigenic cross-reactivity between related viruses may lead to false positive results and (e) less effective in invertebrates which does not produce antibodies.

Microbiological methods are widely used for the diagnosis of bacterial infections and involve culture, isolation and identification of the pathogens. But the procedure is tedious and time consuming and may even take weeks in some cases.

Molecular biology tools involve the detection of genetic material of pathogens using molecular probes. Advantages of Molecular tools include (a) extremely high sensitivity (b) easy to set up and (c) fast turnaround time. Disadvantages are (a) expensive (b) extremely liable to contamination (c) high degree of operator skill required (d) quantitative assay difficult and (e) difficulty in interpreting positive results, especially with latent viruses and (f) though they are more sensitive, are only capable of identifying the presence of genomic material for previously identified agents.

Electron microscopy can be an important adjunct to conventional culture and serologic techniques in diagnosing viral illnesses. Though detection of viruses by E M requires relatively large numbers of virions, and provides no information regarding specific serotypes within a virus family, it has the distinct advantages of being simple and rapid. Also, infectious particles are not required. Some viruses do not grow in tissue culture or grow only after special manipulation, and may not survive if transportation conditions to the lab are not optimal. Naturally, culturing would miss these agents. Additionally, a wide variety of agents can be visualized by E M; because specific reagents such as antibodies, antigens, or nucleic acid and protein probes are not required, one is not limited to the availability of these reagents, and prior knowledge of the virus identity for reagent selection is not required. Diagnostic electron microscopy has two advantages over enzyme-linked immunosorbent assay and nucleic acid amplification tests. After a simple and fast negative stain preparation, the undirected, “open view” of electron microscopy allows rapid morphologic identification and differential diagnosis of different agents contained in the specimen.

The biggest advantage of electron microscopy lies in the fact that it provides direct visual evidence of various pathogens/biological processes, while most of the other techniques are indirect and in some instances non-specific. Electron microscopic diagnosis is uniquely suited for rapid identification of infectious agents. A specimen can be ready for examination and an experienced virologist or technologist can identify, by electron microscopy, a viral pathogen morphologically within 10 minutes of arrival in the electron microscopy laboratory. Once the histopathological observations using light microscopy provides primary information on the target tissues, electron microscopy can

be employed to visualize the the pathogens and study its morphology. Electron microscopy can also provide information on the ultrastructural modifications/changes at sub-cellular levels caused by the pathogen.

So compared with other methods, E M benefits from an "open view", which means that as a "catch all" method it also reveals double infections and the presence of agents that might not otherwise have been considered. Finally, since the test entails the visualization of the virus itself, rather than a color change or agglutination reaction, false positive tests resulting from cross-reactions of reagents with similar materials are not likely. Hence electron microscope can be considered as the ultimate tool in identifying the etiology of emerging diseases.

Two types of preparations are primarily used for routine EM virus identification, negative staining and thin sectioning, although specialized research techniques such as scanning E M, specific antibody aggregation or labeling with electron-dense tags, *in situ* labeling, cryomicroscopy, and high-voltage microscopy have been used to classify viruses and describe virus-host relationships. With the simple negative staining preparation available, E M allows the rapid and direct detection of an etiological agent on a sample from a patient, or from diagnostic cell cultures.

Negative staining of liquid samples is very rapid, and can provide an answer within a few minutes to a couple of hours.. It enables the examiner to view cell particles and organelles in isolation. The isolated cell/particle is placed in a "puddle" of staining material, usually uranyl acetate or phosphotungstic acid, and is then supported on a thin, plastic film. The stain molecules deposit into surface crevices in the specimen during the drying process and typically produce a "ghost" image in which the specimen appears light against a dark background. Sensitivity and specificity of E M may be further enhanced by immuno electron microscopy, which includes classical immunoelectron microscopy and solid phase immuno electron microscopy.

In classical immuno electron microscopy, the sample is treated with specific anti-sera before being put up for EM. The viral particles present will be agglutinated and thus congregate together by the antibody, making them easily visible.

In solid phase immuno electron microscopy the grid is coated with specific anti-sera. The virus particles present in the sample will be absorbed onto the grid by the antibody thus enhancing the visibility under the microscope.

Advantages: The most important among the benefits offered by the electron microscope is undoubtedly the very high resolution. Since timely and accurate diagnosis forms the first step in the health management of farmed fishes and shellfishes, the right diagnosis defines the very success of disease control. Though E M has an important role in the diagnosis of viral infections, it is equally useful in the diagnosis and understanding the pathogens as well as the pathological changes caused by various other pathogenic organisms. As a confirmatory diagnostic method for many of the existing and emerging diseases, especially of viral origin, electron microscopy still remains an indispensable tool in the field of disease investigation and control. To exploit the potential of diagnostic electron microscopy fully, it should be quality controlled, applied as a frontline method, and be coordinated and run in parallel with other diagnostic techniques.

Disadvantages: However, the disadvantages of E M in the diagnosis of infections are (a) detection of viruses by E M requires relatively large numbers of virus particles (b) possibility of false negatives, if concentration is very low (c) provides no information regarding specific serotypes within a virus family and (d). Factors like high cost of operation and infrastructure, need for skilled technical personnel, laborious and time-consuming procedures, thorough knowledge needed for interpretation etc. restricts the use of electron microscopy as a routine diagnostic tool.

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DNA based diagnosis of fish / shellfish pathogens

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Diagnosis forms the first step in any disease control programme, which determines the ultimate success or failure of the programme. The diagnostic procedure may include a single diagnostic test or a battery of tests. However, the diagnosis can often get complicated in the cases of mixed infections, with the involvement of primary, secondary and even tertiary pathogens. The different types of diagnostic methods used in aquaculture include microscopy, histology and histopathology, electron microscopy, culture and biochemical identification, bioassay, serological methods, tissue culture and molecular diagnostics.

Light microscopy: Bright-field, phase contrast or dark field microscopic observation on wet mounts and stained or unstained tissue of abnormal or diseased animals.

- Required resource: Experienced laboratory technicians.
- Nature of diagnosis: Primary, inconclusive.

Histology and histopatholgy: Routine histological and histochemical examination of tissue sections.

- Required resource: Laboratory facilities and experienced laboratory technicians.
- Nature of diagnosis: Secondary. Method provides specific information but poor in sensitivity and speed.

Electron microscopy: Ultrastructural examination of infected tissue sections, negatively stained virus preparations or surface scanning of samples.

- Required resource: Expensive laboratories and expertise.
- Nature of diagnosis: Conclusive, but is time consuming and laborious.

Culture and biochemical identification: Standard culture methods of bacteria and fungi using selected artificial media preparations followed by biochemical tests.

- Required resource: Good laboratories and expertise.
- Nature of diagnosis: Conclusive. But is slow, and time consuming

Bioassay: Laboratory challenge of the candidate species with selected pathogen.

- Required resource: Wet laboratory and expertise.
- Nature of diagnosis: conclusive, but slow and time consuming.

Serological Methods: Use of specific antibodies as diagnostic reagents in immunoblot, agglutination, diffusion, hybridisation etc.

- Required resource: Good laboratories and expertise
- Nature of diagnosis: Conclusive, different levels of sensitivity.

Tissue culture: *In vitro* culture of pathogens in tissue culture systems, or in primary cell cultures.

- Required resource: Sophisticated laboratories with expertise.
- Nature of diagnosis: Conclusive but expensive and time consuming.

Molecular diagnostics: Amplification and detection of unique sections of pathogen' s genome.

- Required resource: Sophisticated laboratories and expertise.
- Nature of diagnosis: Rapid, specific, most sensitive, and conclusive.

Conventional diagnostic procedures listed above have their own limitation especially in speed, specificity and sensitivity. Many molecular techniques are potentially faster or more sensitive than methods such as culture, serology and histology that are traditionally used to identify fish diseases. In addition, the application of molecular tools enables the detection of genetic variations that denote subspecies or strains. A comparison of the above diagnostic techniques shows that each one has its own advantages and disadvantages. But when we consider the specificity and sensitivity of the tests, molecular tools have a definite edge over the others. DNA based new generation diagnostic tools such as PCR has emerged as the solution for rapid diagnosis with speed, specificity and sensitivity. Techniques of major significance include polymerase chain reaction (PCR) amplification of nucleic acids, restriction enzyme digestion, probe hybridisation and nucleotide sequencing. An added advantage of these molecular diagnostic techniques is besides identifying the pathogen species, it has the ability to discriminate below the level of species and identify strains.

Polymerase chain reaction (PCR) and Screening of seed and broodstock/ spawners : In the early asymptomatic stages of viral infections, conventional diagnostic tools fail to detect the carrier hosts. Virus being the smart pathogen with its ultra size, host and tissue preference, reproduction efficiency and lethality, is able to evade the detection levels of conventional diagnostic tools. This is where the DNA based diagnostic technologies emerged as new generation diagnostic tool, which has all the features of a smart diagnostic tool with all the three 'S', Sensitivity, Specificity and Speed, to detect the viral pathogens. Among the DNA-based diagnostic technologies, polymerase chain reaction (PCR) is at the forefront of molecular diagnostic technology toady. PCR technique has been proven to be the most sensitive and specific diagnostic tool for the screening of WSSV in shrimp seeds and mother prawns. Within a couple of hours, PCR technique can amplify from even a single DNA molecule of the pathogen to millions of copies, which can be easily detected using techniques such as electrophoresis.

In India, viral diseases have not been considered to be a significant factor in marine and brackish water finfish culture, but there have been many reports indicating the existence of viral diseases which cause severe mortalities in fin fishes worldwide. Viral encephalopathy and retinopathy (VER), or viral nervous necrosis (VNN) has been reported as a serious disease of larval, juvenile and sometimes in older marine fish that occurs almost world-wide. It is known to cause the disease in groupers, pleuronectids, snappers, white bass, sea bream, Atlantic halibut, large mouth bass and

freshwater aquarium fishes. One of the important sources of the nodavirus is the carrier fish that neither show clinical symptoms nor die due to the VNN. Diagnosis is based on examination of histological sections of brain and retina and by RT-PCR of these organs.

Viral diseases and PCR diagnosis in India : PCR techniques was established as a diagnostic tool in the early nineties. Since 1997, PCR has been in use by the Indian shrimp farmers for the screening of *Penaeus monodon* seeds produced by the hatcheries for WSSV, through the PCR laboratories established by the private promoters, who have used the nested PCR procedures developed in Taiwan and Thailand. Later, institutions such as Mangalore Fisheries college-Mangalore, Central Institute of Brackishwater Aquaculture (CIBA)-Chennai and CMFRI, Cochin, developed the nested PCR for the detection of WSSV in India. PCR labs established by Marine Products Development Authority (MPEDA) at various farming centers further popularized the PCR among the farmers.

Efforts initiated by MPEDA and CIBA with NACA has developed benchmark for PCR harmonization in India with special to the screening of WSSV. Presently, most of the government institutions in India, engaged in the shrimp diseases are equipped with PCR diagnostic facilities, and most of these facilities are available for the use of shrimp farmers. CIBA is planning to set up a referral PCR laboratory at it head quarters at Chennai, to solve the intra and inter laboratory conflicts in PCR results.

Practical issues with PCR detection : Presently, there is no common standard in the PCR diagnosis in the country, among the various PCR laboratories and Government institutions in India. Lack of standardized methodologies can produce inconsistent results which can hamper the reliability and comparability of the diagnosis, and further it will affect the decision making process in health management. The use of PCR technique for health management necessitates a higher level of validity because of the therapeutic and management decisions rests on the outcome of the test. Areas that require standardization are;

Sample collection : Collection of samples for PCR analysis and the method of preservation should be simple and practical. It may not be always possible to bring live or frozen sample to the PCR lab for analysis. Tissue samples or whole larvae for PCR can be preserved in 95% ethanol. Ethanol fixed samples are easy to handle, and the sample can be sent to the PCR labs by courier. Ethanol preserved samples were routinely used for the PCR at CIBA, and the results were always good and comparable to the live/frozen samples.

Tissues used for PCR : The proper selection of DNA sources is critical in obtaining accurate PCR results. Though all the shrimp tissues of ectodermal and mesodermal origin harbours WSSV, tissue has to be selected on the basis of their PCR amplification and consistency. In our laboratory, DNA template from the tissues such as gill, epidermis, stomach wall, eyestalk (without compound eye) and pleopod gave good and consistent PCR amplification. However, the gill tissue or pleopod is the preferred one. Eyestalks, when used along with the compound eyes, PCR inhibition was observed as reported by Lo *et al.* (1998). When postlarvae were used for PCR amplification, their compound eyes were removed to prevent any PCR inhibition.

DNA extraction procedure : Time consuming DNA extraction procedure cannot be suggested in rapid diagnostics. Instead, simple and time saving extraction procedure to produce quality DNA

template has to be used. The extraction procedure should minimize the use of PCR inhibitory substances viz. Phenol, SDS, etc. A simple, rapid and cost-effective DNA extraction procedure developed at CIBA using alkaline lysis coupled with boiling provided good quality DNA for PCR, in just 15 minutes. The DNA quality was comparable to the DNA extracted using standard methods.

DNA template : The amount of template DNA required for a successful PCR amplification has to be standardized with respect to the size and tissue of the shrimp. An excess quantity of DNA template can inhibit the PCR reaction, causing false negative results. When the samples are pooled for PCR analysis, adjustments have to be made in the sample size and DNA-template concentration to avoid possibility of PCR inhibition due to excess DNA.

PCR and risk of contamination : Extreme sensitivity of PCR, can directly lead to the greater risk of contamination. PCR facilities should include separate sample preparation room and amplification room. PCR lab must be clean, and strict discipline should be followed in handling dedicated micropipettes and disposable tips. To prevent cross contamination between samples, disposable tissue homogenizers, centrifuge tubes, and pipette tips have to be used during the DNA-template preparation. Use of low contamination strategies by developing users' friendly PCR kits is required. Positive and negative controls should be included in each reaction to evaluate any false positive/ negative results.

Once a common standard is adopted, the reproducibility and reliability of PCR diagnosis among different laboratories will be high. The PCR data generated among these labs can be used for epidemiological studies in the region, which will be useful in developing the health management strategies for shrimp farming.

PCR and challenges on shrimp health management in India

PCR screening and the hatchery factor : The best expected and useful application of PCR in shrimp farming is its use as a health management tool in the screening of broodstock/spawners, against disease causing pathogens. In the right health management approach, one would expect the PCR testing of the broodstock/spawners and subsequent rejection or acceptance of the mother shrimp in the hatchery, according to the test status. However, this is not happening in the Indian hatcheries. Majority of the hatchery operators use spawners, irrespective of the PCR results. One of the reasons for this attitude is the high cost involved in the purchase of the spawners (Rs. 3000 to Rs. 50,000). However, the high investment in the 'quality broodstock' incurred by the hatchery operators by discarding the infected ones can be recovered by selling 'WSSV free premium shrimp seeds' for high price. Unless and until the hatchery operators adopt the proper (truthful) screening of spawners on the basis of PCR, the testing of seed from the farmers end is just an exercise, without any binding on the real pathogen status on the seeds.

Threat of horizontal virus infection : Water sources such as canals, creeks and estuaries for the shrimp farms in farming areas are already contaminated with WSSV, the purpose of PCR screening of shrimp seed can become simply futile. Further, animals (e.g. dogs and foxes) and common birds (e.g. crows) which freely move in the farming areas, can act as potential agents in horizontal transmission of WSSV.

The purpose of PCR screening can be fruitful, only when the dual possibilities of vertical and horizontal transmission of the virus, mentioned above is prevented. One can imagine the wishful

thinking of using SPF seed and its ultimate fate in the context of potential horizontal transmission of WSSV!

The communication factor : Unlike other animal rearing systems, shrimp farming is a complex and multi faceted activity where so many factors are converging in the three dimensional environment of the culture facility. Hence when a technology such as PCR is applied, there should be a perfect harmony and communication between the hatchery operators, farmers, processors, traders, scientist, social workers and the policy makers. Unfortunately, in India, the communication channels among the above are poor, and many are working in isolated compartments. This will undermine the application of technologies, producing mostly negative results. Formation of an 'Indian shrimp farming consortium (IFC)' linking all the stake holders in Indian shrimp farming, in a level playing field may be a solution, to establish the effective communication, for the progress of shrimp farming.

Change in virulence/viral accommodation : Differential virulence and mortality have become common among the shrimp farms infected with WSSV. Nature of WSSV outbreak and mortality pattern has become most unpredictable and health management effort has become arbitrary. In spite of fully blown up white spot disease, instances are many where farmers were able to harvest fully grown marketable shrimp of 30-40 grams. This can be due to a decreased WSSV virulence level due to viral mutation, or due to an increased tolerance for the virus by the shrimps. Existence of different WSSV strains with pathogenic and non-pathogenic nature is a possibility. Now that the whole WSSV genome is sequenced, development of PCR diagnosis to differentiate pathogenic form from non-pathogenic variant can be a new diagnostic challenge.

Limitations: However, molecular diagnostic techniques have some limitations in terms of appropriate applications, standardized sampling, testing procedures and interpretation of results and are also of limited value to newly emerging diseases where the causative agent is unknown.

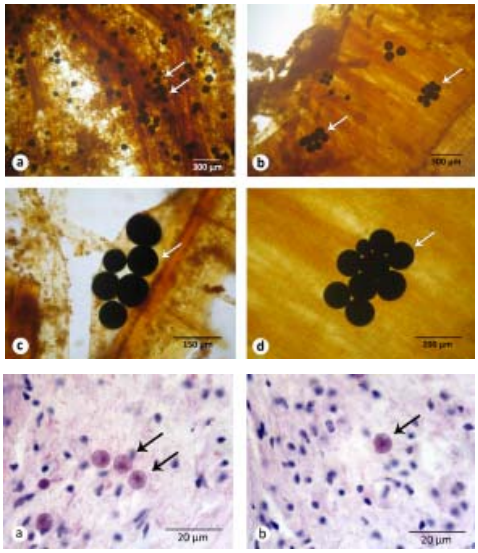
In the case of new and emerging pathogens, conventional methods such as clinical symptoms, light microscopy, histopathology and electron microscopy is required to diagnose the pathogen and purify the pathogen so as to generate genetic details such as sequence information required for molecular diagnosis.

Case study 1 :

Diagnosis of the protozoan parasite *Perkinsus olseni* infection in *Pinctada fucata*.

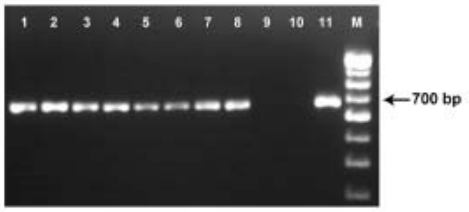
The pearl oyster, *Pinctada fucata* (Gould), is a commercially important bivalve distributed in the Gulf of Mannar along the southeast coast of India. During the past few decades, the natural pearl oyster beds in the Gulf of Mannar have showed a sharp decline leading to the closure of the traditional pearl fishery and was presumed to be due to over-exploitation and pollution.

The pathogen profile of *P. fucata* from the southeast coast of India has not been studied at all. Since *Perkinsus* spp. is known to have destroyed many oyster beds worldwide, the present study was taken up to screen the pearl oyster population along the Gulf of Mannar coast. Samples of *P. fucata* were collected from wild populations at three different locations in the Gulf of Mannar and analysed using Ray's fluid thioglycollate medium (RFTM) culture, histology and molecular diagnostic techniques (PCR).



The current major limitations for identifying the various species of *Perkinsus* are the broad host range encountered and the absence of significant morphological differences among the known species. The standard diagnostic method for *Perkinsus* spp. as per OIE stabdards is the incubation of suspected host tissues in Ray's fluid thioglycollate medium (RFTM) followed by staining with Lugol's iodine. The parasitic stages if present can be visualized as blue-black spheres under light microscope. Though simple and sensitive, it cannot distinguish between various species of *Perkinsus* and some times certain dinoflagellates can give false positive results.

In the present case, Ray's fluid thioglycollate medium assay of the tissues showed enlarged blue-black hypnospores characteristic of *Perkinsus*-like organisms. All the samples collected during the period were found positive showing a prevalence of 100%. But these results cannot be treated as confirmatory. *Perkinsus*-like organisms measuring 4.7 µm to 7.3 µm were observed in the histological preparations. However, typical 'signet ring' stages of trophozoites, a characteristic 'signature' of *Perkinsus* were not observed in the histological preparations studied, again creating some confusion.



The tissues were then screened using the *Perkinsus* genus specific ITS 85 & ITS 750 primers which amplified the product specific to *Perkinsus* sp. (ca. 700 bp) confirming the presence of *Perkinsus* sp. infection beyond any doubt. But again the species level identity of the pathogen was not known. In the next step the PCR products were purified and sequenced and analysed using BLAST. The results showed 99% identity to *P. olseni* with 100% query coverage. The PCR results were in perfect agreement with that of the RFTM and histology studies and confirm the presence of *Perkinsus* spp.

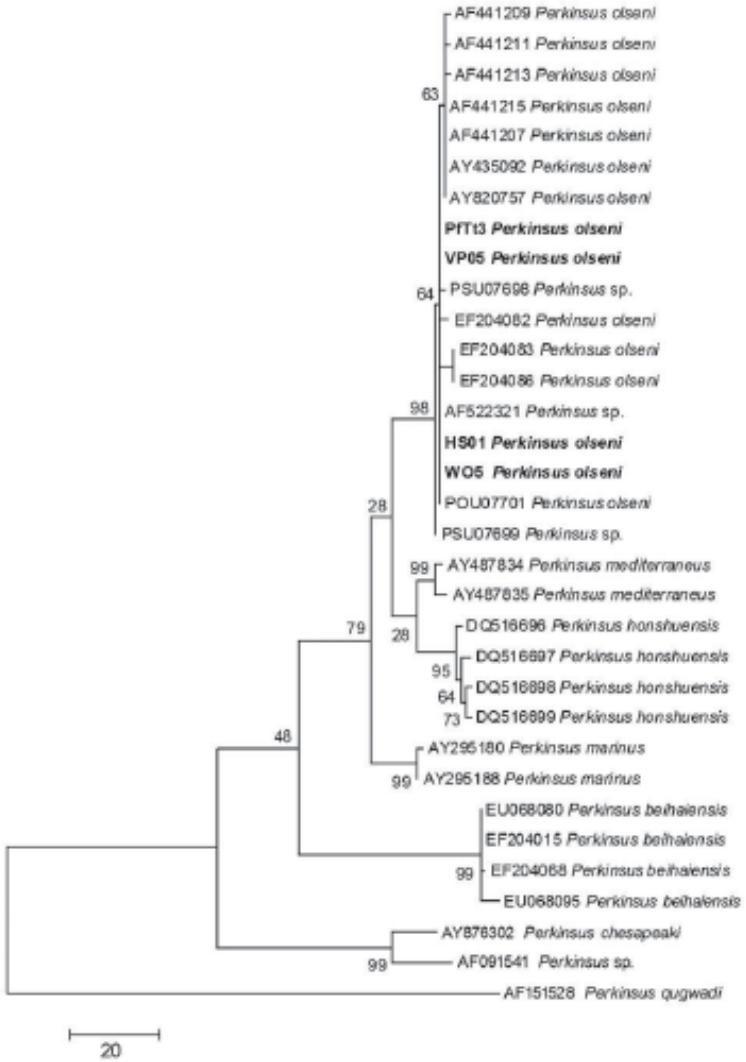
The pairwise genetic distance between the present 4 isolates of *P. olseni* from *P. fucata* appeared to be 0, indicating that they belong to the same species. The pairwise genetic distance between the present *P. olseni* isolates and other members of *P. olseni* group (from different hosts) studied, ranged from 0 to 0.009 (mean, 0.0027), indicating its affiliation to the *P. olseni* clade. On the other hand, the significant variations observed in the pairwise genetic distance between the present isolate and the **other species of *Perkinsus***, clearly showed that they were all taxonomically distinct.

Mean pairwise genetic distances between the various species of *Perkinsus*.

	P. ols	*P. med*	*P. hon*	*P. mar*	*P. bei*	*P. Che*	*P. qug*
Present isolate	0.0027	0.0365	0.0485	0.0480	0.1343	0.1530	0.4280
P. olseni		0.0382	0.0502	0.0498	0.1360	0.1541	0.4275
P. mediterraneus			0.0320	0.0470	0.1230	0.1435	0.4430
P. honshuensis				0.0450	0.1173	0.1395	0.4388
P. marinus					0.1328	0.1475	0.4255
P. beihaiensis						0.1825	0.4858
P. chesapeaki							0.4905
P. qugwadi							

Further, maximum parsimony and neighbor joining analysis of the nucleotide sequences of the ITS region of the present parasite showed it to be *Perkinsus olseni* and sequences from all the 4 samples were identical and were positioned along with the members of the *P. olseni* clade. The topologies of the trees generated with the distance and parsimony analyses were similar. The study has proved that the protozoan parasite infecting the pearl oyster was *Perkinsus olseni* beyond any doubt. This forms the first report on the existence of *P. olseni*, an OIE listed protozoan parasite in *P. fucata* from the southeast coast of India.

This study clearly proved that molecular diagnostic techniques can be extremely useful in the diagnosis of protozoan parasites which are otherwise difficult to diagnose using conventional techniques. Further the specific taxonomic status of the pathogen can also be revealed without any scope for ambiguity. Techniques like nested PCR can enhance the sensitivity of molecular diagnostic techniques greatly.



Phylogenetic tree constructed using maximum parsimony method

Case study 2 :

Diagnosis of WSSV in shrimps

Aquaculture Research

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Virulence status, viral accommodation and structural protein profiles of white spot syndrome virus isolates in farmed *Penaeus monodon* from the southeast coast of India

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Abstract

The objective of this study was to investigate the reason for variation in the virulence of white spot syndrome virus (WSSV) from different shrimp farms in the Southeast coast of India. Six isolates of WSSV from farms experiencing outbreaks (virulent WSSV; *v*WSSV) and three isolates of WSSV from farms that had infected shrimps but no outbreaks (non-virulent WSSV; *nv*WSSV) were collected from different farms in the Southeast coast of India. The sampled animals were all positive for WSSV by first-step PCR. The viral isolates were compared using histopathology, electron microscopy, SDS-PAGE analysis of viral structural proteins, an *in vivo* infectivity experiment and sequence comparison of major structural protein VP28; there were no differences between isolates in these analyses. A significant observation was that the haemolymph protein profile of *nv*WSSV-infected shrimps showed three extra polypeptide bands at 41, 33 and 24 kDa that were not found in the haemolymph protein profile of *v*WSSV-infected shrimps. The data obtained in this study suggest that the observed difference in the virulence of WSSV may not be due to any change in the virus, rather it could be due to the shrimp defence system producing certain factors that help it to accommodate the virus without causing any mortality.

Keywords: WSSV, Indian isolates, virulence, haemolymph proteins, viral accommodation

Introduction

White spot syndrome virus (WSSV) is a major viral pathogen affecting shrimp aquaculture globally. It was first reported in 1992 from an outbreak in cultured penaeids in Taiwan (Chen 1992). The virus has a wide host range and affects almost all species of cultured shrimps and crustaceans (Lo, Lin, Ho, Chu, Chen, Yeh & Peng 1997; Rajendran, Vijayan, Santiago & Krol 1999; Sanchez-Martinez, Aguirre-Guzman & Mejia-Ruiz 2007). It is extremely virulent and cumulative mortality can reach up to 100% within 3–7 days post infection.

White spot syndrome virus represents the type species of a new genus of DNA virus Whispovirus, belonging to the family Nimaviridae (Fauquet, Mayo, Maniloff, Desselberger & Ball 2005). It is an enveloped ovoid-shaped virus with a rod-shaped nucleocapsid (Wang, Lo, Li, Chou, Yeh, Chou, Yung, Chang, Su & Kou 1995; Wongteerasupaya, Vickers, Sriurairatna, Nash, Akarajamorn, Boonsaeng, PanYim, Tassanakajon, Withyachumnarnkul & Flegel 1995). The virus contains double-stranded, circular DNA of about 300 kb, which has been completely sequenced in three different WSSV isolates originating from China (WSSV-CN; Yang, He, Lin, Li, Pan, Zhang & Xu 2001), Thailand (WSSV-TH; van Hulten, Witteveldt, Peters, Kloosterboer, Tarchini, Fiers, Sandbrink, Lankhorst & Vlak 2001) and Taiwan (WSSV-TW; GenBank accession no. AF440570). There have been several reports of differences in the virulence of WSSV isolated

from different geographical locations, and these variations have been attributed to deletions in the variable regions of the WSSV genome (Lan, Lu & Xu 2002; Dieu, Marks, Siebenga, Goldbach, Zuidema, Duong & Vlak 2004; Marks, van Duijse, Zuidema, van Hulten & Vlak 2005).

In India, it has been observed that some farmers in Tamil Nadu and Andhra Pradesh (South India) are able to obtain a reasonably good harvest despite the presence of WSSV infection characterized by severe white spots on the carapace. Similar observations have also been made in China (Lan *et al.* 2002). The epizootiological investigations carried out on other viral diseases of *Penaeus monodon* have revealed that the severity of epizootics declined within 1.5–2 years from the first appearance of the new virus (Fegan, Flegel, Sriurairatana & Waiakrutra 1991; Flegel 1997; Owens, Haqshenas, MeElnea & Coelen 1998). These observations suggested the existence of possible genetic variants of WSSV (i.e. 'virulent' and 'non-virulent' strain) by the researchers and farmers. The work reported here, therefore, aimed to compare the putative 'virulent' and 'non-virulent' strains of WSSV and verify whether a strain variation does exist in WSSV in South India. The clinical, light microscopic and electron microscopic characteristics of shrimp infected with these strains are described. Protein profiles of haemolymph of *P. monodon* infected with 'virulent' and 'non-virulent' WSSV are also compared to determine the host response that may be responsible for the resistance of *P. monodon* to WSSV infection.

Materials and methods

Sample source

Shrimp samples were obtained from nine farms located in India (Table 1) during February 2002–December 2002. Of these, six farms experienced white spot disease outbreak and crop losses. The viral isolates obtained from these farms were tentatively named as 'virulent' WSSV (*v*WSSV). The remaining three farms had successful harvests, although the shrimps were infected with WSSV. The viral isolates obtained from these farms were named as 'non-virulent' WSSV (*nv*WSSV).

Detection of WSSV in shrimp tissues by PCR

DNA was extracted from the gills and pleopods of shrimps. The tissue was homogenized in DNA extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 500 mM NaCl and 1% SDS), boiled for 10 min and then centrifuged at 10 000 $\times$ *g* for 5 min. The supernatant was precipitated in ethanol and the DNA pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). A two-step PCR amplification was performed using the primer sets reported by Kimura, Yamano, Nakano, Monoyama, Hiraoka and Frousp (1996).

Histopathological study

Tissues (gills and stomach) were preserved in Davidson's alcohol–formalin–acetic acid (AFA) and processed according to the standard procedure of Bell and Lightner (1988). Tissues were sectioned at 4–5 μm thickness and stained with haematoxylin and eosin. The stained sections were observed under an Olympus light microscope (Olympus, Tokyo, Japan) and photomicrographs were taken using a WILD MPS 46 camera (Wetzler, Germany) fitted to a Leitz Laborlux S microscope (Wetzler, Germany).

Electron microscopy

For transmission electron microscopy (TEM) preparation, small pieces (1–2 mm^3) of the gills and stomach were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 24 h at 4 °C, followed by three rinses (10 min each) with 0.1 M PBS buffer;

Table 1 Places of collection of different WSSV isolates from the southeast coast of India

Collection location	Host species	Life stages of shrimp	Cultured or wild	'Virulent' or 'non-virulent'
Chidambaram (TN)	*P. monodon*	Sub-adult	Cultured	'Virulent' (V1)
Marakkanam (TN)	*P. monodon*	Sub-adult	Cultured	'Virulent' (V2)
Mahabalipuram (TN)	*P. monodon*	Adult	Cultured	'Non-virulent' (NV1)
Kovalam (TN)	*P. monodon*	Adult	Cultured	'Non-virulent' (NV2)
Kattur (TN)	*P. monodon*	Adult	Cultured	'Virulent' (V3)
Gudur (AP)	*P. monodon*	Sub-adult	Cultured	'Virulent' (V4)
Nellore (AP)	*P. monodon*	Adult	Cultured	'Virulent' (V5)
Kakinada (AP)	*P. monodon*	Adult	Cultured	'Virulent' (V6)
Bhimavaram (AP)	*P. monodon*	Adult	Cultured	'Non-virulent' (NV3)

the specimens were post fixed in buffered osmium tetroxide for 2 h. After rinsing it again in the same buffer, the specimens were dehydrated in graded ethanol and embedded in Epon 812 resin (Merck, Darmstadt, Germany). Both 1-μm-thick and ultra-thin sections were cut on a Reichert-Jung ultra microtome (Wetzler, Germany) fitted using a diamond knife. The ultra-thin sections were stained with 0.5% uranyl acetate, followed by lead citrate (Reynolds 1963). These sections were examined and photographed under a JEOL-JEM 100SX transmission electron microscope (Jeol, Tokyo, Japan).

Isolation of intact WSSV viral particles

To purify the virus, gill tissues were removed from each of the nine WSSV-infected shrimps. The tissues were homogenized in TNE buffer containing protease inhibitor 1 mM PMSF (phenyl methyl sulphonyl fluoride) and centrifuged at 3000 × *g* for 5 min at 4 °C. The supernatant was centrifuged at 100 000 × *g* for 1 h at 4 °C (Beckman Coulter ultracentrifuge, SW41 rotor, Beckman Coulter, CA, USA). Then, the pellet was resuspended in 1 mL of TNE buffer. The suspension was overlayered on the top of a 10–50% (w/v) continuous sucrose gradient and centrifuged at 123 000 × *g* for 90 min at 4 °C. The viral band was collected and the fraction was diluted in the ratio 1:10 using TNE buffer and centrifuged at 123 000 × *g* for 1 h. The pellet was then resuspended in 100 μL of TNE buffer and stored at − 70 °C until further use. The degree of purity of virus isolated was evaluated by negative-staining TEM. For TEM examination, each virus suspension was mounted on a carbon-coated nickel grid (400 μm mesh) and negatively stained with 2% phosphotungstic acid, and the specimens were examined under a Hitachi H600 transmission electron microscope (Hitachi, Tokyo, Japan).

SDS-PAGE analysis of purified WSSV virions

Purified intact WSSV virions were analysed by SDS-PAGE. The total protein was estimated using the standard method of Lowry, Rosebrough, Farr and Randall (1951). About 30–50 μg of protein was separated by 12% SDS-PAGE and visualized using Coomassie Brilliant Blue R-250 staining.

Haemolymph protein profile

The haemolymph was collected from infected shrimps (both *v*WSSV and *nv*WSSV) under sterile conditions. The haemolymph was allowed to clot, and the serum was separated by centrifugation at 3000 × *g* for 5 min. The serum was transferred to a fresh tube and the sample was separated by 10% SDS-PAGE.

Cloning, sequencing and computer analysis of viral envelope protein VP28

The vp28 gene of WSSV is of considerable significance in WSSV pathogenicity, among the other important viral proteins. Subunit vaccines (Witteveldt, Cifuentes, Vlak & van Hulten 2004; Witteveldt, Vlak & van Hulten 2004) and dsRNA and siRNA targeting this gene (Robalino, Bartlett, Shepard, Prior, Jaramillo, Scura, Chapman, Gross, Browdy & Warr 2005; Westenberg, Heinhuis, Zuidema & Vlak 2005) have been shown to confer significant protection in shrimp against WSSV. To determine whether any mutational changes in this gene could contribute to the observed difference in virulence, vp28 was amplified from each of the 'virulent' and 'non-virulent' isolates using gene-specific primer designed from the vp28 sequence in GenBank (GenBank accession no. DQ007315). The PCR primers used were forward primer 5′-AGA*GA ATTC*ATGGATCTTTCTTTCAC-3′ (*Eco*RI site in italics) and reverse primer 5′-CAC*GTCGAC*TTACTCGGTCTC AGTGC-3′ (*Sal*I site in italics). PCR was carried out using the following profile: 5 min at 95 °C, 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The amplified product was digested and cloned into the plasmid vector pET20b (+) (Novagen, Darmstadt, Germany). One of the clones obtained was sequenced. Sequencing was carried out on one end of the cloned fragment using the universal T7 terminator primer. The sequencing was performed by a commercial sequencing company (Microsynth, Switzerland). The deduced amino acid sequence was analysed for homology to other proteins contained in the public database Genbank (BLASTP). The amino acid sequences of vp28 obtained in the present study and other published sequences of vp28 from GenBank: Vietnam (accession no. CAD83839), the Netherlands (accession no. AAF29807), Korea (accession no. AAP87278), Japan (accession no.AAP06670), Indonesia (accession no. AAP06668), China (accession no. AAY27882) and the United States of America (accession no. AAP06661) were subjected to multiple alignments using CLUSTAL W (1.82).

Shrimp culture

Healthy *P. monodon* postlarvae (PL) (15–20 days old), belonging to an individual broodstock that tested

negative for WSSV and MBV, were obtained from a commercial shrimp hatchery in Chennai, India. Representative animals were again screened for WSSV using the PCR method described in 'Detection of WSSV in shrimp tissues by PCR' and for MBV using a standard squash mount preparation described by Lightner, Redman and Bell (1983). After confirming the WSSV- and MBV-free status, the larvae were reared in a 2000-L concrete tank (salinity 22 g L^{-1}, temperature 27–29 °C) on a commercial diet in the laboratory (CIBA, Chennai, India) until the animals gained 5–6 g body weight.

In vivo shrimp infectivity test

An inoculum of WSSV was prepared from the gills of both *v*WSSV- and *nv*WSSV-infected shrimps. From each shrimp sample, 100 mg tissue was homogenized in 1 mL TNE buffer (50 mM Tris-HCl, 400 mM NaCl, 10 mM EDTA, pH 7.4) and centrifuged at 1500 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.45 µm filter (Sartorius, Germany), and the filtered suspension was diluted in the ratio of 1:50 with 0.9% NaCl.

A total of 300 juveniles (5.5 ± 0.5 g) were used for the infectivity tests. The animals were divided into 30 groups, comprising 27 test groups and three control groups, with three replicates for each WSSV isolate, and stocked in 10-L aquaria (10 animals $tank^{-1}$). Each shrimp from the test group was injected with 50 µL WSSV inoculum at a point between the second and the third tergal plates on the lateral side. The control groups were injected with extracts from the gills of WSSV-negative *P. monodon* prepared in the same manner as described for the test groups.

Results

Gross morphology

Gross morphology of shrimps infected with *v*WSSV and *nv*WSSV showed typical clinical signs of WSSV infection. Virulent WSSV-infected shrimps showed white spots on the carapace, reddish discolouration on the body surface and appendages, lethargy, loss of balance, reduced feeding and they finally died. No clinical signs, other than apparent white spots on the carapace, were observed on the shrimps infected with *nv*WSSV. Furthermore, these animals were found to be healthy with normal feed intake and survived until harvest.

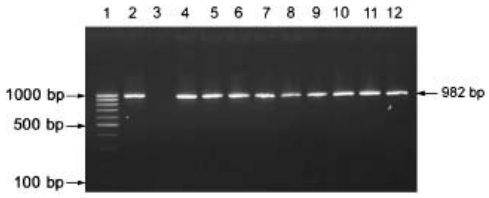

Figure 1 Detection of WSSV in the shrimp samples collected at different places. All samples were positive by first-step PCR. Lane1: 100 bp DNA ladder, lane 2: positive control, lane 3: negative control, lanes 4–12: shrimp tissue samples in order of collection site (please see Table 1 for order).

Polymerase chain reaction (PCR)

In PCR amplification, all the nine isolates were found to be first-step PCR positive for WSSV (Fig. 1). A band corresponding to 982 bp was detected after electrophoresis of the PCR products of all the nine isolates.

Histopathology

Histopathological changes in the tissues of *v*WSSV-infected (Fig. 2a) and *nv*WSSV-infected (Fig. 2b) shrimps were similar. These changes were characterized by nuclear hypertrophy, chromatin margination, variable multifocal necrosis and haemocyte encapsulations. In *nv*WSSV-infected shrimps, histopathological changes were less severe and widespread cellular degeneration, as noticed in *v*WSSV-infected shrimps, was not observed.

Transmission electron microscopy

Transmission electron microscopic observations of *v*WSSV- (Fig. 3a) and *nv*WSSV-infected (Fig. 3b) tissues showed similarity in size and morphology. The size of the larger virion ranged between 112 and 268 ± 34 nm in length, whereas the smaller virion ranged between 98 and 260 ± 30 nm in length. The size of the nucleocapsid varied from 82 to 246 ± 35 nm. A paracrystalline array of virus was observed within the nucleus. The virion is typically characterized by an apical envelope extension. Rod-shaped to elliptical virus particles surrounded by a trilaminar envelope were found in the nuclei of affected cells.

Isolation of WSSV

After sucrose gradient centrifugation, a white band thought to contain the purified virus was observed in the middle of the gradient. The viral band was

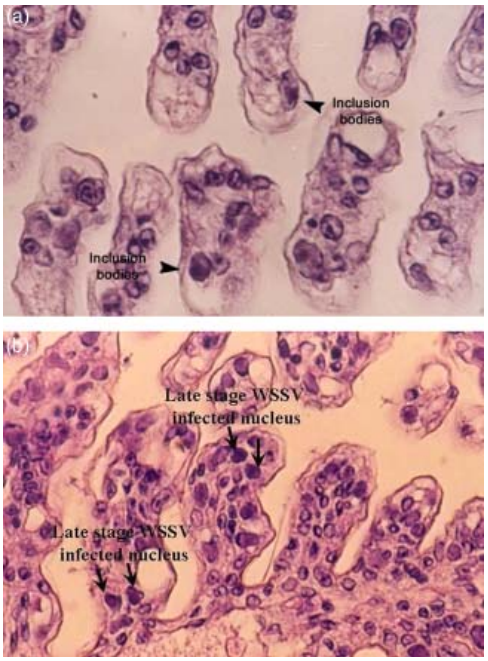

Figure 2 (a) Light photomicrograph of histological section through a region of non-virulent WSSV-infected gills of *Penaeus monodon*. Cells showing eosinophilic intracellular inclusions (arrow heads) surrounded by marginated basophilic chromatin are observed (b) Histological section through a region of virulent WSSV-infected gills of *P. monodon*. There is cellular degeneration with nuclear hypertrophy. Late stage WSSV-infected nuclei are seen.

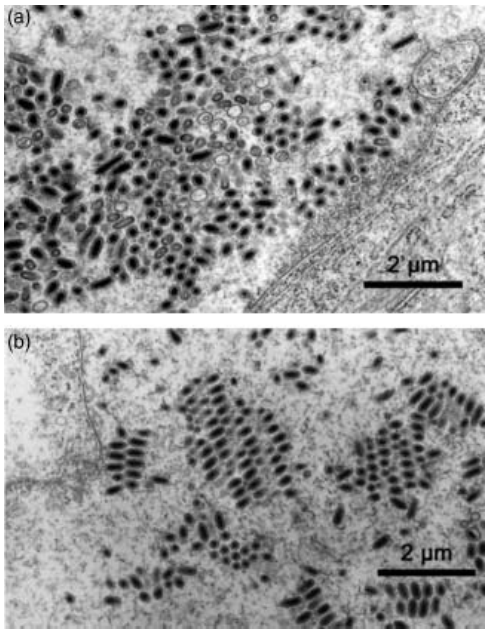

Figure 3 Transmission electron micrograph of ultrathin section of WSSV-infected stomach epithelial cells of *Penaeus monodon*. (a) *v*WSSV-infected shrimp tissue section. (b) *nv*WSSV-infected shrimp tissue section. Note the arrangement of viral particles in paracrystalline array.

collected. The purity of the virions was determined by TEM. The shape of the negatively stained intact WSSV virions was rod-shaped to elliptical. Naked viral nucleocapsid cores were observed (Fig. 4a), and each intact virion had a long tail-like extension at one end (Fig. 4b). Both *v*WSSV and *nv*WSSV virions were alike.

Viral structural protein profile

More than 20 bands of different intensities were observed in all the groups on staining with Coomassie Brilliant Blue R-250. The protein profile of all the nine isolates was identical (Fig. 5). Eight major bands at 75, 69, 34, 27.5, 24, 18, 13.5 and 11 kDa were clearly observed. Of these, the 75 and 69 kDa bands corresponded to shrimp haemolymph proteins that were co-purified with the virus (van Hulten, Westenberg, Goodall & Vlak 2000). The protein profiles obtained from the *v*WSSV and *nv*WSSV were similar, and no variation was observed between the two.

Haemolymph protein profile

We found three additional major bands corresponding to 41, 33 and 24 kDa in the haemolymph of *nv*WSSV-infected shrimp (Fig. 6), which were not present in the haemolymph protein of shrimp affected with *v*WSSV. These additional bands were not found in any of the other lanes. The other protein bands were similar in virulent- and non-virulent virus-infected shrimp. Haemolymph protein profiles from control shrimp were comparable, except for the three additional proteins detected in *nv*WSSV shrimp protein.

Sequencing and comparison of VP28

The viral coat protein VP28 is a major structural protein of WSSV (van Hulten *et al.* 2000) and has been implicated in the systemic infection of shrimp by

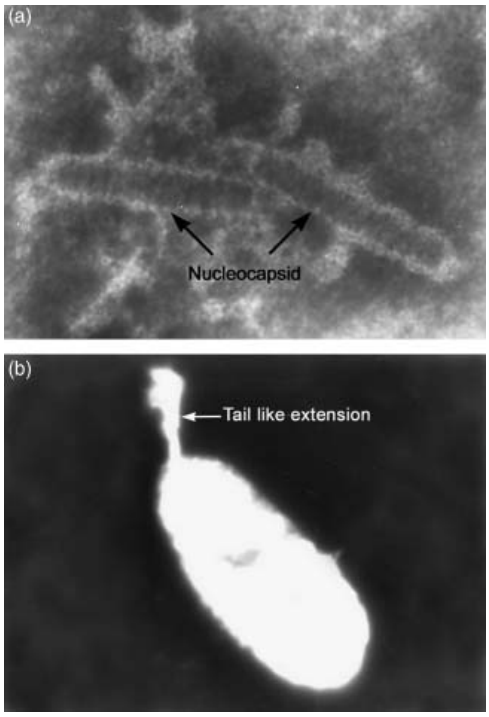

Figure 4 Electron micrograph of negatively stained WSSV purified by sucrose gradient. (a) Enveloped rod-shaped nucleocapsid is clearly observed. There is a distinct vertical helix located horizontally along the axis of the nucleocapisd core. (b) Complete virion with the tail-like extension is observed.

WSSV (van Hulten, Witteveldt, Snippe & Vlak 2001; Yi, Wang, Qi, Yao, Qian & Hu 2004). In order to determine whether there were any differences in the gene sequence of *vp28* of these two isolates, the gene was amplified from one *v*WSSV and one *nv*WSSV isolate and sequenced. The *vp28* sequences of the 'virulent' and 'non-virulent' isolates were 100% identical to each other. Thus, there was no difference in the *vp28* sequences of 'virulent' and 'non-virulent' isolates. When compared with the sequences in GenBank, our sequence showed 100% similarity to those obtained in Vietnam, the Netherlands, Korea, Japan, Indonesia, China and the USA. The deduced amino acid sequence of *vp28* in this paper and the previously published sequences were aligned using CLUSTALW (1.82) multiple sequence alignment (EMBL-EBI). All the sequences showed 100% homology, except the USA isolate, which differed at amino acid 40, where histidine is replaced by arginine (data not shown).

In vivo infectivity studies of the nine WSSV isolates

To understand the difference in the pathogenicity of the two types of virus isolates, challenging studies were undertaken to determine whether the difference in infectivity could be attributed to changes in the virus itself. Challenging the *v*WSSV and *nv*WSSV isolates to juvenile *P. monodon* showed that all the nine isolates were highly pathogenic to the animals. The cumulative mortality reached 100% within 5 days post infection in all the test groups. No mortality was observed in the control groups. White spot syndrome virus infection of all moribund shrimps in the test groups was confirmed by WSSV-nested PCR, and all the infected animals were found to be first-step PCR positive. The control groups were negative for WSSV by second-step PCR.

Discussion

In the study presented here, the possible existence of a 'virulent' and a 'non-virulent' WSSV was analysed using histopathological, electron microscopical and molecular data.

Histopathological observation showed that both *v*WSSV- and *nv*WSSV-infected tissues had symptoms of WSSV infection, as reported earlier (Rodriguez, Bayot, Amano, Panchana, de Blas, Alday & Calderon 2003), although there was a difference in the severity of symptoms. The histopathological changes in the *v*WSSV-infected tissues were markedly severe, which is to be expected because the shrimps were collected during the outbreak time and many animals had died in these farms due to the disease. Study of the WSSV latency-related gene in specific pathogen-free (SPF) shrimp (Khadijah, Neo, Hossain, Miller, Mathavan & Kwang 2003) reported the presence of WSSV in a latent state in the SPF stock. In this study, however, it cannot be argued that the virus in *nv*WSSV-infected shrimps was in a latent state because the animals tested positive for WSSV by first-step PCR showed obvious signs of infection such as white spots on the exoskeleton and typical intranuclear inclusions of WSSV in the affected tissues. Electron microscopic observation of the infected tissue sections and purified *v*WSSV and *nv*WSSV virions showed similar morphologies, with no discernible difference in size or shape.

In the present study, we found that both *v*WSSV and *nv*WSSV isolates caused similar mortality patterns in *P. monodon*, with 100% mortality caused by

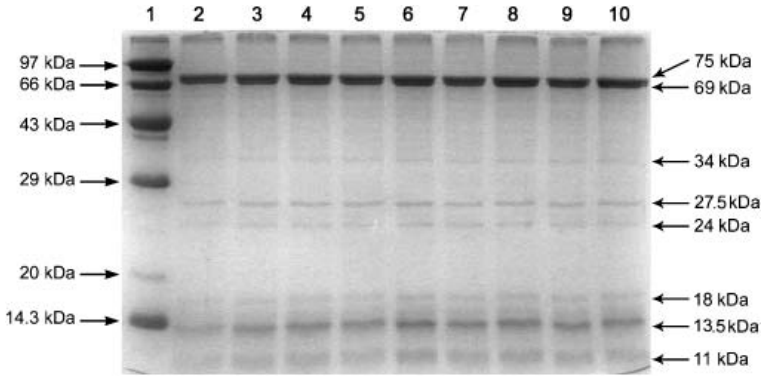

Figure 5 SDS-PAGE protein profile of the nine WSSV isolates. Lane 1: protein molecular weight marker; Lanes 2, 3, 6, 7, 8 and 9: V1, V2, V3, V4, V5 and V6 respectively; Lanes 4, 5 and 10: NV1, NV2 and NV3, respectively, where V, virulent isolate and NV, non-virulent isolate (see Table 1 for details). Eight major bands corresponding to 75, 69, 34, 27.5, 24, 18, 13.5 and 11 kDa were clearly observed. Of these, the 75 and 69 kDa bands correspond to shrimp haemolymph proteins.

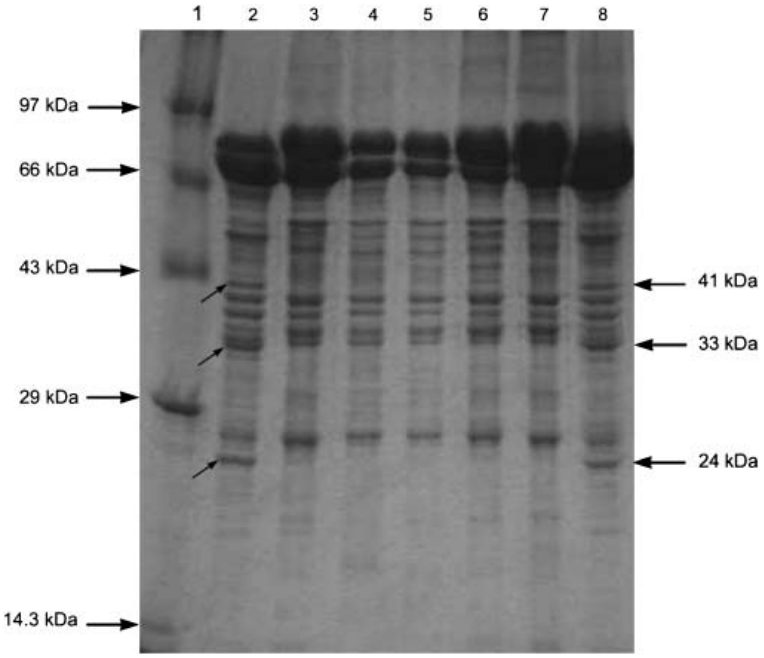

Figure 6 SDS-PAGE profile of shrimp haemolymph from *v*WSSV- and *nv*WSSV-infected animals. Lane 1: medium range protein molecular weight marker, Lanes 2 and 8: haemolymph of shrimp infected by *nv*WSSV, Lanes 3–7: haemolymph of shrimps infected by *v*WSSV. The three additional protein bands obtained in the haemolymph of animals infected with *nv*WSSV are indicated by arrows.

both isolates within 5 days. This is in marked contrast to the finding of Marks *et al.* (2005), wherein there was a significant difference in the median lethal time of the less virulent TH-96-II isolate (14 days) and the more virulent WSSV-TH isolate (3–5 days). The authors had suggested that WSSV-TH, which has a smaller genome (~ 293 kb), may possess a replication advantage when compared with the TH-96-II isolate, which has a larger genome (~ 312 kb), and this could be the reason for its higher virulence.

Analysis of the viral protein profile of the two isolates showed identical profiles with no discernible difference. Mutational changes in amino acids of ma-

jor viral proteins that play an important role in infectivity have been shown to cause a change in the pathogenicity of the virus. Recently, a new variant of the Taura syndrome virus (TSV) called the Belize isolate has been described that varies in virulence when compared with the Hawaii isolate and was found to belong to a distinct group on performing phylogenetic analysis of a major capsid protein-encoding gene (Tang & Lightner 2005). It has been reported that a single amino acid change in the E2 spike protein of a virulent strain of Semliki Forest virus, which is lethal to mice, attenuates pathogenicity and causes the virus to become avirulent when given to adult mice (Glasgow, Killen, Liljestrom, Sheahan & Atkins 1994). Several WSSV viral proteins have been implicated in the infectivity in shrimp including VP28, VP31, VP36B, VP68, VP76, VP281 and VP466 (van Hulten, Witteveldt, Snippe *et al.* 2001; Huang, Xie, Zhang & Shi 2005; Li, Xie & Yang 2005; Wu, Wang & Zhang 2005; Li, Yuan, Cai, Gu & Shi 2006). To understand whether a mutation in the genes encoding these proteins could contribute to the difference in virulence observed in this study, we amplified and sequenced the *vp28* gene from both the isolates. *vp28* encodes a major structural protein of WSSV that has been implicated in the systemic infection of shrimp (van Hulten, Witteveldt, Snippe *et al.* 2001) and has been shown to be involved in the binding and entry of WSSV into shrimp cells by an *in vitro* binding assay (Yi *et al.* 2004). The sequence of *vp28* from both our isolates showed 100% similarity, ruling out any mutations in the *vp*28 amino acid composition. However, it is necessary to examine all WSSV proteins involved in the infectivity process to rule out the possibility of any mutations in the genetic make-up of the isolates.

On analysing haemolymph protein profile, it was found that the haemolymph of shrimp infected with the *nv*WSSV isolates revealed three bands corresponding to 41, 33 and 24 kDa, which were not observed in any of the other lanes. These bands may correspond to some protein expressed only by shrimp that have developed some resistance or tolerance to WSSV. This could explain why the same isolate could produce rapid mortality upon experimental infection in the laboratory. The experimental animals were raised from the PL stage under closed laboratory conditions without any exposure to infectious agents and cannot develop any tolerance to the viral infection. Substances with broad anti-viral activity in tissues of crustaceans have been reported (Pan, Kurosky, Xu, Chopra, Coppenhaver, Singh & Baron 2000). An earlier publication has reported the anti-viral nature of an approximately 28 kDa protein called *Pm*AV in *P. monodon* and has suggested that this protein may play an important role in the defensive mechanism of *P. monodon* against viruses (Luo, Zhang, Shao & Xu 2003). In another report, the resistance against WSSV observed in 'immune' kuruma shrimp was attributed to the virus-neutralizing factor(s) in the haemolymph, which appeared 3 weeks after exposure to the virus and lasted for about 1 month (Wu, Nishioka, Mori, Nishizawa & Muroga 2002). In a recent report, two anti-viral factors were identified in haemocytes of WSSV-resistant *Penaeus japonicus*, of which one was an interferon-like protein (InHP) and the other was a (2′–5′) oligo(A) synthetase-like protein (He, Qin & Xu 2005).

An earlier report had proposed a new concept of 'active viral accommodation' for crustacean response to viral pathogens (Flegel & Pasharawipas 1998). This concept was proposed to explain the lack of a tissue response or apoptosis in response to viral pathogens in crustaceans that enables the host to tolerate viral infection without mortality. It further proposed that accommodation is characterized by the absence of active defence against the viral pathogen. It was proposed that shrimp had a specific 'recognition mechanism' by which they could acquire 'tolerance' to the new viral pathogen during their larval development. By accommodating viruses in persistent infections without mortality, there would be positive selection of viral variants with the least negative effect on the host (Flegel 2007). The present study finds resemblance to the concept of 'active viral accommodation' proposed by Flegel and Pasharawipas (1998). Furthermore, it is also possible that the '*nv*WSSV-infected shrimps' could have encountered WSSV in an innocuous form early in their larval stages during stocking in the ponds, which may have given them some tolerance to the virus. In this context, it is proposed that the three extra protein bands observed in the haemolymph protein profile of *nv*WSSV-infected shrimp may be a similar anti-viral protein or may be some factor(s) associated with resistance or tolerance to WSSV.

Considerable progress has been made in the characterization of WSSV, but the understanding of shrimp's defence system in response to viral infection is still poor. It is essential to consider the virus–host interaction while studying the change in virulence of WSSV rather than looking at the virus or shrimp alone. Such a holistic approach can further our understanding of shrimp response and adaptation to

viruses and may, in the long run, help us to find new treatment methods for viral diseases in shrimp.

Acknowledgments

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Immune Responses in Fishes

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The term immunity means exemption. The meaning originated from exemption granted to certain citizens in the case of compulsory military services. This term was further extended to the disease resistance seen in people recovered from epidemic diseases. This knowledge led to the development of small pox vaccine by Edward Jenner in1798. In animals, the mechanism for self-recognition and non-self discrimination evolved some 400 million years ago and continues as the basic immune response mechanisms. The basic pattern of protein molecules involved in the self/nonself-recognition remained without much alteration, while the diversification of these molecules to suit the emerging challenges was super imposed. We have several examples of self recognition in invertebrates. Aggregation of dispersed colonies of sponges is regulated by species-specific surface glycoproteins. Failure of adhesion of unrelated species amounts to a primitive graft rejection. The cells lining the cavity of sponges are able to capture microorganisms. In the insect larval metamorphosis, phagocytosis of dead and disintegrated cells occurs by the same mechanism. Sea stars and corals reject grafts from unrelated forms. The same mechanism enables fertilization of ova in marine fishes and other organism by the spermatozoa of same species. Hence, the self-recognition mechanism enables the millions of species to maintain their integrity, while excluding invasion of their body from align cells and changed self- constituents (mutated cells including neoplastic cells).

The immune system, vested with the role of defense is composed of various cell types, tissues and organs. The mechanisms of defense executed by the immune system are of non-specific and specific types. The former is encountered in almost all living organisms including fish. It is non-specific because the same immune response can be elicited by a number of unrelated foreign particles. Specific or acquired mechanism of immunity is found only in vertebrates and the reactions are directed against specific molecules that stimulate such reactions. Though these two mechanisms appear as distinct, in fact they function in conjunction with each other, making, the study of immunology a complex and vast one.

Basic pattern of Molecules

The cell surface molecules that are markers of self/non-self recognition are either carbohydrates or carbohydrate terminal groups on glycoproteins. The recognition factors themselves are proteins. The marker proteins and recognition proteins have evolved from $\hat{a}_2$ micro globulin or Thy-1 protein. The basic structures of immunoglobulin, histocompatibility antigens and phagocyte receptors indicate this. Each $\hat{a}_2$ micro globulin consists of a polypeptide chain with 110 amino acids of molecular weight

12000 (approximate). Vertebrates including fish have improved upon this primitive recognition mechanism and evolved a highly efficient system to deal with potential invasion from the co-existing biological world as well as aberrant or rogue cells evolved through spontaneous mutations (eg. nepolastic cells). This system has retained all the primitive mechanisms such as, phagocytosis, agglutination of heterologus cells, lysis etc., while developing specific molecules and specific cellular mechanisms against foreign molecules and cells.

Innate Immunity: Non-specific Immunity

The innate immune mechanisms are non-specific. The main determinants of innate immunity are genetically controlled, varying widely with species, strain and to a lesser extent between individuals

Surface barriers:

- **Mucus**: a layer of mucus forms the interface between body and environment. It is rich in glycoproteins, proteoglycans proteins and humoral factors. Mucus entraps microorganisms and mucus is continually replenished by mucus secreting cells, which inhibits the colonization of integument. The rate of secetion of mucus increases in response to infections or due to action of irritants. Lyszyme, bacteriolysin and complement cascade present in the fish are anti-microbial.
- **Skin:** The skin surface of fish differs from that of higher vertebrates in that the epidemis is composed of non-keratinized living cells, which are continuously sloughed off preventing colonization of microbes. Epidermal integrity is vital to fish in maintaining osmotic balance and excluding microorganisms. Epidermal healing response in fish is extraordinarily rapid, even at low temperatures. It involves a migration of Malpighian cells from the periphery of wound surface rapidly closing the lesion, and is quite different from the scab formation, which occurs in mammals. Epidermis has resident migratory phagocytes. Malpighian cells are also capable of phagocytosis.
- **Gills:** The gill is a route entry for pathogens and has a large surface area of delicate epithelium. The epithelial layer secretes copious amount of mucus, which protect the gill. The epithelium responds to infections by hyperplasia. The pillar cells lining the brachial blood sinuses are also phagocytic.
- **Gastro-intestinal tract**: The mucus membrane of the tract secretes abundant mucus. The digestive tract environment is hostile to many pathogens. (1) Acidic pH in stomach. (2) Action of lytic enzymes (Pepsin, trypsin amylase peptidase etc.). In teleost M cells and Peyer's patches are absent. However, intra epithelial lymphocytes are seen. Macrophages lymphocytes and eosinophilic granular cells are found in lamina propria.

Non-specific humoral factors: These include (1) growth inhibitors (2) inhibitors of enzymes or toxins produced by pathogen (3) lysins (4) precipitins and (5) agglutinins.

- **Growth inhibitors**: These substances either deprive essential nutrients to microbes or interfere with their metabolism.
- **Metal ion binding proteins**: These occur in the serum of all vertebrates including fish. Iro binding proteins (siderophilins) such as apotransferrins, ceruloplasmin and metallothionein have been identified in fish. They deprive essential metal ions and inhibit growth of microbes.

Apotransferrin binds two ferric ions. Ceruloplasmin oxidizes ferrous ions to ferric ions and metallothionein binds to copper, zinc, cadmium and mercury ions. Metallothionein specifically binds to macrophage plasma membrane, initiating respiratory burst activity and signal transduction. Apotransferins, which are also acute phase proteins, display anti-microbial properties by limiting the amount of endogenous iron available to pathogens including intracellular bacteria/protozoan.

- **Acute phase proteins**: Plasma proteins collectively termed as acute phase proteins increase in response to infections, and tissue injury. These include C-reactive proteins, serum amyloid A protein, á$_1$ antitrypsin, á$_2$ macroglobulin, ceuloplasmin, C$_9$ and factor B.
- **Cytokines:** Cytokine related molecules are detected in fish and invertebrates. These are interleukin-1 (IL-1), IL-2,IL-3, IL-4, IL-5, IL-6,tumor necrosis factor (TNF), Chemo tactic protein-1, macrophage migration inhibition facto (MIF) and other peptide factors, which are involved in modulation of immune response and inflammatory reactions. Cytokines mobilize the host immune response and inflammatory reactions. Cytokines mobilize the host immune response, activate inflammatory reactions, and mediate bi-directional communication among various organs/tissues and between cells. Colony-stimulating factors (glycoproteins and peptides) regulate haematopoiesis and haematopoietic cell function, and transforming growth factors profoundly affect wound healing and cellular differentiation.
- **Interferons**: are proteins that inhibit intracellua viral replication. The interferon has been reported from fish. They are classified into class I interferon (á and â interferons) and class II interferons (ã interferon). In each type there are several different forms. Á interferons are produced mainly by ly+
- **Lymphocytes and other nucleated cells:** Interferon â is produced by fibroblasts and interferon ã is produced by T-lymphocytes and natural kille cells (NK orNC cells). Interferon producing cells, when infected with virus (stimuli for interferon synthesis are nucleic acids, bacterial cell walls double stranded RNA and poly synthetic nucleotides) synthesize and secrete interferons into extra cellular fluid. The interferons bind to specific receptors of uninfected cells. The antiviral effect is produced by derepression of two genes leading to synthesis of two specific enzymes. One enzyme catalyses the phosphorylation of ribosomal protein and initiation factor elf-2, which is necessary for protein synthesis. This reduces the m-RNA translation in cells. The other enzyme catalyses the formation of short chain polymer of adenylytic acid, which activates a latent endonulease, thi in turn degrades viral and host cell m-RNA. This establishes a cordon of uninfected cells around the site of viral infection restraining its spread. In addition to these effects it has several other immunological functions such as major histocompatibility class II protein molecule (MHC II) expression on macrophages, increased phagocytosis by neutrophils and macrophages. It also enhances activity of natural kille cells, T-lymphocytes B-lymphocytes and other immune cells
- **Eicosanoids**: are important group of compounds derived from 20 carbon poly unsaturated fatty acids. In fish, eicosanoids are generally produced in organs rich in blood cells after ionophore stimulation. These eicosanoids include prostaglandins, thrombaxans lipoxins and leuktrines. They regulate blood clotting, MHC II expression, inflammation and phagocytosis.

Enzyme inhibitors: Many pathogens produce enzymes in order to gain access to host body. Host tissue fluid and blood contain many factors, which neutralize these lytic enzymes. The á$_2$ macro globulin is able to entrap and form covalent linkages with proteins such as transforming growth factor (TGF) B$_1$, Il-1B and platlet derived growth factor BB. The á$_2$ macro globulin thus regulates the action of coagulation cascades and complement cascade.

Lysins: There are several enzymes, which cause lysis of heterogenous cells. These include complement cascade, pro-phenol oxidase, lysozyme and trypsin.

Complement: is an enzyme cascade consisting of several protein components. In fish twelve proteins have been detected. In mammals about 26 proteins are involved. These proteins are heat labile and are inactivated at 45«%C in fish and at 55«%C in mammals. The function of complement include

- Making bacteria more susceptible to phagocytosis
- Directly lysing some bacteria and foreign cells
- Producing chemotactic substances
- Increasing vascular permeability
- Causing smooth muscle contraction and promoting mast cell degranulation.

Complement cascade is activated two ways; the classical pathway and alternate pathway. Once initiated, a cascade of events ensues, providing the functions listed above. Most of the components are numbered (C1, C2, C3, C4, C5, C6, C7, C8 AND C9). Some are referred as fators (factorB,D,P,etc.).

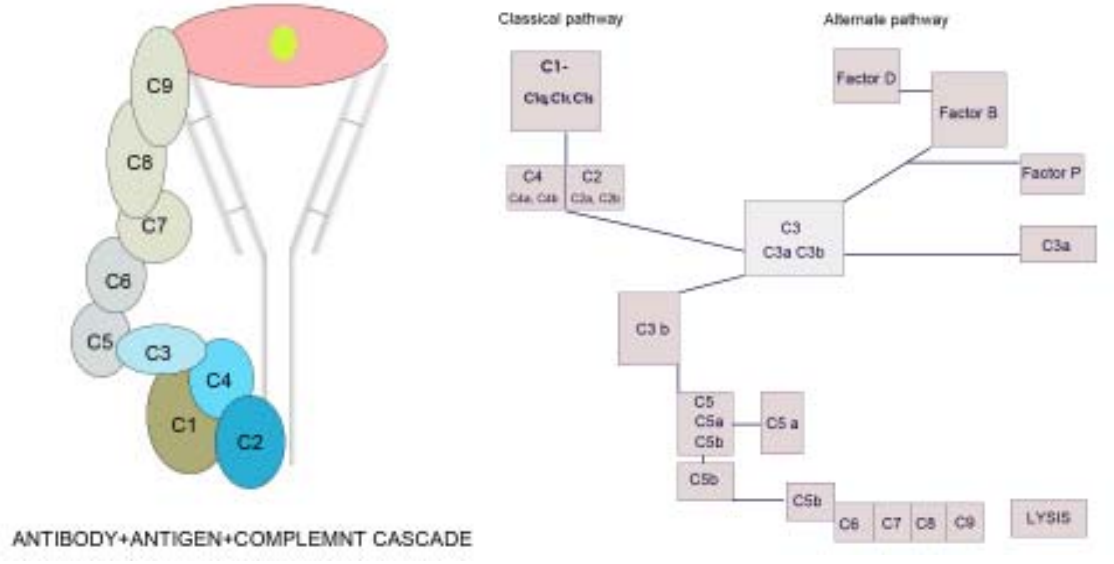
Components of Classical Pathway (Antigen Antibody Complex mediated pathway)

Native component	Active component	Functions
C1	C1q	Binds to antibody bound antigen, activate C1r
	C1r	Cleaves C1s to activate protease function
	C1s	Cleaves C2 and C4
C2	C2a	Unknown
	C2b	Active enzyme. Cleaves C3 and C5
C3	C3a	Mediates inflammation
	C3b	Activation of alternate pathway and binds cell surface-opsonization
C4	C4a	Mediates inflammation
	C4b	Binds C2 for cleavage by C1s. Binds cell surfaces for opsonization.
Components of the Membrane-Attack Complex (same for both classical and alternate pathways)		
C5	C5a	Mediates inflammation.
	C5b	Initiates the assembly of the membrane-attack complex (MAC)
C6	C6	Binds C5b, forms acceptor forC7
C7	C7	BindsC5b67, inserts into membrane forms acceptor forC8
C8	C8	bindsC5b67, initates C9 Polymerization
C9	C9n	Polymerizes around C5b678 to form channel that causes lysis

Components of the Alternate Pathway

Native component	Active component	Functions
C3	C3a	Mediates inflammation, anaphylotoxin
	C3b	Binds cell surfaces for activation of alternate pathway
FactorB	B	Binds membrane bound C3b cleaved by factor D
	Ba	Unknown
	Bb	Cleaved form stabilized by P produces C3 convertase
FactorD	D	Cleaves factor B when bound to C3b
Propedin	P	Binds and stabilizes membrane bound C3bBb

In fish complement is found in serum and mucus



In the alternate pathway, the bacterial endotoxins, polysaccharides like zymosan and inulin, which activate properdin or factor B that convert Factor C3 into C3b and C3a and thus setting the entire cascade into action.

Pro-phenol oxidase system: This system consists of an enzyme cascade leading to the activation of pro-phenol oxidase and other compounds with related activities. Pro- phenol oxidase on activation by â-1, 3 glucans and zymosan cleaves to phenol oxidase, which catalyses oxidation of tyrosine to quinones. Quinones polymerize to form melanin deposits. Melanin deposition is a common immune response seen in invertebrates. Conversion of pro- phenol oxidase to phenol oxidase is catalyzed by a protease named phenol oxidase activating enzyme. This enzyme is kept in check by a protease inhibitor.

Lysozyme: is a low molecul;ar protein found in the blood of vertebrates, haemocytes of invertebrates, serum, haemolymph and mucus. It functions as a mucolytic enzyme, splitting sugars off the glycopeptides of the cell wall of many gram-positive bacteria resulting in their lysis. Lysozyme also plays a role in the intracellular destruction of some gram- positive bacteria.

Trypsin: trypsin and trypsin containing cells are found in epidermis, gills and intestine indicating local production and its secretion into mucus.

Precipitin and agglutinins

C-reactive proteins (CRP) and Serum amyloid protein A: These are plasma proteins and forms part of acute phase proteins. CRP binds to phosphoryl ester groups of bacterial cell wall, which contain phosohoryl choline. This binding is Ca^{++} dependent and activates complement. It has structural analogy to inulin of horse shoe crab. CRP enhances the leukocyte migration, phagocytosis and respiratory burst of phagocytes. CRP can act as opsonin and cause precipitation of heterologus proteins/ carbohydrates in non immunized sera.

Serum amyloid protein A has got lectin binding property by which they bind to bacterial cell wall glycoproteins.

Agglutinins: These are a group of phylogenically conserved substances that are present in fish serum, mucus, bile and haemolymph of invertebrates. This group of substances includes lectins and other receptor specific substances. They act as opsonins and cause aggregation by binding to protein/ glycoproteins and or carbohydrate moieties that are free in solution or are constituent of microbes. Lectins are usually constitutive proteins or glycoproeins, which posses binding activity towards carbohydrate residues.

Cellular factors

Phagocytosis and the inflammatory response: Various circulating and tissue fixed phagocytes rapidly engulf any foreign particles, which enter animal body. In vertebrates they are polymorphonulear leukocytes and macrophages; where as haemocytes constitute the phagocytes of crustaceans and mollusks. The phagocytes contain digestive enzymes that degrade the ingested material. The phagocytes recognize, bind and ingest particulate material. Recognition and binding take place though the interaction of cell surface glycoproteins and cell wall carbohydrates of microorganisms. More recently evolved mechanisms utilize receptors in the phagocyte cell membrane for a part of the antibody molecule (Fc portion of antibody) and for a component of the complement (C3b). Microorganisms coated with antibody and complement thus adhere to the phagocyte and can then be ingested. Antibodies that enhance phagocytosis are the opsonins.

Inflammation: Inflammation is the dynamic process occurring in a viable tissue. It is the reaction of tissues to irritants/ disease causing agents. Inflammation begins following sub lethal injury to tissues and ends with the repair or healing of injured/ damaged tissue. Following an injury the first sequence of changes occur in local vascular system. These vascular changes are the result of release of pharmacodynamic amines from injured mast cells. The eosinophilic granular cells found in fish are analogues to mast cells found in higher vertebrates. These cells are abundant in the connective tissue of blood vessels as well as the stromal connective tissue, which form the structural framework of many organs and tissues. The bacterial products, physical and chemical trauma, products of damaged cells and the complement factors released by immune response can produce injury to mast cells, which release the vaso-active amines like histamine and serotonin. These amines induce increased blood flow (*rubor*). Dilatation of capillaries causes stretching of capillary fenestration, which allows colloids of the plasma to escape into the interstitial space. These results in increase in

colloidal osmotic pressure that attracts fluid content of blood to tissue spaces, leading to swelling of the area (*tumor*) and the fibrinogen escaping with the plasma proteins will be initiated to form fibrin mesh work. Dilatation of arterioles and pre-capillary sphincter cause more capillaries to be opened. An increase in capillary and venule blood pressure is associated with dilation of vessels. Increased permeability of capillaries and venules leads to retardation of the flow and drop in blood pressure. The endothelial cells are activated to produce a lectin on their surface - selectin. The retardation of blood flow allows the heavy elements of blood to be distributed evenly. The leukocytes have on their surface the selectin receptor, which cause them to adhere to the endothelial layer. Then inside out signaling causes certain integrins on the leukocytes ($\hat{a}_1$ and $\hat{a}_2$ subunits) to gain affinity for molecules of immunoglobulin family: particularly those called **ICAM** (Inter Cellular Adhesion Molecules) on endothelial cells. These attachments help the leukocytes to stop, squeeze between endothelial cells and cross the blood vessel wall into the damaged or infected tissue.

Exudation of plasma / serum

The changes in blood flow and the dilatation of capillaries and venules following enlargement of of afferent arterioles lead to retardation of blood flow. This causes increased permeability and osmotic pressure change. Injury to cells leads to breakdown of macromolecules and they enter intercellular fluid resulting increased osmotic pressure. Loss of colloids into interstitial space through increased vascular permeability leads to fall in osmotic pressure of blood. Hydrostatic pressure at venules is increased due to vasodilatation of arterioles. Hence, there is increased accumulation of fluid at the tissue side, where as the re-absorption of fluid from tissue is retarded due to the fall in vascular osmotic pressure. The exudates formed will have plasma proteins including fibrinogen.

The exudates have following functions.

1. Dilute irritants
2. Globulins are brought in contact with irritants, which may neutralize them
3. Fibrinogen in the exudates forms fibrin scaffolding around the irritants, which will contain the spread of infection. The fibrin mesh will act as an anchor for leukocyte migration and phagocytosis.

Migration of leukocytes: Leukocytes emigrate to the tissues by amoeboid movement. The chemotaxis initiate this. Lipo-polysccharrides of bacterial cell wall released at the site are the major chemotactic agents. The cleavage products of complement, such as C3a, C5a C567 lymphokines produced from stimulated lymphocytes and the product of granulocytes and monocytes all act as chemotactic agents. Fatty acid derivatives derived from injured cell membranes such as leukotrienes 5 hydroxyeiosateraenoic acids (5HETE) are all chemotactic. White cells actively migrate through the fenestrae to enter the affected tissues. The cells penetrate junctions between endothelial cells and between basement membrane. They escape to the tissues at the points where basement membrane slits to accommodate pericyte. The collagenase enzyme of leukocytes digests collagen. Lymphocytes are pinocytosed at the basement membrane region. Erythrocytes also leave through the fenestrae. The cells, which leave blood, are 1) neutrophils, 2) monocytes, 3) eosinophils, 4) lymphocytes and 5) thrombocytes.

Neutrophils: These are the first cells to migrate. They contain numerous cytoplasmic granules, which are lysosomes containing number of enzymes capable of destroying the ingested organisms. They are hydrolytic enzymes, oxidative enzymes, proteolytic enzymes, phagocytin and lysozyme.

The proteolytic enzymes are two categories; acid proteases and neutral proteases. Acid proteases act within phagosomes; where as neutral proteases degrade collagen, basement membrane, fibrin, elastin and cartilage. The neutral proteases are responsible for the tissue destruction and this may release kinin and split complement factors C3 and C5 that in turn induces chemotaxis

Opsonic serum factors coating on particles enable phagocytosis. They are complement fragments in fish. Immunoglobulin coated opsonization is weak in fish. The phagocytosis by neutrophils release some quantity of enzymes to the tissues because the fish neutrophils are not efficient phagocytes. The phagocytosis and subsequent digestion are energy dependent. There are two types of digestion, oxygen dependent and oxygen independent systems. In oxygen dependent system there are two types. The superoxide system is characterized by increase in hexose monophosphate shunt activity (This is called respiratory burst, which can be demonstrated by Nitroblue tetrazolium staining (NBT). This generates superoxide anions O_3^-, H_2O_2, ^-OH and O^-. These radicals affect (peroxidation) macromolecules of living organisms like bacteria. In the myeloperoxidase - peroxidase system, myeloperoxidase enzyme increases efficiency of H_2O_2 generating system by releasing halide ions (free halide). This system is more efficient in killing. In oxygen independent system -Hydrgen ions (H^+) reduce pH. Hydrolytic enzymes hydrolyze macromolecules; lysozyme slits off sugars of bacterial cell walls. Fish neutrophils have very similar morphological and histochemical properties to mammalian neutrophils. They are present in kidney, spleen, blood and inflammatory lesions.

Monocytes

These cells appear in an inflammation in later stages. They are actively mobile and have numerous pseudopodia. The monocytes nucleus is ovoid, kidney shaped or indented. Nucleus is usually eccentrically placed. Nucleoplasm is condensed near its membrane. Cytoplasm is abundant and contains mitochondria, Golgi apparatus, and rough and smooth endoplasmic reticulum. Once these cells reach tissues after leaving the blood stream they divide and mature. They are known as mononuclear macrophages. Their main function is phagocytosis by engulfing large particles. They can recognize complement coated cells and particles through specific receptors that assist in phagocytosis. They also recognize molecules that have altered or denatured membranes and engulf them. They can secrete hydrolytic enzymes. Some macrophages mature into secretary cells with abundant cytoplasm and become closer to each other with indistinct boundaries. They are called epithelioid cells. Some macrophages fuse their cytoplasm while attempting to ingest large particles and become multi-nucleated giant cells. Differentiation into epithelioid cells and giant cells occurs in chronic inflammation.

Macrophages are wide spread in tissues but their concentration is more in reticulo-endothelial system. Reticulo-endothelial cells are found in interstitial tissue of kidney, parenchyma of spleen and endocardial lining of heart. Many macrophages in fish contain melanosomes within lysosomes and are termed melano-macrophages. Melanin plays a role in bactericidal mechanism involving the release of free radicals. Melano-macrophages form aggregates in parenchymal organs, which are called melano-macrophage centres

Natural Killer Cells/ Natural Cytotoxic Cells (NK/ NC Cell)

They are large granular lymphocytes. They recognize structures on high molecular weight glyco-proteins, which appear on the surface of infected cells. This recognition occurs through receptors

on NK or NC cells' surface, which bring killer and target into close opposition. Activation of NK cells ensues and leads to release of granular contents into the space between the two cells. The important factor perforin or cytolysin insert into the membrane of the target cell and produce an annular pore, inducing cell death. The granules contain two serine esterases.

Eosinophils

Eosinophils are evolved in killing parasites. A major basic protein (MBP) is located in the core of the granules. Cationic protein and peroxides are present in the matrix of granules. Other enzymes are arylsulphatase B, Phospholipase D and histaminase. It also contains dopaminase. Eosinophils have receptors for C_{3b} which allows eosinophils to adhere and the activated eosinophils secrete MBP and cationic protein

Haemocytes

These are invertebrate blood (haemolymph) cells and have the same enzymes described for leukocytes and perform the phagocytic and degradation function seen in vertebrates.

Acquired Immunity

Acquired immunity has two wings. (1) humoral immunity (2) cellular immunity or cell mediated immunity (CMI). Fish has developed both these systems.

Cell mediated immunity (CMI)

Lymphoid cells may be induced, by prior exposure to antigen, to react subsequently directly with inducing antigen and bring about cytotoxic effects, as for example destruction of foreign cells from a graft. In both type of immunity the executors of the reaction are lymphocytes. Lymphocytes are found in the circulation, lymphoid organs and other tissues. In mammals and birds there are two distinct type of lymphocytes (1) originating from or primed in thymus - T lymphocytes, (2) bone marrow or bursa of *Fabricius* derived cells - B-lymphocytes. T lymphocytes are responsible for cell mediated immune responses (CMI), and providing assistance for antibody production (helper function) and suppression of immune reaction.

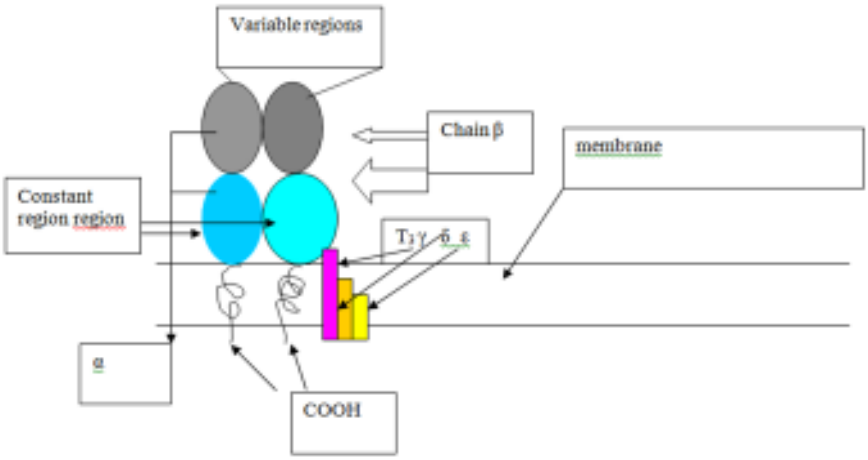
B-lymphocytes are executors of humoral immunity. They transform into antibody or immunoglobulin secreting cells on antigenic stimulation. Subsets of T&B cells perform another function i.e. memory of antigen encounter. In fishes dichotomy of immune system is also present but the details are not fully worked out. Evidence for T&B lymphocyte is also available.

Lymphocyte subpopulation

The T lymphocytes have surface antigen receptors, which are á and â receptors in adult and ä ã receptors in embryonic stage. T lymphocyte receptor is a heterodimer composed of á and â chain each of molecular weight 40-50 kD. Each chain is folded into two domains, one having a relatively constant structure, the other exhibiting far more variability. The variable region has the job of binding to antigen and MHC.

Both á and â chains are required for antigen specificity. In all immuno competent T lymphocytes, the antigen receptor is non-covalently but still intimately linked in a complex with T_3, molecule composed of three peptide chains (ã ä å), which transduces the antigen recognition signal received

by the á â heterodimer to the inside of cell. The complete receptor is thus consisting five peptides. In fish, thymocytes have a portion of Immunoglobulin heavy chain. In mammals no heavy chain receptor is found in thymocytes, where as they bear light chain related molecule.



Structure of antigen receptor in T lymphocyte

T lymphocytes are differentiated in thymus. In higher vertebrates the priming of lymphocytes to T lymphocytes occurs in the thymus. Thymus is protected against the ingress of foreign antigens by specialized endothelial lining of the blood vessels. The role of thymus in fish is probably similar to that of mammals. The thymus consists of mainly lymphocytes in various stages of development and a few epithelial cells and macrophages. There is no differentiation into cortex and medulla. Thymus is a paired organ occupying dorsal region of the brachial cavity and is extremely superficial, being situated within epithelium, external to basement membrane. The blood vessels of thymus have specialized endothelium with tight junctions. In the embryo this is the first organ to become lymphoid. A single layer of epithelium covers the thymus up to post fingerling stage. There are fenestrations of 20 ìm diameter in the epithelial layer. In older fish these fenestrations close and epithelium become thickened or thymus becomes deeply embedded in underlying tissue. Involution of thymus starts at sexual maturity, but it is a slow process in fish. Even in older fish some amount of thymic tissue will be discernable. Thymus is a primary non-executive lymphoid organ. Foreign particulate matter and protein antigen present in circulation are not able to enter thymus. Lymphocytes also do not enter thymus. There is high mitotic activity in thymus and there is migration of thymocyte from thymus to spleen, anterior kidney and intestine. In adult fish, thymus is responsible for regulating antibody response to T dependent antigen and suppressor activity. The developing thymocytes exhibit membrane antigen, which decide their future roles. These antigens are extensively studied in mouse. The mouse T cell antigens are termed Thy (ô) TL and Ly antigens. TL antigen is lost and the amount of Thy antigen is reduced during differentiation. Ly antigen appear later in the development. Ly-1, 2, 3 antigens expressing cells are the immature T cells. Ly-1, 2, 3 cells give rise to Ly-1 cells, Ly2, 3 cells and Ly-1Qa^{+} cells (60% Ly-1 cells are bearing Qa^{+}). Ly-1 cells are having helper function and Ly-1Qa $^{+}$ cells control the generation of suppressor cells. Ly-2, 3 cells have suppressor function on helper cells and B-cells. Ly-2, 3 cells also function as T-killer cells or cytotoxic cells. In other vertebrates the helper function is associated with CD_4 receptor bearing cells and killer and suppressor function

is associated with CD_8 receptor. CD4, CD8 cells are immature T cells. Though clear-cut division based on T cell antigen has not been studied in fish thymus cells, helper, killer and suppressor function are noticed in sub population of lymphocytes of fish. Antigen difference between thymus lymphocytes and lymphocytes of anterior kidney are also evident. In mammals T lymphocyte antigen cross reacts with brain tissue antigen. In fish the lymphocytes, which are responsive to T cell mitogens have antigens cross-reacting with brain tissue antigens.

B-cells have antigen receptors as single heavy chain of ì (Ig M). The B cell is produced in lymphoid tissues like anterior kidney and spleen. The B lymphocyte on biding with respective antigen through the receptor is stimulated to undergo multiplication and many of these cells acquire immunoglobulin secretary function.

Response to mitogens: Certain plant lectins are found to specifically stimulate division of lymphocytes. Phyto-haemagglutinin (PHA) and Concavalin-A (Con.A) are T_cell mitogens, where as Lipo-polysccharrides specifically stimulate B-lymphocytes. In fishes it is found lymphocyte cross-reacting with brain tissue antigen are responsive to PHA and Con.A, where as they are not responsive to Lipo-polysaccharides. Lipo-polysaccharide responsive cells bear Ig M heavy chain molecule on their surface; where as PHA, and Con.A responsive cells bear a part of heavy chain Ig molecule.

The carrier hapten effect: One method to detect T-helper function is to estimate carrier hapten effect. Certain low molecular weight simple substances when injected into an animal will not produce any antibody or immune response. This low molecular weight substance is chemically linked to large molecule. Then the Linked compound is injected to an animal; will induce antibody response against the low molecular substance. The molecule, which is attached to the large molecule, is the hapten and the molecule, which is carrying it, is the carrier. Dinitro-phenyl (DNP) molecule will not induce immune response, where as; if it is linked to bovine serum albumin (BSA), it will elicit antibody response against DNP. In carrier-hapten effect, T-cell co-operation with B cell is required. Carrier-hapten effect has been demonstrated in a number of fish. It is also noticed that T lymphocytes of fish are capable of forming rosettes with sheep erythrocytes.

Cell Mediated Immune Reactions in Fish

The markers of CMI are allograft versus host reaction and delayed hypersensitivity. Both these reactions are found in fish. In graft versus host reaction, an organism rejects organ/ tissue transplants from individuals of the same species as well as phylogenically different species. The rejection process will be faster, if donor and recipient are not genetically related. In this type of reaction no antibody is involved but only lymphocytes and macrophages. Once an animal reject a tissue transplant, it will reject another transplant from the same donor at a short duration of time, thus rejection reaction induces immunological memory in recipient.

Scale transplantation (both allograft and xenograft) have been attempted in fish. In all cases rejection and immunological memory have been noticed. The lymphocyte of the recipients have been shown to retain sensitivity to donor antigens

Delayed hypersensitivity reactions are lymphocyte mediated reactions and lymphokines play a major role. These are specifically provoked by slowly evolving mixed cellular reactions involving lymphocyte and macrophages. The reaction is not brought about by circulating antibody but by

sensitized lymphocytes. It can be transferred in experimental animals by means of such cells not by serum. The classical example is the tuberculin response. The animals infected with *Mycobacterium tuberculi* are given 0.1 ml of 1 in 100 dilution of protein extract of *M. tuberculi* intradermally. An indurated inflammatory reaction in the skin appears about 24 hours later and it may persist for weeks. The injection site is infiltrated with large number of lymphocytes and macrophages, most of these cells are seen around small blood vessels. Among circulating lymphocytes there are a few sensitized lymphocytes, which on contact with antigen produce lymphokines and influence other lymphocytes and monocytes to aggregate at the site of antigen concentrations; and lymphocyte multiply at the site.

Delayed hypersensitivity can be demonstrated in fish by injecting tuberculin or T dependent antigens like BSA and making lymphocytes sensitized. Later an intradermal injection of antigen will produce locsl inflammatory reaction like one described above. In chronic disease like bacterial kidney diseases (*Renibacterium* infection) of trout delayed hypersensitivity has been demonstrated

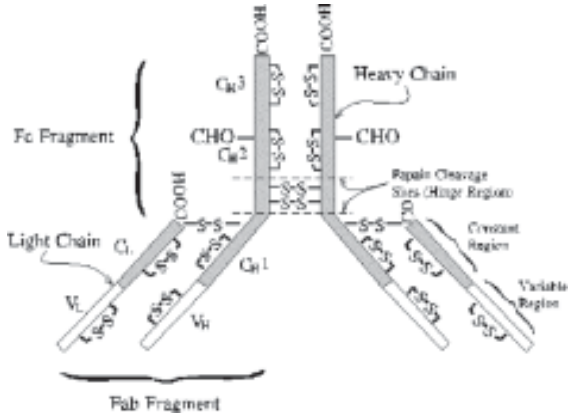
Thus it is clear fish has got a very good CMI response and cells analogues to T lymphocytes are present in fish. The production of lymphokines in fish can be demonstrated by test like macrophage migration inhibition test and demonstration of chemotaxis in special chambers. Macrophage activation by the lymphokines can also be demonstrated. *In vitro* tests like specific contact cytotoxicity, mixed leukocyte reaction and antigen induced blatogenesis of lymphocytes indicate fish has a strong CMI.

Humoral immunity

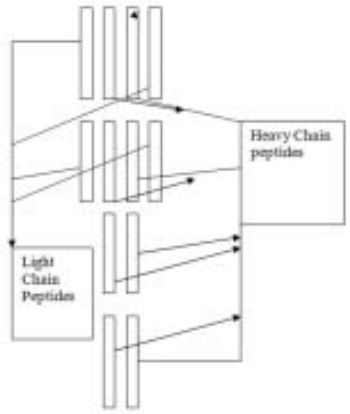
The characteristic form of this immunity is the appearance of globulins-immunoglobulin or antibodies in blood and lymph. These antibodies combine specifically with the antigen, which stimulate their production and lead to remarkable consequences. Induction of the humoral response begins with the recognition of antigen by specific B-cells. This process requires the intervention of T cells bearing CD4 B-cells which recognize the antigen via immunoglobulin receptors on their surface. T cells recognize the antigen through their receptors in association with CD4 and MHC II molecule. T-Cells Produce lymphokines on antigen recognition, resulting in the proliferation of B-Cell leading to antibody secretion. In addition to T helper cells, several other cells present antigens to B-cells and stimulate antibody production. They are macrophages, B cell itself and the spleen ellipsoids. The basic structure of immunoglobulin can be discerned from the structure of vertebrate Immunoglobulin G or Ig ã. It is formed of two heavy chains which acquire the form of 'Y' when unfolded and two light chain one on each side of the arm of 'Y'.

The tips of the Y and light chains enclose the hyper variable areas, (V_H and V_L respectively) which are antigen binding site and designated as Fab fragment while the other area is called Fc region , which is responsible for complement binding, attaching to macrophages and other activities (C_3b mediated inflammatory reactions) resulting from antigen antibody complexes. Each heavy chain is formed by four peptides and light chain formed by two peptides. These peptides are derived from â$_2$ micro-globulins of 110 amino-acids. This is depicted below.

Presence of antibodies can be demonstrated in fish sera by agglutination, precipitation and complement fixation tests. In mammals and birds there are five classes (isotypes) of immunoglobulins based on the antigenic difference in heavy chains. The heavy chain classes are ì, á, ã, å and ä (IgM, IgA, IgG, IgE andIgD). The light chains are two types' ë (lambda) and ê (kappa). In mammals, serum IgM is a pentamer, which consists five basic units linked in the form of ring attached with a protein



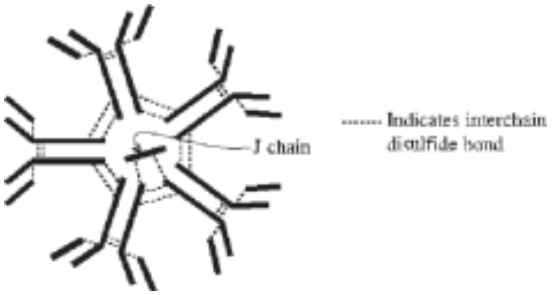
Structure of antigen receptor in T lymphocyte



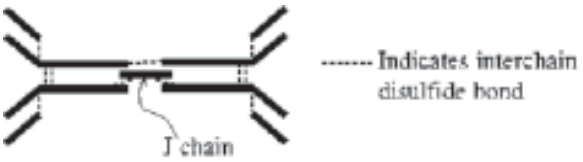
Basic Structure of Immunoglobulion

called J protein segment. In teleosts only IgM isotype is found. The serum IgM is tetrameric. However monomeric, and dimeric forms are found in mucus, bile, skin and eggs.

In mammals and birds functional specialization is associated with heavy chain classes. In teleosts, antibodies can execute most of the reactions observed in mammals and birds, indicating heterogeneity. There is also increasing evidence of local synthesis of secretary antibodies in bile, cutaneous and gastrointestinal mucus. Though immunological methods demonstrated only one type of antibody in fish, there is difference in amino-acid composition of heavy chains. The difference is not amplified enough to recognize them as separate epitopes in immunological reactions to classify them into different idiotypes.



IgM Pentameric structre



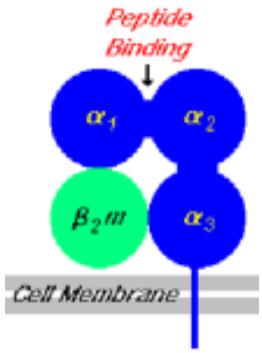
Ig Dimeric form

Cell co-operation and major histocompatibility complex

The precise mechanism by which immuno-competent cells co-operate involves cell surface antigens. These antigens are glyco-proteins, involved in the rejection of grafts to unrelated recipients and are recognized as foreign. The cell surface antigens are known as histocompatibility antigens or **Major Histocompatibility Complex (MHC)**. The genes controlling MHC are closely related to immune response genes (Ir genes). They are situated very close to Ir genes in the same chromosome. The MHC genes have been studied in many animals including trout and carps.

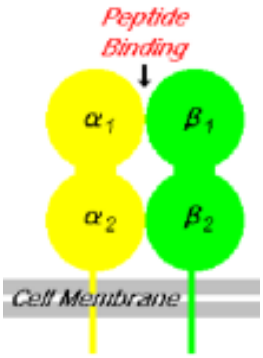
The histocompatibility genes/antigens belong to two classes class I and class II. The genes coding for C2, C4 and factor B, have made their way into the MHC region and are referred to as

class III genes. Both class I and class II molecules are membrane bound heterodimers. Class I molecule consist of a heavy chain of 43 kD non covalently linked to a 11kD peptide, â2 micro globulin. The heavy chain has the globular domains á$_1$ á$_2$ á$_3$, which protrude from cell surface. The hydrophobic section anchors the molecule into cell membrane and short hydrophilic end, which is C terminus, enters cytoplasm.



MHC Class I Molecule

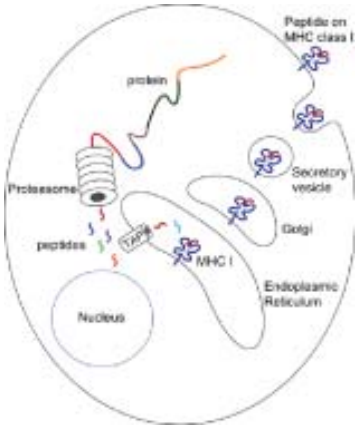
The class II MHC is also trans-membrane glycoprotein having á and â polypeptide chains with molecular weight of 34 kD an 28 kD respectively. Both chains are folded to give two domains, the ones nearest to the membrane having considerable homology with â$_2$ micro-globulin and the characteristic Ig domain. It is seen that I-J region code for more number proteins that it can hold. This includes the suppressor and helper T lymphocyte receptors, immunoglobulins and other peptides. Probably the gene alleles mediate the selection of structurally elated molecules indirectly, perhaps through idiopathic interactions involving T cell receptors. In the immunoglobulin system, variability is achieved in each individual with highly polymorphic system based on multiple alleles. It is very high in class I molecules. Multiple allelic forms generated by variety of mechanisms, point mutation, recombination and homologous but unequal crossing over and gene conversion. The mechanism has been detected in mice. Most of the mutations contain clusters of multiple amino acid substitution and seen to arise by transfer of up to 95 nucleotides from class I Qa genes to á$_1$and á$_2$ domains of H 2 K. these findings have indicated that the large number of functionless Qa genes may represent a stockpile of genetic information for the generation of polymorphic diversity in the working class I molecules. Evidence for gene conversion has also been obtained for the class II genes.



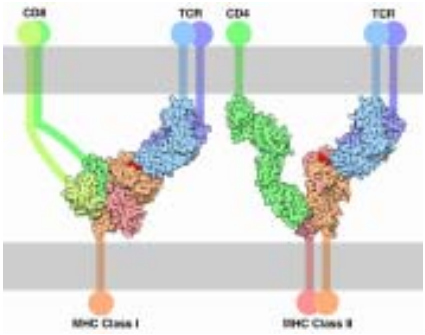
MHC Class II Molecule

All nucleated cells express class I molecules. These are abundant on lymphoid cells, less so on liver, kidney and only sparsely on brain and striated muscles. Class II molecules are restricted to B lymphocytes, macrophages and antigen presenting cells; however, when stimulated by cytokines, capillary endothelium and many epithelial cells express class II molecules. MHC molecules have several physiological functions in addition to immunological functions. It is a known fact that detection non-self MHC activates the T lymphocytes. The T cells can recognize an antigen in association with MHC. The Class I molecule synthesized in rough endoplasmic reticulum and transported to cell surface in a transport vehicle. The MHC molecules have a groove which carries a native peptide (â$_2$). The cells with CD8 receptors search the MHC I molecules. In viral infected cells the peptide is replaced by viral encoded peptides or in cancer cells and old cells the peptide has a different constitution. The detection of changed configuration in MHC leads to the multiplication and secretion of cytotoxic molecules by CD8 t cells. Hence, destruction of the cell occurs. The MHC class II also has a groove. The molecule is synthesized in RER but held in cytosol. In B cell the antigen is captured by surface antibody receptor. It is delivered inside the cell. The antigen is broken down into small peptides; the class II molecule grab the peptide and moves to surface (macrophage also degrades the antigens and fragments are exhibited on MHC II). The CD4 T lymphocytes combine with the antigen presented on MHC II molecule. In nan infection the B cells and macrophages

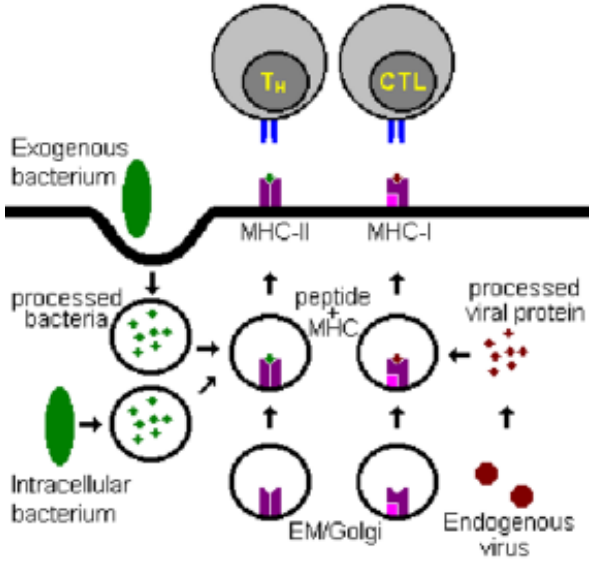
express another molecule B-7. This combines with CD 28 on the T cells. These bindings trigger T cells to secrete cytokines, which initiate B lymphocyte proliferation and antibody secretions. The production of B-7 is essential for activating the T cell secretion of lymphokines and proliferation of B lymphocytes..



MHC Class I Molecule Processing in a Cell



Association of CD 8 and CD4 Molcules with Mhc I and MHCII



The Processing of Antigen in Relation to MHC Classes (I &II)

Clonal Selection Theory of Immune Response

This theory proposes that the cells of the antibody forming system have developed from random mutations resulting in the emergence of small numbers of cells which differentiate so as to be capable of producing one or very small number of specific antibodies. Contact by such differentiated cells with self or foreign antigens during foetal life before cells have reached maturity, would lead to suppression because cells are annihilated by apoptosis.

After intial contact with antigen the cells of immune system retain memory, subsequent contact with antigen lead to quicker as well as amplified immune response. The immune system becomes more skilled with continued experience with the same antigen. The antibodies increase its combining capacity-avidity. This is due to the expansion of the clone. Memory involves generation of long lived T and B lymphocytes and changes in receptor involving generation of high affinity receptors bearing cells.

The antibody has a variable region, which is situated in Fab end and comprises both heavy chain and light chain peptides. The genes for these variable regions are inherited as fragments and these fragments are joined together to form complete genes in individual lymphocytes as they develop. The enzymes that combine the gene fragments add random DNA bases to the ends and as a result new genes are formed. Further diversity results from the assembly of protein chain into a complete receptor. Antibodies are made from two pairs of protein chains a heavy chain and light chain. Each B cell produces one kind of light chain and heavy chain so that B cell makes unique receptor. The genes for receptors of T and B cells mutate extremely rapid, when antigens activate them

Each heavy chain and light chain has variable (V) and constant (C) domain. The V domain is in the N terminal of the both chains. V domain chain is formed by the recombination of four gene fragment- J (joint), D (Diversity) and V (Hyper variable segment). J segment forms the joint with the constant region C. The V region forms hyper variable region and has 100 alleles in mouse, D12 and J 4. Thus in mouse heavy chain itself 4800 varieties can be produced. Considering the light chain variability the antibody types are innumerable.

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Immuno - Diagnostic Techniques in Aquatic Animal Diseases

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Diagnosis plays an important role in any disease control programme. The realization that techniques applied for human medicine and farm animal medicine can be equally used for aquatic animal disease management led to the application of all diagnostic tests in this field also. The immuno-diagnostic tests utilize the body's reaction against foreign substances. These reactions are of two types, one that secrete the specialized globulins (immunoglobulins) and the other generating sensitized cells. Though both these mechanisms can be utilized for diagnosis, the first one is extensively used because of its easy interpretation, convenience and simplicity.

When an antigen comes in contact with the specific antibody they interact with each other forming a union. This union occurs between the Fab fragment of antibody and the particular grouping of antigen (antigenic determinant). The molecules are held together by non-covalent intermolecular forces. The methods used to demonstrate the antigen antibody reactions form the basis of immuno-diagnosis.

The antigen antibody reaction can be detected at two levels. The primary union of these reactants can be demonstrated by labeling one of the reactants with a suitable marker. Such markers are, fluorescent dyes, enzymes and radioisotopes. The union is detected by the reaction exhibited by these markers. Another method employs the physical changes that occur in the antigen-antibody complex after their union. These changes are termed secondary phenomena. The initiation and development of secondary effect involve a series of many events, which involve a number of variables. These secondary effects are broadly put under agglutination, precipitation, activation of complement cascade and release of histamine from mast cells.

Agglutination tests

In this reaction the antigen is part of the surface of some particulate material. Antibody added to a suspension of such particles combines with the surface antigens and links them together to form clearly visible aggregates. There are a number of tests using agglutination. These are slide agglutination, tube and micro plate agglutination, latex bead agglutination, haemagglutination and haemagglutination inhibition.

Slide agglutination test

Two drops of saline containing 0.5% phenol are placed on a slide. Suspension of the test bacterium is prepared in this saline. One drop of antiserum is added to one drop of bacterial

suspension. The slide is rocked to mix the antibody with the bacterial suspension. Visible agglutination develops within 5 minutes. A set of positive controls and negative control are to be used for checking the results.

Tube or micro titre agglutination

Serial dilutions of antibody are prepared in 0.5 ml volumes in test tubes and the same volume of antigen is added to the tubes and mixed. Instead of test tubes micro-plates having 96 wells are also used; 50 ml saline is taken in each well of 96 well of micro-titre plates. Serial doubling dilutions of the serum are prepared by adding 50 ml serum to first well, mixing and transferring 50ml to the second well and continuing to the third and so on. A saline control is also set up. 50ml bacterial suspension is added to each well; mixed and plates are incubated overnight at ambient temperature in moist chamber. Agglutination results in irregular settling of antigen. The reciprocal of the antiserum dilution at the end point is known as titre of serum. If agglutination occurs at a dilution of 1/3000, the titre is 3000. To make agglutination clear the antigens can be stained with dyes. When reaction take place at a high dilution of antibody /serum, the reliability of test increases many fold.

Haemagglutination

In this test the agglutination is made more visible using erythrocytes. The soluble antigens are adsorbed on the cell-membranes of mammalian erythrocytes. Commonly used cells are sheep erythrocytes, which are treated with tannic acid and subsequently with the antigen. The antibodies cross link the erythrocytes and produce agglutination. Blood cells after saline washing are suspended in PBS (0.6 ml packed cell are suspended in 10 ml PBS). 10 ml of 1/20000 tannic acid + 10 ml erythrocyte suspension are incubated for 15 minutes at 37°c. The cells are washed in PBS. A portion of cells is kept as negative control. To 1 ml of treated RBC suspension, 1 ml buffer containing antigen to be coated to the cells is added. The mixture is incubated at 37° C for 30 minutes. The cells are centrifuged, washed 3 times in buffer containing 1% rabbit serum. The coated and uncoated cells are suspended in PBS containing 1% rabbit serum to give 1% cell suspension.

Agglutination test is performed using coated RBC suspension in place of antigen suspension. Plates are left for 2 hours at ambient temperature and left at 4° C overnight. The settling of RBC in compact button form indicates negative test. Positive reaction indicated by formation of irregular clumps. Uncoated RBC suspension forms negative control.

Haemagglutination inhibition test

Certain viruses agglutinate RBCs of several animals. Mixing the virus with the appropriate red cell in the presence of antiserum inhibits haemagglutination. This test can be used for testing suspected patient's serum for specific antibody and for proving identity of virus using known antiserum

Latex agglutination

This is used to detect toxins released by microbes. Polystyrene latex particles are sensitized with antiserum. The cultures are freed of cells by centrifugation and supernatant diluents are prepared in wells of micro titre plates by serial doubling dilutions. To the wells add an equal quantity of sensitized latex suspension. Mix the contents of plates by rotating the plate. The plate is kept in a humid chamber at room temperature for 20-24 hours. Irregular settling of latex particles indicates the positive reaction.

Precipitation tests

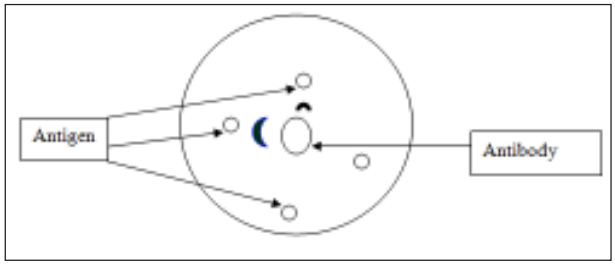
When antigen molecules are in soluble form, they combine with antibodies to form insoluble complexes. These complexes will precipitate and produce cloudiness in solution. This aggregate formation will reach maximum, when the antigen and antibodies are in optimal proportion. If one component is in high proportion the precipitation will be inhibited. The precipitation can be performed in test tubes. This test is used for detecting and identifying antigens, for typing of microbes. The technique is also used in forensic studies for detecting adulteration of foodstuffs. The modifications of this test are the immuno-diffusion techniques. The antigen and antibodies are allowed to diffuse and a concentration gradient forms in gel. The precipitation bands form in the gel in the position where the antigen and antibody reach optimal proportion after diffusion.

Simple test tube precipitation

Antiserum is taken in a test tube. Layer an extract of antigen/ microbe without mixing. Incubate the tubes at room temperature. Haziness will start appearing in the junction where the two suspensions meet.

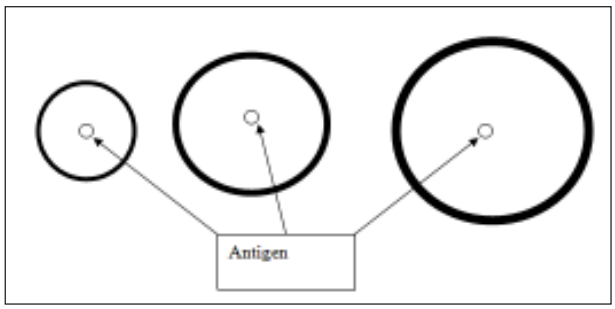
Agar gel precipitation (Ouchterlony's immuno diffusion)

1% Agrose gel is prepared in phosphate buffer and poured in to a petri dish. Once the gel cools, wells are cut in gel layer. Antiserum antigen solutions are placed opposite to each other in the wells. The gels are incubated at 37° C in a damp chamber for a few days. Precipitin bands will form, where antigen and antibody meet.



Single radial immuno-diffusion

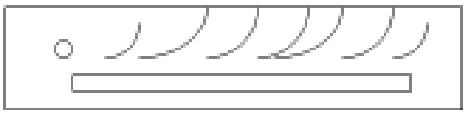
Antiserum is incorporated in agrose gel prepared in PBS (antiserum: gel = 1: 9). The gel is melted in water bath. When it is cooled to 50° C add the antiserum and mix it with agrose. The gel is poured in Petri dish. Wells are cut. Place antigen solution in the well and incubate at 37° C. The formation of a ring of precipitation around the well indicates positive reaction. The precipitation ring forms at a distance from the rim of the well, where the optimum concentration of antigen is attained. Hence the diameter of ring of precipitation is directly proportional to antigen concentration. Using different known concentrations of antigen in single radial immuno diffusion test, the diameters of precipitation rings can be plotted in a graph against concentrations of antigen. In this way the concentration of antigen in a test sample can be directly found from the plotted graph.



Increasing concentration of antigen

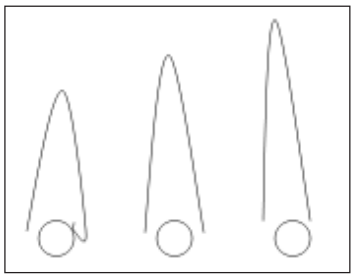
Immuno-electrophoresis

A number of immuno diffuson tests use the help of movement of colloids in an electric field. The electrophoresis separates the antigen mixtures. These separated antigens are used to perform precipitation reactions with antibody. This is commonly used for studying serum proteins. A well is made in electrophoretic agrose gel 1/3 distance away from one end of the gel in the midline and a narrow trough is made parallel to the well extending 2/3 length of the gel. The well is filled with the antigen mixture with indicator dye, and the electrophoresis performed till the indicator dye reaches 2/3 distance of gel (the gel is oriented with the antigen well near the cathode). After termination of electrophoresis the trough is filled with the antiserum and the gel incubated in a moist chamber as in immuno-diffusion. Several precipitation arcs will form in the gel, each representing one antigen.



Rocket electrophoresis

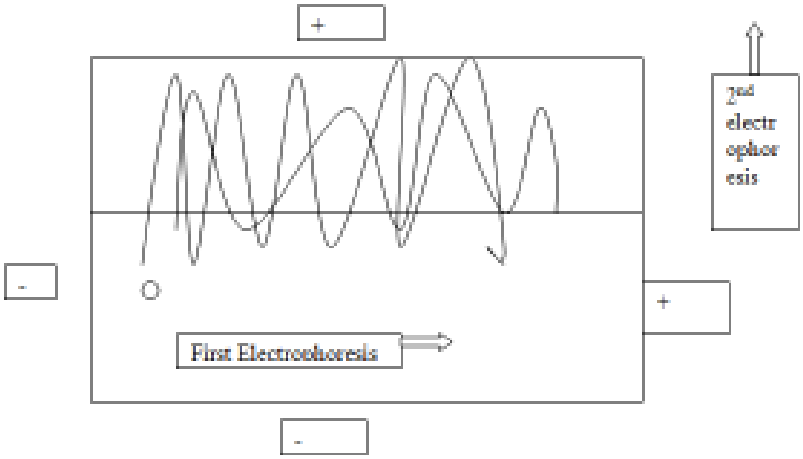
Antiserum is incorporated in the agrose gel (prepared in electrophoresis buffer) as in single radial immuno-diffusion. Then wells are cut in the gel, filled with antigen and electrophoresis is performed. Rising arc will be formed in the gel. The height of arc is proportional to antigen concentration. Like single radial immuno-diffusion a standard curve can be plotted for estimation of antigen.



Increasing concentrations of antigen

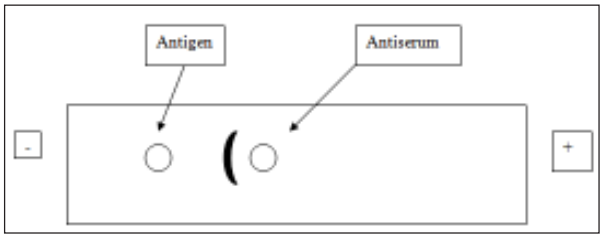
Two dimensional immuno-electrophoresis

A glass plate area is divided in two halves by holding another glass plate vertically. In one half ordinary electrophoretic gel is poured and allowed to solidify. The glass plate is removed after solidification of gel. The other half is filled with antiserum-incorporated gel. In the ordinary gel, a well is cut and antigen is filled. Electrophoresis is performed connecting the ends of ordinary gel to buffer tanks after this electrophoresis, the plate turned 90°. The ordinary gel portion is connected to buffer in cathode tank, while the antibody incorporated gel end is connected to anode tank. The electrophoresis is again performed, which result in development of arcs corresponding to each component of antigen mixture.



Counter current electrophoresis

This is a test, which demonstrate even minute quantity of antigen, hence a powerful diagnostic technique. Two wells are made in the gel in longitudinal direction the well near the cathode is filled with suspected antigen and the anode end well is filled with antiserum. Electrophoresis is run for one hour. In positive cases precipitation arc develops between two wells.



Activation of complement

Complement fixation test detects the presence of antibody in an animal or group of animals. Antibody combining with antigen exposes its FC portion, which makes the receptor to combine with the complements. Activation of complement system results in the lysis of cells bearing antigen. The test system consists of antigen (virus), dilutions of antiserum (serum is heated at 55 ˚C for 15 minutes) and a complement source (guinea pig serum). This system is incubated at 37 ˚C for 45 minutes. Indicator system consisting of SRBC and anti sheep erythrocyte serum (haemolysin) is added, absence of haemolysis after incubation indicate positive reaction. Positive and negative controls have to be run along with the test. This is highly effective for virus detection. The test has to be performed with different dilutions of antiserum and haemolysin.

Labeling techniques

Radioimmunoassay.
Fluorescent antibody technique.
Enzyme-Linked Immunosorbent Assay. (ELISA)
Immuno-Peroxidase

Fluorescent antibody technique

Fluorescein isothiocynate, and rhodamine isothiocynate produce fluorescence under UV light. Antibodies are conjugated with these dyes. Infected tissue smears are prepared, dried and fixed. A drop of conjugated antibody solution is placed over the smear. Incubate at room temperature for 15 minutes. Positive and negative controls are set up in parallel. The slide is washed thrice in PBS allowed to dry in dark. The slide is observed in a fluorescence microscope using appropriate UV light. 'Coombs' test is an indirect method and is more specific. Label an anti-globulin serum with the fluorescent dye. The slide/ smear is treated with the specific anti-sera, followed by labeled serum. The antigen in smear will combine with the antibody of first sera, which will be the antigen for the antibodies in the second sera.

Radioimmunoassay

Mostly used in the estimation of hormones. It is also used in the estimation of immunoglobulins like IgE. The radioiodine labeled antigen competes with non-labeled antigen of a sample under test, for the antibodies with which the labeled antigen and test sample are mixed. The more of antigen (hormone) in the test sample the less chance the labeled hormone has of combining with the limited number of antibody molecules that are available in the anti-hormone serum. Measuring quantity of

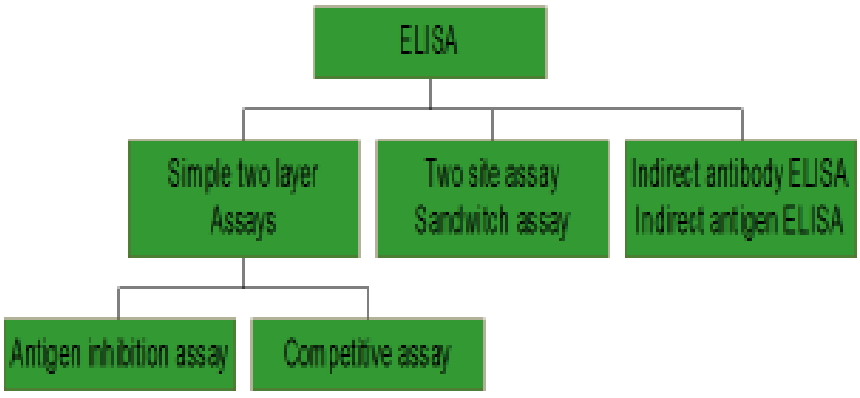
labeled hormone combined with antibody a measure of the hormone in the test sample can be obtained. Scintillation counter is used. Hormone antibody complexes are separated by electrophoresis or the antibody linked to an insoluble support (cellulose).

Enzyme labeling

- Enzyme Linked Immunosorbent Analysis (ELISA)
- Immuno-Peroxidase

Enzyme Linked Immunosorbent Analysis (ELISA)

Most antigens bind spontaneously to plastic surfaces like polystrene. Antibodies also attach while retaining their antigen binding activity. In subsequent steps, one or more layers of the solid phase captured immune complexes are formed. A variety of enzymes can be chemically coupled to either antibody or antigen without any change in their biological activity. An enzyme conjugate in antigen antibody complex leaves enzyme component available for substrate action. Addition of the substrate with the chromogen results in colour change. The reaction can be stopped at an appropriate stage and the colour signal determined by visual comparison with an appropriate standard or by optical density measurement. When the test requires the solid phase itself, the substrate should form an insoluble coloured precipitate. ——Dot. Enzymes commonly used are horseradish peroxidase, alkaline phosphatase and streptavidin (Avidin biotin complex).



Antigen inhibition assay

Coat the wells with standard antigen. Antibody enzyme conjugate is diluted to get optimal binding. The conjugate so diluted can be quantitatively inhibited by traces of free antigen in test samples. The diluted conjugate is then re-incubated with different dilutions of positive test samples in coated wells (the reactions are carried out in antigen coated wells). After washing the substrate is added there is inhibition of colour development. This inhibition is inversely proportional to the concentration of antigen in test sample.

Competitive assay

Wells are coated with specific antibody. Enzyme labeled standard antigen is titrated on the antibody to determine optimal dilution, which is easily competed for by free antigen. Standard free antigen dilutions are then used in competition with labeled antigen to construct a standard competition curve. Antigen from test samples is estimated by the inhibition.

Two-site (sandwich) assay

Antibody is coated on wells. Test samples are added to the antibody coated wells. The complex is exposed to diluted reference antibody conjugate to the same antigen. The test is carried out under the assumption each antigen molecule has multiple epitopes for antibody attachment, hence free antibody combining sites will be available after combination with coated antibody.

Indirect antibody ELISA or indirect antigen

Valuable tool to detect specific antibody in epidemiological studies. Antigen is coated in wells, followed by application of antiserum. Specific antibody is detected by the use of anti-globulin conjugate directed against first immunoglobulin (first antibody). It can utilize a "Universal" anti-species immunoglobulin.

ELISA-Avidin- biotin system

The low molecular weight coenzyme, biotin can be linked to the antibody using biotinyl-N-hydroxysuccinimide. It has less risk of steric interference with the binding site. The enzyme is brought into solid phase by using enzyme-coupled streptavidin, which has high affinity for biotin. One molecule of avidin bind with 4 molecules of biotin.

Immuno-histochemistry and immunoperoxidase

Permanent preparation can be made. Chemical and molecular components of cells can be studied. Enzymes such as horseradish peroxidase, alkaline phosphatase and glucose oxidase are used for conjugating antibodies. These reactions can be performed on histological section. Hence it is widely used for demonstration of macromolecules, microbial antigen in tissues/cells. The reaction products are insoluble coloured precipitates. These precipitates can be viewed under conventional microscope. The precipitate can also made electron dense by osmium fixation, hence electron microscopic studies are possible. Conjugating antibodies with gold and treating the section with conjugated antibodies enables electron microscopic detection of antigens. Direct and indirect methods are available.

Immuno-Dots or Dot blots

Modification of ELISA for practical field application. Antigen or antibody is spotted on to nitrocellulose paper using a fine pipette. The strip is then incubated in a blocking solution. Enzyme conjugated antibody or antigen is applied to the spot along with substrate.

Western blot analysis

This method combines enzyme-linked immunoassay with electrophoresis. Sample subjected to electrophoresis to separate components. The separated antigens are transferred to immobilising medium — nitrocellulose paper. Enzyme linked immunoassy is carried on the paper. This test is useful for characterization of antigens and antibodies.

It can detect presence of antigens in tissue fluids, sera; blood etc. The test is also used for detection of antibodies in a population following exposure to pathogens.

Poly acryl amide gel electrophoresis of suspected antigen source is done first. Blotting of separated proteins to nitrocellulose paper follows this. Either capillary blotting or electro-blotting achieves this.

Blotting of separated proteins to nitrocellulose paper

Capillary blotting

2-3 sheets of absorbent filter paper are saturated with transfer buffer and connected to buffer reservoirs. The gel is placed on top of the filter paper and the nitrocellulose paper is placed on the top of gel. 2-3 dry layers of dry blotting paper and several layers of paper towels are placed above the transfer paper and gel. A one-litre flask filled with water is placed over the paper towels. Blotting take 24- 48 hours.

Electro-blotting

It is done as per instructions of the manufacturers**.** Two or three layers of Whatman filter paper soaked in cathode buffer are placed over cathode plate of transfer system. The gel is placed over the filter papers and the nitrocellulose paper moistened with distilled water placed over the gel. Layers of the filter paper soaked in anode buffer follow this. Anode plate is placed in position and the system is connected to power supply. Electrophoretic transfer can be carried out at 100 volts for about 1.5 hours. After transfer the nitrocellulose paper is placed in an appropriate blocking buffer. Nitrocellulose paper is washed with tween tris buffered saline. Nitrocellulose paper is placed in diluted antibody for 1 hr at room temperature. Washing of nitrocellulose paper followed by placing the paper in conjugated antibody solution.

Though cell mediated reactions are of diagnostic value, they cannot be routinely used because of high technical skill required and the non-specific nature of many of these tests. However delayed hypersensitivity reactions are useful in diagnosing chronic bacterial diseases (tuberculin reaction in Mycobacterial infections) and systemic fungal infections.

Monoclonal antibodies

In conventional methods of antibody production the anti-serum contains antibodies wide range of specificity. This is because the molecule used as antigen has several antigenic determinants, each producing an antibody. This leads to non-specificity and cross reactions. This can be overcome with the use of monoclonal antibodies reacting with single antigenic determinant.

The principle of the method is fusing antibody producing cells with cells of a cultured myeloma cell line. Polyethylene glycol is the agent used to fuse the two types of cell. The myeloma cells are selected for their ability to grow in 8-azaguanine because they lack the enzyme hypoxanthine guanine phosphoribosyl transferase. This enzyme is required for rapid growth in tissue culture medium containing hypoxanthine, aminopterin and thymidine (HAT medium).

After fusion the cells are grown in HAT medium in a number of wells for10-14 days. The supernatants of each well are tested for antibody activity. Clones that secrete the require antibodies, usually only a small proportion of cultures, are selected and grown in bulk. This is done either in cultures or grown in peritoneal cavity of mice; the antibodies are secreted into tissue culture fluid or the peritoneal fluid of mice.

1. Mouse infected with antigen.
2. Spleen cells removed.
3. Fused to myeloma cells.

4. Hybrids grown in HAT medium in wells.
5. Supernatants tested for antibody.
6. Clones secreting require antibody selected and grown in bulk

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Appendix I

Case History Record

CASE #:_____ **DATE REC:**_____ **REC BY:**_____

FACILITY:_____ **STATE:**_____

☐ OTHER:_____

SERVICE:

Type: ☐Complete ☐Virology ☐Bacteriology ☐Diagnostic

☐OTHER:_____

FISH SPECIES:

1)_____ 2)_____ 3)_____ 4)_____

5)_____ 6)_____ 7)_____ 8)_____

MEMO:_____

_____

_____

TYPE & NUMBER OF SAMPLES:

_____

_____

_____

_____

_____

_____

_____

ALL DIAGNOSTICIANS NOTIFIED: Initials_____

Possession Assumed/Initials:

☐Virology_____ ☐Bacteriology_____ ☐Parasitology_____ ☐DFAT_____

☐ELISA_____ ☐PCR_____ ☐Other_____

Appendix II

LABORATORY FINDINGS:

□ VIROLOGY:_____

_____

_____

Completed/Date/Initials:_____ Sample Disposition:_____

□BACTERIOLOGY:_____

_____

_____

Completed/Date/Initials:_____ Sample Disposition:_____

□PARASITOLOGY/plankton centrifuge, digest,
PCR:_____

_____

_____

Completed/Date/Initials:_____ Sample Disposition:_____

□(BKD) *R. salmoninarum* /DFAT:_____

_____

ELISA:_____

_____

PCR: _____

Completed/Date/Initials:_____ Sample Disposition:_____

Results and Recommendations (if applicable):_____

_____

_____

About CMFRI

The Central Marine Fisheries Research Institute (CMFRI) was established by the Government of India under the Ministry of Agriculture in 1947. Over the period, the Institute has grown significantly in its size, stature & research infrastructure and currently enjoys the status of a premier research organization in the country. CMFRI has three Regional Centres at Mandapam Camp, Visakhapatnam & Veraval and Research Centres at Mumbai, Karwar, Mangalore, Calicut, Vizhinjam, Tuticorin and Chennai with excellent infrastructure and research facilities.

Adapting to the fast changing marine fisheries scenario the Institute has been carrying out pioneering research and development work in the field of fisheries with the objective of ensuring fisheries resource, ecosystem and livelihood sustainability in the country.

Marine Biotechnology Division

Marine Biotechnology Division has an R&D focus with a proper mix of expertise in different areas of marine biotechnology viz., genetics & genomics, fish nutrition & feed biotechnology, fish health including molecular diagnostics, and marine bio-prospecting.

The division is involved in basic, applied, and strategic research with special emphasis on mariculture and marine fisheries. The division has full fledged laboratories in genetics, genomics & molecular biology, nutrition, feed biotechnology, fish health including molecular diagnostics, microbiology, cell culture, electron microscopy, biochemistry & bio-prospecting. This division has developed two low-cost diagnostic PCR kits for WSSV which were commercialized. Under the division, the fish nutrition unit has developed an array of ornamental fish feeds that has been field tested & patented and is under the process of commercialization. Marine bioprospecting unit is dedicated to developing value added products from marine sources and a neutracutical product `GMe' has also been patented and launched.

Conducting regular training programmes and refresher courses for up grading the skills of scientists, teachers, researchers and R&D personnel in the areas of fish health & diagnostics, genetics, biotechnology, nutrition and bio-prospecting forms a part of the activities of the division. Marine biotechnology being an emerging area, the division looks towards new R & D areas in fisheries & marine sciences, focussing on the institutional & national priorities.