Microalgae as live feed

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Introduction

The development of mariculture is fully dependent on the availability seed of the fish or shell fish to be cultured. For this, hatchery production of the seed is imperative owing to the uncertain nature of natural seed resources. At present the seed production technology of some species with mariculture potential have been developed in India, which includes Cobia, *Rachycentron canadum*, Silver/Snubnose pompano, *Trachinotus blochii,* Indian pompano, *T.mookalee*, Orange spotted grouper, *Epinephelus coioides*, Sea bream, *Lethrinus lentjan*, Asian sea bass, *Lates calcarifer* etc. The larviculture of all these fishes invariably comprises of a stage during which only live feeds can be fed to them since their internal organ system is not equipped to effectively digest any artificial feed /pellet feed. Hence live feeds form an integral part of the seed production of these fishes. Live feeds used in the hatchery can be broadly classified into two groups, viz; Phytoplankton and Zooplankton.

Phytoplankton, which forms the base of the food chain includes the microalgae. Microalgae are microscopic organisms, typically found in freshwater, estuarine, and marine environment. These are required for larval nutrition during a brief period, either for direct consumption, in the case of molluscs and Penaeid shrimp, or indirectly as food for the live prey fed to the larvae. They are typically photosynthetic autotrophic organisms, which can produce complex compounds using simple substances available in their surroundings. Besides chlorophyll, they also show various carotenoid pigments which impart different colours to them.

According to the nature of photosynthetic pigments, algae are further classified into three divisions such as Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae). Brown and red algae are mostly marine forms while green algae i.e. Chlorophyta is mostly freshwater and free floating type. Nearly 16 genera of microalgae are commonly employed for aquaculture purposes. They are generally free living, pelagic and in the nannoplankton range (2-20µm). All algal species may not be equally successful as a live feed. Suitable algal species have been selected on the basis of their mass-culture potential, cell size, digestibility, and overall food value for the feeding animal.

Today, micro algae is used as an essential food source for rearing all stages of marine larvae of fishes (cobia, pompano, cod, halibut, tilapia ect.,) bivalve mollusks (clams, oysters, scallops), gastropods (abalone, conch), and shrimps (*Penaeus* sp., *Macrobrachium* sp.,). Micro algae also constitute an important source of food for live food organisms (rotifers, copepods, cladocerans, brine shrimp etc.) used in aqua hatcheries.

Major classes and genera of micro-algae cultured in aquaculture (FAO, 1996).

Class	Genus	Examples of application
Bacillariophyceae	*Skeletonema**	PL,BL,BP
	Thalassiosira	PL,BL,BP
	Phaeodactylum	PL,BL,BP,ML,BS
	*Chaetoceros**	PL,BL,BP,BS
	Cylindrotheca	PL
	Bellerochea	BP
	Actinocyclus	BP
	Nitzchia	BS
	Cyclotella	BS
Haptophyceae	*Isochrysis**	PL,BL,BP,ML,BS
	Pseudoisochrysis	BL,BP,ML
	dicrateria	BP
Chrysophyceae	*Monochrysis (Pavlova)*	BL,BP,BS,MR
Prasinophyceae	*Tetraselmis (Platymonas)**	PL,BL,BP,AL,BS,MR
	Pyramimonas	BL,BP
	Micromonas	BP
Cryptophyceae	*Chroomonas*	BP
	Cryptomonas	BP
	Rhodomonas	BL,BP
Cryptophyceae	*Chlamydomonas*	BL,BP,FZ,MR,BS
	Chlorococcum	BP
Xanthophyceae	*Olisthodiscus*	BP
Chlorophyceae	*Carteria*	BP
	*Dunaliella**	BP,BS,MR
Cyanophyceae	*Spirulina**	PL,BP,BS,MR

PL- penaeid shrimp larvae; BL- bivalve mollusc larvae; ML- freshwater prawn larvae; BP- bivalve mollusc postlarvae; AL- abalone larvae; MR- marine rotifers (*Brachionus*); BS- brine shrimp (*Artemia*); SC- saltwater copepods; FZ- freshwater zooplankton.

Cellular dynamics and reproduction

Microalgae may have different types of cell organization: unicellular, colonial and filamentous. Most of the unicellular cyanobacteria are nonmotile, but gliding and swimming motility may occur. Baeocytes, cells arising from multiple fission of a parental cell, may have a gliding motility. Swimming motility occurs in a Synechococcus sp., even if flagella are not known. Unicellular microalgae may or may not be motile. In motile forms, motility is essentially

due to the presence of flagella. The movement by the secretion of mucilage is more unusual. Gametes and zoospores are generally flagellate and motile. Some pennate diatoms have a type of gliding motility, as well as the red alga Porphyridium and a few green algae.

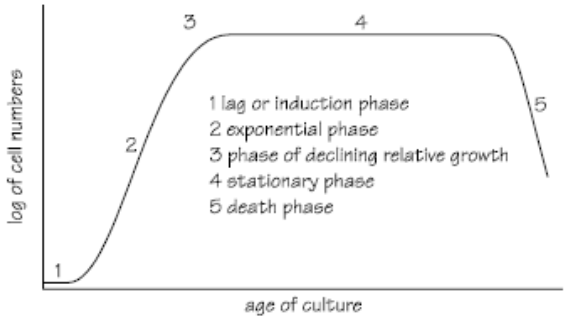
In unicellular microalgae the cell size generally doubles and then the cell divides into two daughter cells which will then increase in size. The cell cycle in eukaryotic algae involves two phases: mitosis and interphase. During the interphase the cell grows and all cellular constituents increase in number so that each daughter cell will receive a complete set of the replicated DNA molecule and sufficient copies of all other constituents and organelles. During the mitosis the nuclear division occurs. Vegetative reproduction by cell division is widespread in the algae and related, in many species, to an increase in cell or colony size. Other types of asexual reproduction occur by fragmentation and by production of spores, named zoospores if flagellate and aplanospores or hypnospores if nonflagellate. Autospores are also produced by various algae and are like aplanospores lacking the ontogenetic capacity for motility. Although sexual reproduction occurs in the life-history of most of the species, it is not a universal feature in algae. It involves the combination of gametes, often having different morphology and dimension, from two organisms of the same species (isogamy, anisogamy or oogamy). When the culture environment is favorable and all nutrients required for cell growth are present in a non-growth limiting quantity, i.e. at sufficiently high concentrations so that minor changes do not significantly affect the reaction rate, most unicellular algae reproduce asexually. The size and biomass of individual cells increase with time, resulting in biomass growth. Eventually, the DNA content is doubled in quantity and cell division ensues upon complete division of the cell into two progenies of equal genome and of more or less identical size. Population number is thereby increased, and population growth is therefore referred to as increase in population of the number of cells in a culture. The time required to achieve a doubling of the number of viable cells is termed doubling time. It is also termed generation time, as it is the time taken to grow and produce a generation of cells.

Growth cycle

A basic understanding of the growth cycle of micro algae would be helpful for successful operation of micro algae culture in a hatchery. The growth of micro algae passes through the following phases:

1. **Lag phase or induction phase**: This is the acclimation phase just after inoculation, where there is little or no growth is seen. The cells begin to absorb the nutrients and increase in size

2. **Log phase or exponential phase**: The cells reproduce very fast and population growth is exponential
3. **Transitional phase or phase of declining relative growth:** Here the cell division rate slows down as the light penetration through the culture reduces and nutrients becomes limiting.
4. **Stationary phase:**The number of cells remain constant as the growth and cell increase is compensated by cell death. This can last for many days in case of flagellates whereas for diatoms it can last for a short time.
5. **Decline phase or death phase:** The cell number decreases since the death rate exceeds growth.



Growth phase of micro algae

It is advisable to harvest the microalgae during their log phase, since in the new culture they will grow more rapidly and will yield a more viable population.

Micro algal culture Techniques

The micro algal culture in a hatchery can be broadly divided into two components:

- Stock culture of the pure strains
- Mass culture of the desired species

Stock culture

Stock culture of the pure strains of desired species of micro algae have to be maintained in a hatchery in a dedicated room under asceptic handling protocols to avoid any chances of contamination. This is essential to provide the desired species at any time of the hatchery operation. The stock culture forms the starter culture from which the quantity is scaled up to mass culture levels to meet the live feed requirements of the hatchery. Unlike natural seawater which contains many species of microalgae , in hatcheries, monoculture is to be maintained to scale up the desired species according to the nutritional requirements of the larvae or the zooplankton. Some of the common species of micro algae being used in mariculture seed production include

those belonging to the genus *Pavlova, Dicrateria, Thalassiosira, Isochrysis, Chaetoceros,Dunaliela, Nanochloropsis, Tetraselmis, Chlorella and Nannochloropsis.* The pure strains should be obtained from reputed, established and running hatcheries or repositories of R&D institutes. The traditional methods for obtaining pure culture involves isolating pure strains from raw sea water by various methods as listed below:

Enrichment: Twenty litres of water is collected from the water body and enriched with nutrients and left under low light (100 to 300 lux) until algal bloom occurs. The nutrient added for enrichment should be appropriate for the species to be isolated. The isolation of a single algal cell from the bloom can be accomplished by any one of the following methods:

2. Simple capillary pipette isolation Method: The mixed plankton sample is kept in a petridish under a binocular microscope. The desired species is isolated using a capillary pipette and transferred to culture tubes having suitable sterile culture medium.

3. Centrifuging method: By repeated centrifuging the water samples and then by inoculating the deposits, we can isolate several microalgae.

4. Serial dilution Method: This method is used mainly for the isolation of phytoflagellates (i.e. motile species). This involves systematic dilution of the inoculaum in five stages (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} or 4 steps 0.001, 0.01, 0.1 and 1ml) so that the subject species is well separated from any contaminant. The species thus isolated is transferred to the culture tubes.

5. Agar plating Method: Agar medium is prepared by adding 1.5 gm of agar to one litre of suitable culture medium. This agar medium is sterilized in an autoclave for fifteen minutes under 120 lbs pressure and 120ºC temperature. Now the medium is poured in sterilized 15 cm petri dishes and kept for 24 hrs. The required species can be picked by platinum needle or loop under microscope and streaked on the surface of agar plate. After inoculation, these petridishes are placed in an incubation chamber for 7-8 days providing light (1000 lux) and constant temperature (25ºC). Within this time, the required species, if it has grown into a colony is removed by platinum loop under microscope and transferred to culture tubes. Further from the culture tubes to small conical flasks and larger flasks, the algae can be grown on a mass scale.

The primary requirement for stock culture is the availability of pure cultures of the desired micro algae. The basic principle involved here is to culture and maintain the pure strains in sterilized sea water by adding nutrients and providing adequate light for the photosynthesis. The nutrients required for growth of micro algae is supplied by the culture media being used which is composed of chemicals, trace metals and vitamins. The common culture media used for marine micro algal culture includes Conway or Walne's media (Walne,1974), F/2 medium (Guillard, R. R. and Ryther, J. H. 1962), Erd-Schreiber's medium and Miquel's media (Miquel, 1892). The

algal pure strains are kept under standard controlled environment in conditioned rooms or in especially designed incubators, in which routine work can be performed under strict hygienic control. The pure strain cultures are usually kept in small glass containers, such as 10 to 25 ml test tubes or 100 ml glass Erlenmeyer flasks, closed by a sterile stopper (screw cap or a folded aluminium foil). Pure-strains cultures should be maintained at a steady or resting stage, i.e. under environmental conditions which allow them to reproduce, but not to increase exponentially in number. In this way, their sexual reproduction is fostered, thus increasing their genetic variability, and the growth of unwanted organisms such as other algal species, bacteria and ciliate protozoa is prevented. Culture parameters are therefore kept below the values adopted for mass production. In particular, only half dose of nutrients is used, water temperature is kept at around 14-16°C, light intensity ranges from 300 (test tubes) to 1000 lux (flasks) and no aeration and carbon dioxide are provided. Under routine conditions, strain cultures are usually renewed every month. In the replication process, an inoculum of 0.1-0.2 ml (from test tubes) or 0.5 to 1 ml (from Erlenmeyer flasks) is taken from the best old culture which is free of contamination, to inoculate three new vessels of the same size to start a new strain. The old culture is then either utilised for upscaling, or is discarded. Strain culture vessels should be stirred at least once a day by hand, paying attention not to stir bottom debris up.

Inoculation of flasks from test tube

Follow the same procedure as previously described for pure strains. Each 0.1-l flask will receive 50 ml of enriched medium and 0.5 ml of inoculum. At this stage, no aeration is required. When mature, each small flask will inoculate a new 2-l flask.

Inoculation of 2 and 5-l flasks from another 2 or 5-l flask:

- Prepare the necessary amount of new flasks filled with sterilised seawater, as well as all equipment required for the operation (pipettes, nutrients, cotton stoppers, aluminium foil, glass tubing, etc.).
- Select the mature culture that will be used as inoculum, checking a sample under the microscope for contaminants.
- Remove its cap and flame its neck; then close with a flamed aluminium foil stopper and let it cool.
- In the meanwhile, add the fertilizing working solutions to each new flask, at a rate of 1 ml/l; using a new sterile pipette for each solution.
- Flame their necks and aluminium caps thoroughly.
- When cool, remove the stopper and pour some algal culture of the old flask into the new

vessels at a rate of about 10% of the receiving volume, avoiding to wet their neck with the inoculum, then gently shake flasks to mix the new culture.

- Flame thoroughly their necks, introduce the sterilized glass tubing for aeration and close tightly with sterilised cotton stopper (or any other type of sterile cap).
- Write date and algal species on the new flask.
- Place the flasks on the lighted shelf and connect to the air delivery system, adjusting its flow to a gentle bubbling.
- After an hour, check all new vessels for a proper air bubbling.

Note: Use only the upper layer of the old culture, leaving dead cells and debris in the flask used to inoculate the newones. The size of the inoculum for small volumes is only 10% of the new volume because of the high cell density. Remember to label the flask properly and get rid of every contaminated flask. The above mentioned procedure applies to the upscaling of the other medium size vessels.

A general set of conditions for micro algal culture		
Parameters	**Optimum values**	**Range**
Temperature (^{0}C)	18-24	16-27
Salinity (ppt)	20-24	12-40
Light intensity (Lux)	2500 -5000	1000 – 10,000 (depends on volume and density)
Photoperiod (L:D; hours)	16: 8 (minimum) 24:0 (Maximum)	
pH	8.2 -8.7	7-9

Micro algal Culture Media

Media for the culture of marine phytoplankton consist of a seawater base (natural or artificial) which may be supplemented by various substances essential for micro algal growth, including nutrients, trace metals and chelators, vitamins, soil extract and buffer compounds. The term 'nutrient' is colloquially applied to a chemical required in relatively large quantities, but can be used for any element or compound necessary for algal growth.

Seawater base

The quality of water used in media preparation is very important. Natural seawater can be collected near shore, but its salinity and quality is often quite variable and unpredictable, particularly in temperate and polar regions (due to anthropogenic pollution, toxic metabolites released by algal blooms in coastal waters). The quality of coastal water may be improved by ageing for a few months (allowing bacterial degradation of inhibitory substances), by autoclaving (heat may denature inhibitory substances), or by filtering through acid-washed

charcoal (which absorbs toxic organic compounds). Most coastal waters contain significant quantities of inorganic and organic particulate matter, and therefore must be filtered before use.

Media preparation

The salinity of the seawater base should first be checked (30-35‰ for marine phytoplankton), and any necessary adjustments (addition of fresh water/evaporation) made before addition of enrichments. Seawater, stock solutions of enrichments and the final media must be sterile in order to prevent (or more realistically minimize) biological contamination of unialgal cultures.

Sterile filtration is done to sterilizing seawater without altering the chemistry of the seawater, as long as care is taken not to contaminate the seawater with dirty filter apparatus. Sterilization efficiency is, however, to some extent reduced compared with heat sterilization methods. Membrane filters of 0.2µm porosity are generally considered to yield water free of bacteria, but not viruses. 0.1µm filters can be used, but the time required for filtration of large volumes of culture media may be excessively long. Most stock solutions of culture medium additions can be sterilized separately by autoclaving, although vitamin stock solutions are routinely filtered through 0.2µm single use filter units, since heat sterilization will denature these organic compounds. Filter sterilization of all additions may reduce uncertainties about stability of the chemical compounds and contamination from autoclave steam, but absolute sterilization is not guaranteed. Stock solutions were stored in ultra clean sterile glass bottles. In order to minimize effects of any microbial contaminations, all stock solutions should be stored in a refrigerator at 4°C, except vitamin stocks which are stored frozen at –20°C and thawed immediately prior to use.

Conway' or Walne's medium	
Solution A.	**In 1 litre Dist. water**
Potassium nitrate	100 gm
Sodium orthophosphate	20 gm
EDTA (Na)	45 gm
Boric acid	33.4 gm
Ferric chloride	1.3 gm
Manganese chloride	0.36 gm
Solution B.	**In 1 litre Dist. water**
Zinc chloride	4.2 gm
Cobalt chloride	4.0 gm
Copper sulphate	4.0 gm
Ammonium molybdata	1.8 gm
Solution C.	**In 100 ml Dist. water**
Vitamin B1 (Thiamin)	20 mg
Vitamin B12 (Cyanocobalamine)	10 mg

Solution D (for culture of diatoms-used in addition to solutions A and C, at 2 ml per liter of culture)	
Sodium metasilicate (Na2SiO3.5H2O)	40.0 g
Make up to 1 litre with distilled water; Shake to dissolve	
Prepare stock solution A, B and C (each) in different reagent bottles. Add 1 ml of solution A, 0.5 ml of solution B and 0.1 ml of solution C and 1ml of D (only for diatoms) to 1 litre of filtered and sterilized seawater.	

f/2-Si *(Guillard, 1975)*Medium

To 996ml of sterile seawater aseptically add

Quantity	Compound	Stock solution (sterile)	Final conc. in medium
1.0ml	$NaNO_3$	75.0g/litre H_2O	884µM
1.0ml	$NaH_2PO_4.H_2O$	5.0g/litre H_2O	36µM
1.0ml	f/2 trace metal solution	(see recipe below)	(see below)
1.0ml	f/2 vitamin solution	(see recipe below)	(see below)

f/2 trace metal solution

To 950ml distilled H_2O add:

Quantity	Compound	Stock solution	Final conc. in medium
3.15g	$FeCl_3.6H_2O$	-	11.7µM
4.36g	$Na_2EDTA.2H_2O$	-	12µM
1.0ml	$CuSO_4.5H_2O$	9.8g/litre H_2O	0.04µM
1.0ml	$Na_2MoO_4.2H_2O$	6.3g/litre H_2O	0.03µM
1.0ml	$ZnSO_4.7H_2O$	22.0g/litre H_2O	0.08µM
1.0ml	$CoCl_2.6H_2O$	11.9g/litre H_2O	0.05µM
1.0ml	$MnCl_2.4H_2O$	178.2g/litre H_2O	0.9µM

Make up to 1 litre with distilled H_2O, sterilize (autoclave or filter) and store in fridge.

f/2 vitamin solution.

To 950ml distilled H_2O add

Quantity	Compound	Stock solution	Final conc. in medium
1.0ml	Vit.B_{12} (cyanocobalamin)	0.5g/litre H_2O	0.37nM
1.0ml	Biotin	5.0mg/litre H_2O	2.0nM
100.0mg	Thiamine HCl	-	0.3µM

Make up to 1 litre with distilled H_2O, filter sterilize into plastic vials and store in

freezer
Silicate is specifically used for the growth of diatoms which utilize this compound for production of an external shell. **Silicate Solution** Working Stock: add 30 g Sodium silicate (Na_2SiO_3) to 1 liter distilled H_2O**.**

Mass culture of micro algae

The mass culture is carried out to supply the algae for use in the hatchery for culturing the zooplankton. It is also required for the green water technique being used for larval rearing of marine fin fishes. The technique involves in scaling up of the stock culture to mass scale sufficient to meet the requirements of the hatchery. This can be done in outdoor or indoor. In outdoor it can be carried out with FRP tanks, cement tanks, Acrylic tanks, Polythene bags etc. Indoor mass culture can be done by providing sufficient lighting apart from the nutrients. This is usually carried out in acrylic tanks, Polythene bags, plastic carbuoys etc.

Outdoor mass culture: The outdoor mass culture can be done using commercially available fertilizers as nutrient supply source to reduce the cost. The proportion of the same is given below:

Media for outdoor mass culture		
Ammonium sulphate	**Urea**	**Super phosphate**
100g/tonne of water	10g/tonne of water	10g/tonne of water

Mass culture facilities for microalgae

Algae are cultured in a dedicated sector of the live feeds production section, which is made of three working areas inside the hatchery building: a lab for duplicating small cultures, a conditioned room to maintain small culture vessels and pure strains and finally a large area for the mass cultures in FRP tanks. Small volume cultures are kept in vessels ranging from 20-ml test tubes up to 18 l carboys. They can be made of borosilicate glass, polycarbonate, PET or any other material able to stand a sterilization process. These vessels are placed on glass shelves lightened by fluorescent tubes and equipped with a CO_2 enriched air distribution system. The unit also stores the special equipment to process pre-treated seawater, such as fine filters and sterilizers, as well as a laboratory where nutrients and glassware are prepared and stored, and where the necessary monitoring operations are performed. Standard cleaning procedures have to be strictly followed to maintain proper hygienic conditions.

Maintaining monoalgal culture in outdoor tanks

The major difficulty in operating *Chlorella sp.* and *Nannochloropsis sp*. outdoors cultures completely open to air-borne contaminants is that cultures become, too often, contaminated thereby becoming useless. Open cultures may be operated, therefore (either batch wise or in continuous cultures), providing a working protocol has been developed to control growth of foreign organisms. The greatest damage to these cultures is caused by grazers; most serious of which is *Paraphysomonas imperferata*, a non-specific heterotrophic flagellate, 7–12 m, with very short generation time. Appearing as soon as temperatures become high, it may crash a dense culture of *Nannochloropsis sp.* in 24 h. Another grazer is *Euplotes sp*., a 50–90 µ ciliate, which may graze on *Nannochloroposis sp*. cells as well as other organic matter in the culture, including bacteria. The presence of this protozoon is always associated with aggregation of the host's cells and reduction of cell numbers. Species of algae, e.g. diatoms mostly *Amphora* sp., may also become established in an open *Nannochloropsis* culture. Although diatoms are not regarded as direct competitors to *Nannochloropsis*, their cells excrete polysaccharides which cause *Nannochloropsis sp*. cells to stick together. Relatively low numbers of diatoms can cause serious damage affecting such cell conglomerates, arresting thereby culture growth, making the culture useless. Other contaminating microorganisms are colorless microflagellates, bacteria, as well as *Uronema sp*., 20–25 µ ciliates grazing on bacteria.

Controlling contaminants

Different treatments may be applied to eliminate contamination, thereby facilitating cultivation of *Chlorella sp.* and *Nannochloropsis sp.* in open culture system:

(a) **Lowering the pH**: This represents a useful tactic by which to arrest development of some contaminants. pH 6 is low enough to eliminate diatoms in a *Nannochloropsis sp*. culture; the aggregates dissolve and within a few hours the culture regains its normal appearance. Elimination of *Paraphysomonas* cells and similar contaminants requires the pH to be lowered to 2.5 for a couple of hours. *Nannochloropsis sp*. cells lose their photosynthetic capacity at this pH, which should thus be soon raised to 5.5 or 6.0 at which pH cells regain their normal functioning and after a short period of photosynthesis, the pH rises to the 8.0–8.5 at which it is maintained.

(b) **Chlorination**: Chlorinating contaminated *Chlorella sp.* and *Nannochloropsis sp.* cultures using concentration of 4 to 10 ppm of active chlorine is quite effective in eliminating grazers. Density of the cultured cells, the organic load (dissolved and particles) and the temperature represent the parameters to be considered in applying an adequate chlorine concentration dose

for effective treatment. High doses of chlorine would be used for dense cultures of high organic loads and when culture temperatures are high, thus in summer, there is a need to chlorinate up to twice a week, whereas one application may be sufficient for two months during the cold season. Tank walls should be cleaned every five to six days in winter and after every successful culture in summer.

Harvesting

For both *Chlorella sp.* and *Nannochloropsis sp*. mass culture system, the desired volumes are pumped out every day either directly to the consumers or to an industrial centrifuge, creating a 40% solid paste, which is preserved for later use. After harvest, a mixture of fresh sea and tap water, including nutrients, is introduced in the tanks, bringing the culture back to its original volume.

Production quality

The harvested microalgae is used for culturing rotifers and for maintaining the rotifers' nourishing value in the larvae rearing tanks of marine fish (Green Water). It is essential to focus on the algae quality, particularly the total lipid content and polyunsaturated fatty acids (PUFAs). Cell density in an open culture of *Nannochloropsis sp*. may exceed 200 x 10^6 cells ml^{-1}. At this cell concentration, however, productivity would be much below maximal. For maximal sustainable yield, the optimal cell concentration of 100–120 x 10^6cells m^{-1}, depending on the season and cultures depth, should be maintained. The output rate (in dry weight) of cell mass varies greatly with the season. In summer, 25–30% of the culture is harvested per day, yielding an average of 6g (dw)/t. In winter, due to shorter daylight and lower temperatures, only 15% of the culture is harvested daily, averaging 4 g (dw)/t.

Methods of culturing micro algae:

The micro algal culture can be done as batch culture or continuous culture

Batch culture

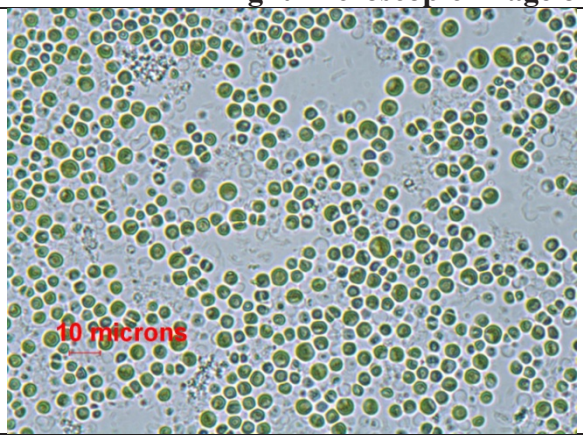
This is the most common method for cultivation of microalgal cells. In limited volume (batch) cultures, resources are finite. When the resources present in the culture medium are abundant, growth occurs according to a sigmoid curve, but once the resources have been utilised by the cells, the cultures die unless supplied with new medium. In practice this is done by sub culturing, i.e. transferring a small volume of existing culture to a large volume of fresh culture medium at regular intervals. In a simple batch culture system, a limited amount of complete culture medium and algal inoculum are placed in a culture vessel and incubated in a favorable environment for growth. Some form of agitation, such as shaking or impeller mixing, is necessary to ensure nutrient and gaseous exchange at the cell–water interface. The culture vessel can be a simple conical flask or an environment controlled fermentor. Batch culture is widely

used for commercial cultivation of algae for its ease of operation and simple culture system. Since the process is batch wise, there is low requirement for complete sterilization. For mass algal culture production, a portion of the culture could be retained as inoculum for the next culture batch. The different phases, which may occur in a batch culture, reflect changes in the biomass and in its environment.

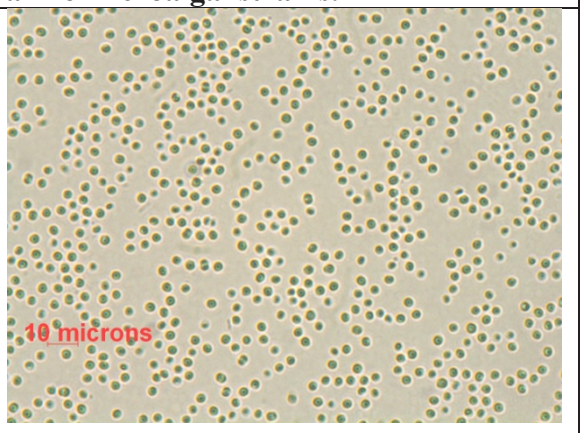
Continuous cultures

In continuous flow cultures, fresh culture medium is supplied to the homogeneously mixed culture and culture is removed continuously or intermittently. Here the resources are potentially infinite: cultures are maintained at a chosen point on the growth curve by the regulated addition of fresh culture medium. In practise, a volume of fresh culture medium is added automatically at a rate proportional to the growth rate of the algae, while an equal volume of culture is removed. The approach is based on the observations that substrates are depleted and products accumulate during growth. Eventually, culture growth ceases due to depletion of the growth limiting substrate or accumulation of a growth-inhibiting product. To sustain cell growth, the growth-limiting substrate needs to be replenished and the growth inhibitory product needs to be removed or diluted by adding fresh culture medium.

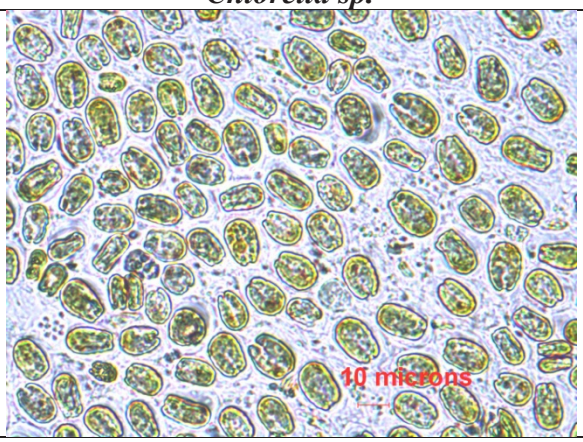
Light microscopic image of marine microalgal strains.



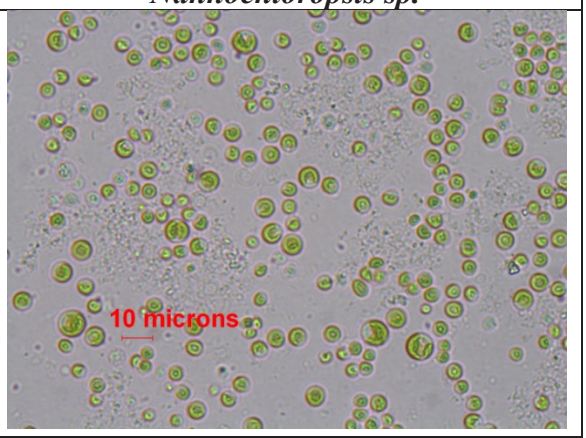
Chlorella sp.



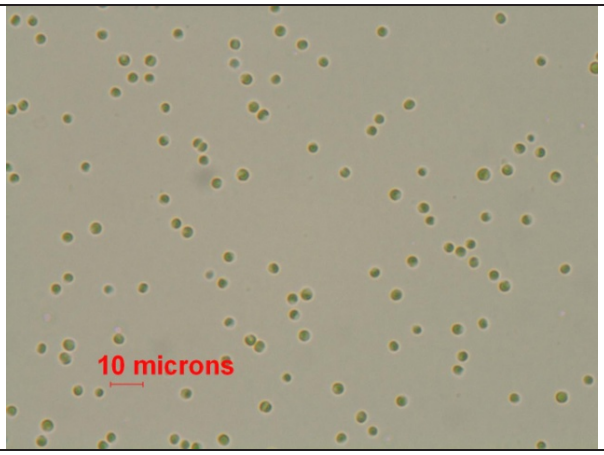
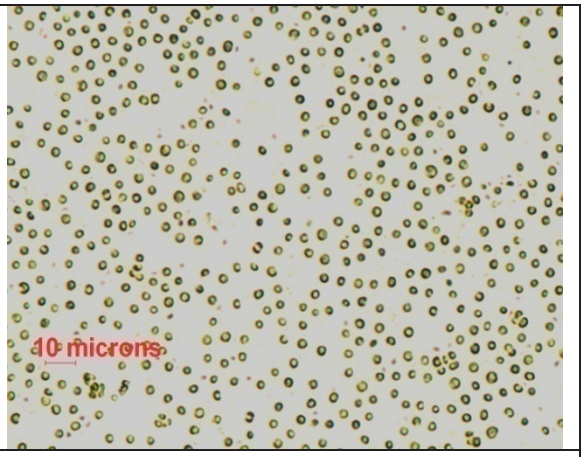
Nannochloropsis sp.



Tetraselmis sp.



Dunaliella sp.

	
Pavolova sp.	***Isochrysis sp.***

Hygenic handling protocols:

The cardinal principles involved in micro algal culture section is to avoid any chances of contamination of the cultures, for which the personnel manning the section shall be aware of the hygenic handling protocols which are detailed below:

Work in clean place or preferably in a laminar flow cabinet (cabinet must be turned on at least 30 minutes before transfer; if equipped with UV lamps, leave on overnight prior to use). Clean the working surface with 70% alcohol (ethanol/isopropanol) prior to and after use. Clean hands with disinfecting soap and rinse with 70% alcohol prior to all operations. When not using a laminar flow cabinet (and to be safe even when using a cabinet), sterilise (flame) the neck of vessel of origin before and after transfer (not possible with some plastic vessels, which must, therefore, be opened in a laminar flow cabinet). Pipettes must be clean and sterile; use autoclavable tips for repeating pipettes, pre-wrapped sterile single use plastic/glass pipettes, or if using non-sterile glass pipettes (with cotton plugs), sterilise in the flame before use.

The preparation of glass wares and containers is a vital step in the microalgal culture: The step by step protocol is given below:

- wash with detergent
- rinse in hot water
- clean with 30% muriatic acid
- rinse again with hot water
- dry before use.

Alternatively, tubes, flasks and carboys can be sterilized by autoclaving and disposable culture vessels such as polyethylene bags can also be used.

Cleaning procedure

The culture flaks have to be scrubbed (abrasive brushes not appropriate for most plastics) and soaked with warm detergent (not domestic detergents, which leave a residual film on culture ware-use laboratory detergent such as phosphate-free Decon); rinse extensively with tap water; soak in 10% HCl for 1 day-1 week (not routinely necessary, but particularly important for new glass and polycarbonate material); rinse extensively with distilled and finally double distilled water; leave inverted to dry in a clean, dust-free place.

Sterilization

Sterilization can be defined as a process which ensures total inactivation of microbial life (not the same as disinfection, which is defined as an arbitrary reduction of bacterial numbers). The primary purpose of sterilization is to prevent contamination by unwanted organisms, but it may also serve to eliminate unwanted chemicals. There are several sterilization methods and the choice depends on the purpose and material used:

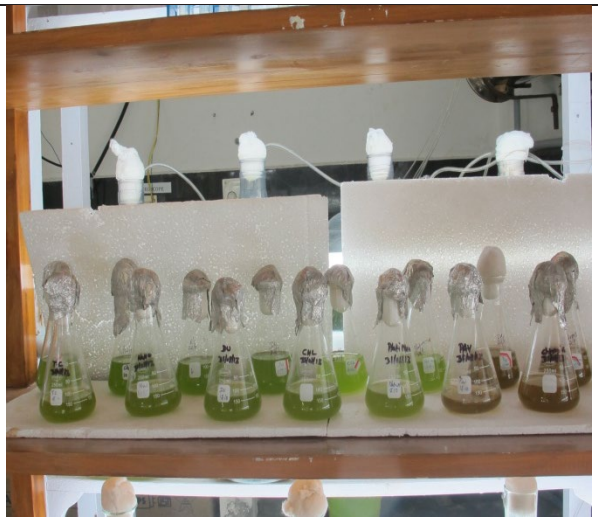
Moist heat

Autoclave or pressure cooker (1-2 Bar, 121°C, in pure saturated steam). For sterility the steam must penetrate the material (wrap in material that allows access of steam-kraft paper or aluminium). Autoclave steam may introduce chemical contaminants; glass and polycarbonate vessels should be autoclaved containing a small amount of double distilled water which is poured out (thus diluting contaminants) under sterile conditions immediately prior to use. Never close vessels (risk of implosion); use cotton wool bungs, or leave screw caps slightly open.

Mixing-Mixing of micro algal cultures may be necessary under certain circumstances: when cells must be kept in suspension in order to grow in concentrated cultures to prevent nutrient limitation effects due to stacking of cells and to increase gas diffusion. Mixing has to be given through gentle bubbling with air.

Microalgal indoor and outdoor culture systems.

Miacroalgal stock culture in test tubes



Miacroalgal stock culture in conical flasks

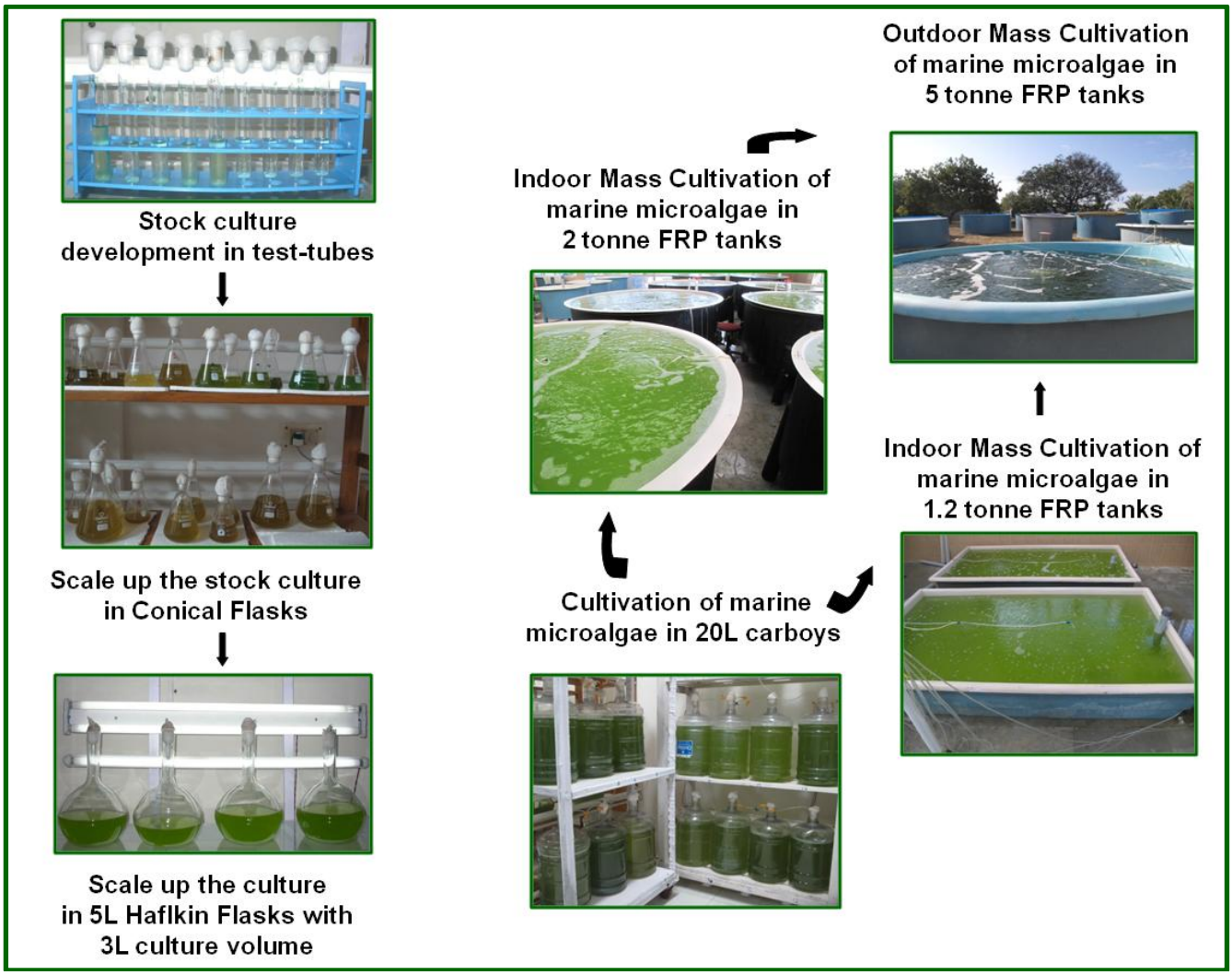
***Nannochloropsis sp.*in haffkine flask**

***Isochrysis sp.* in haffkine flask**

***Nannochloropsis sp.* in 20L carboys**

Outdoor mass algal culture unit

Figure 4. Marine microalgal scale up, indoor and outdoor mass cultivation process.



Measurement of cell growth and culture productivity

Net growth may be estimated quickly by measuring changes in the overall turbidity of the culture. This, however, provides only a rough estimation of growth and should be followed routinely with other measurements such as cell count, dry weight or total organic carbon (TOC). Cell chlorophyll and protein may be suitable for expression of growth in algae, but should be used with caution, particularly in outdoor cultures, being strongly affected by environmental conditions.

Turbidity

The turbidity can be measured at three different nanometers 540nm, 640nm, and 660nm respectively along with culture medium in sterile seawater as a blank by using a spectrophotometer.

Counting Cells in Cultures with the Light Microscope *using a Haemocytometer*

Haemocytometer

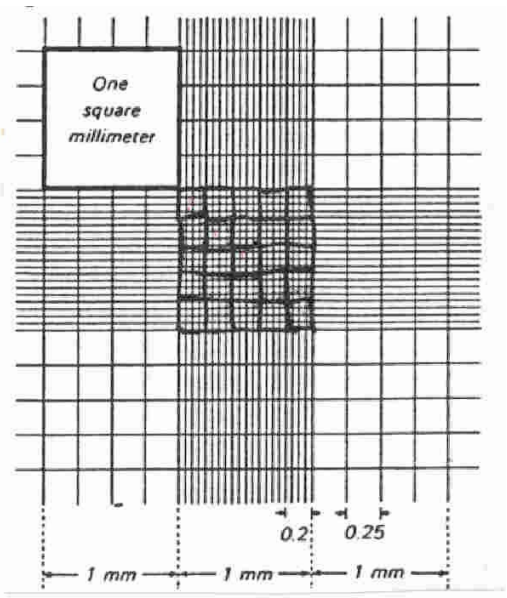
As the name suggests these counting chambers have been developed for counting blood cells but they can be used to calculate the cell density of an algal culture provided the cells are relatively small (~ 5-50µm) and either single cells or short chains. The size of these chambers can vary with manufacturer but we use a Neubauer brand which consists of two chambers, each with a volume of $0.1mm^3$, containing a marked counting grid $1mm^2$ in area. The haemocytometer can be used where cell densities are in the range 5×10^4 - 10^7 cells / mL.

Method

1. Algal Sample –algae is non-motile cells which do not need fixing. Counting has to be done as soon as the sample is collected. Algal growth is measured by counting the cells in a 24hr interval.

2. To fill haemocytometer chambers, thick cover glass is placed over both grids and take a Pasteur pipette and fill its tip by capillary action with sample. Hold the pipette at an angle of 45^0 (higher or lower to control flow rate) and place the tip at the leading edge of the cover slip. With very gentle pressure, allow the sample to flow quickly and evenly into the chamber, exactly filling it. The chamber surface in the Neubauer brand is a flat mirror-like rectangle and the sample must cover this rectangle but not flow over its edges. It is useful to rest your hand on a bench and steady the pipette tip with a finger. If flooding occurs, rinse haemocytometer and cover slip with distilled water, and repeat procedure. Refill the pipette for each chamber. The time taken to fill the chamber should be short, to minimize setting of cells in the pipette.

3. Allow cells to settle (1min) and check the grid under the microscope for satisfactory distribution of cells, i.e. evenly spread.



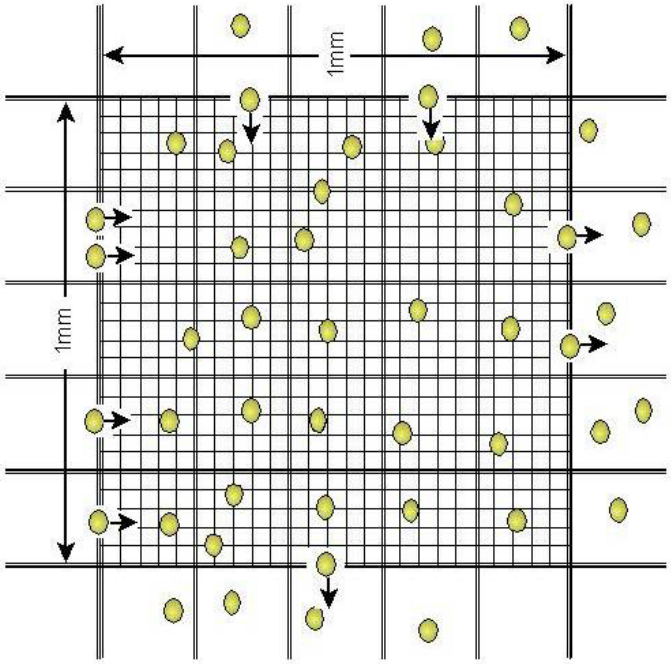
The Haemocytometer grid in detail

The grid is divided into 9 large squares, each 1mm x 1mm, by triple lines. Each large square is divided into 25 medium squares, each 0.2mm on a side, and each medium square is further divided into 16 small squares, each 0.05mm on a side.

Haemocytometer (Neubauer brand).

For all haemocytometers, the fundamental measurement is the average number of cells per 1mm^2 so the centre large square is usually counted. To obtain the total number of cells in this large square, the number of cells in each of the 25 medium squares are counted, recorded then added.

Note: When counting cells bordering on triple rulings, the convention is to count only those cells touching the top and left-hand side rulings of each square



After counting each of the two haemocytometer chambers, the haemocytometer and coverslip are rinsed with distilled water. Usually the procedure is repeated twice more to give a total of 6 counts. The cell density was obtained by calculating the average cell count and multiply by the conversion factor (for Neubauer = No of cells x10^4 cells/ml).

Biomass estimation

Dry weight of algal cells can be determined by filtering and drying algae from aliquots of culture of known concentration

1. Filter an exact volume of culture on pretared glass-fiber filters (1 µm pore size) using a filtration setup connected to a vacuum pump.
2. Wash the filter with a solution of ammonium formate (0.5 M) to remove salts.
3. Follow the same procedure with control filters on which an equal volume of 0.22-µm filtered seawater is filtered.
4. Dry the filters at 100 °C for 4 h to volatilize the ammonium formate
5. Weigh on an analytical balance.
6. Calculate the dry weight per algal cell according to the formula:

$$DW\ (g.cell^{-1}) = (DWA - DWC) / (N \times V)$$

Where,

DWA = average dry weight retained on algal filter (g)

DWC = average dry weight retained on control filter (g)

N = algal concentration (cells/ml)

V = volume of algal culture and filtered seawater filtered on algal and control filter, respectively (ml)

In order to improve the correction for salt residues and the variation among samples, cellular dry weight can be determined in triplicate from regression analysis of DW retained on the filter versus number of algal cells filtered.

CONCLUSION

In mariculture seed production technology, microalgae culture is an inevitable component. It forms the food for growing the zooplankton apart from being used for the green water technique commonly employed in larviculture of marine finfishes. The microalgal section is the starting point of life in a hatchery. So it is the most important section with regard to observance of strict hygienic protocols to avoid any type of contamination, because any contamination at this stage would spread to all other sections. It is always advisable to assign different personnel for indoor stock culture maintenance and outdoor mass culture, so that cross contamination does not occur. The growth and quality of the algae has to be monitored regularly by well trained and qualified personnel. Periodically the stock cultures should be renewed with new stock, since over a period of time, the quality of the same strain/stock may deteriorate. The uncertain nature of weather conditions and risks of contamination is a major bottleneck in the successful mass production in outdoor tanks. This could be overcome to a great extend by establishing mass production facilities indoors by providing artificial lighting.