INVESTIGATIONS ON THE STRESS EFFECTS OF PETROLEUM HYDROCARBONS ON *METAPENAEUS DOBSONI* (MIERS)

THESIS SUBMITTED TO THE
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY IN PARTIAL
FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MARINE BIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

BY

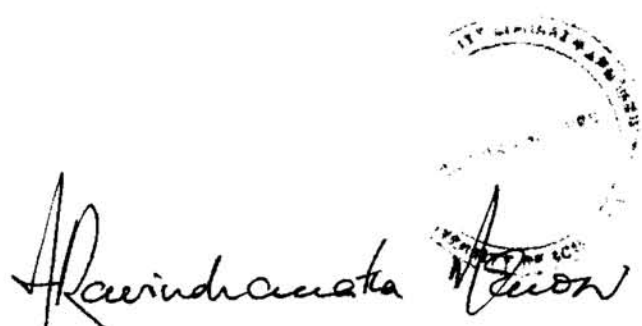
MIRIAM PAUL

DIVISION OF MARINE BIOLOGY, MICROBIOLOGY AND BIOCHEMISTRY
SCHOOL OF MARINE SCIENCES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI 682 016

2001

CERTIFICATE

This is to certify that the thesis entitled " Investigations on the stress effects of petroleum hydrocarbons on *Metapenaeus dobsoni* (Miers)" is an authentic record of the research work carried by Ms. Miriam Paul under my scientific supervision and guidance in the School of Marine Sciences, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy of the Cochin University of Science and Technology and that no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.



PROF. DR. N. RAVINDRANATHA MENON,

Director

School Of Marine Sciences,

Cochin University of Science and Technology,

Kochi 682 016

Kochi 16

5 June 2001

DECLARATION

I hereby declare that this thesis entitled " Investigations on the stress effects of petroleum hydrocarbons on *Metapenaeus dobsoni* (Miers)", is a genuine record of the research work done by me under the scientific supervision of Prof. Dr. N. Ravindranatha Menon, Director, School of Marine Sciences, Cochin University of Science and Technology, Kochi and that this has not previously formed the basis of the award of any degree, diploma or associateship in any University.

MIRIAM PAUL

Kochi 16

5 June 2001

ACKNOWLEDGEMENTS

It is with great pleasure that I express my sincere gratitude to my esteemed supervising teacher, Prof. Dr. N. R. Menon, Director, School of Marine Sciences, Cochin University of Science and Technology, Kochi, for his invaluable guidance, constant encouragement and the rare degree of intellectual freedom that he nurtures.

I take this opportunity to thank Prof. Dr. Babu Philip, Head, Department of Marine Biology, Microbiology and Biochemistry and Prof. Dr. R. Damodaran, Dean, School of Marine Sciences, CUSAT, for their advice and support.

I am also grateful to the Directors of the Central Marine Fisheries Research Institute, Kochi, Dr. M. Devaraj (Retd.), Dr. V. N. Pillai (Retd.) and Dr. Mohan Joseph Modayil, for their kind permission to complete this work.

I express my thanks to Dr. A. Mohandas, Dean, School of Environmental Science, CUSAT, for facilitating the preparation of the photomicrographs. The scientists and staff of the electron microscopy unit of the Fishery Environment Management Division, CMFRI and the Regional Microscopy Facility, All India Institute of Medical Sciences, New Delhi, have rendered much assistance in the preparation of the electron micrographs. I am very grateful to Prof. Dr. G. G. Martin, Occidental College, Los Angeles, California (USA) for his critical evaluation of some of the electron micrographs.

I thank Shri. C. I. Roy and Shri N. Cherian Punnoose of the Kochi Refineries Limited, Kochi for the supply of crude oil for the experiments.

I thank the boat crew of *R V Kingfisher*, Shri Shaji of the Central Brackishwater Research Institute, Narakkal and the fishers of Vypeen for their sincere efforts and patience on sample and specimen collection trips. I would like to take this opportunity to pay tribute to their deep understanding of the dynamics of the Vembanad estuary and its biota.

I express my sincere gratitude to Dr. Ajith Kumar, Assistant Professor and my respected in-laws, Prof. Dr. L. Prema and Prof. Dr. R. S. Iyyer (Retd.)

of Kerala Agriculture University, Vellayani, for help with statistical analysis of results.

I gratefully recall the assistance and encouragement given to me by the research scholars and staff of the School of Marine Sciences and the School of Environmental Studies. A special thanks is due to my respected teachers Dr. Rosamma Philip of the School of Marine Sciences and Dr. Bright Singh, School of Environmental Studies, for help and encouragement with the microbiology component. I thank my friends Giri Kumar and Radhika Sehgal for logistic support in New Delhi. And for their infectious enthusiasm, empathy and prompt assistance, Soumhya Venkatesan and Dr. Jonathan Wolfe at Cambridge University, U. K. and Anu Idikula at Rhodes University, RSA.

Last but not least, I thank my family for their support and endurance, especially my brother Rajesh Paul Aalapat. My heartfelt appreciation to my in-laws for their encouragement and blessings, my cousin Arun Paul A. C for help with thesis preparation and Unny for assistance with outdoor chores. I apologise to my little girl Shama, for being an absentee mother and not spending quality time with her in her infancy. Words are insufficient to express my gratitude for the affection, understanding and support rendered by my mother, Lalitha Paul and my husband, Lt. Cdr. S. Sreeram.

This thesis has been made possible by the generosity of all those mentioned above.

MIRIAM PAUL

To my beloved Father for bequeathing me with the Joy of Nature

CONTENTS

PREFACE

During the past four decades, the environmental agenda has included, in succession, depletion of the Earths resources (1960's), regional pollution (e.g. acid rain – 1970s'), global change (e.g. stratospheric ozone hole – 1980s') and sustainability (1990s'). In the immediate future, international environmental agendas would focus on global changes, pollution, waste disposal, biodiversity, natural and man made risks and hazards, sustainable management of resources, including land and marine resources, fresh water supplies and coastal zones.

The history of ecotoxicology has a clear-cut chronology. And it developed in a sequence lasting for about fifty years, commencing from discovery and passing through different stages like alarm, recognition and maturation, culminating in "Eco" concerns. Regulatory ecotoxicology and scientific ecotoxicology are complimentary. The toxic effects are studied qualitatively to identify the fate of chemicals in organisms, the organs and the physiological functions affected and to assess the molecular mechanisms involved. In general comparative studies are required in order to learn whether experimental studies with a limited range of laboratory models also apply to species living in the natural environment. Assessment of dose response relationship is the most important qualitative aspect of toxicology.

Several petroleum hydrocarbons are priority rated pollutants and are present in high concentrations in certain coastal areas. To understand their impact biological assays are carried out with suitable organisms in the laboratory. The investigated biomarker could be a

xenobiotically induced physiological variation, expressed as cellular, sub-cellular or biochemical components. Changes in physiological processes or alterations in structure and function put together, cellular injury, deformation of cell structures, parasitism and changes in biomass are some of the most important histological and behavioural parameters looked into by investigators to assess oil toxicity on marine biota.

The present investigation has addressed the effects of PHC contaminated culture medium on the morphology, physiology and behaviour of shrimps[1]*. The shrimp Metapenaeus dobsoni is an important member of the crustacean animal community abounding the oil contaminated benthic regions of Cochin backwater system. Since it is known that true pollutants can disrupt the sustainability of ecosystems by its effect on species, populations and communities, a representative species was used for the study. The results discussed in this work is bound to help in understanding the ecotoxicant resistance that the animal may display under toxic conditions compared to dynamic steady-state systems in nature.*

[1] Paul M., Menon N. R. and Philip R., 2000. Stress response and vibriosis induced by petroleum hydrocarbons in the penaeid shrimp *Metapenaeus dobsoni* (Miers). In: Oil & Hydrocarbon Spills II: Modelling, Analysis and Control. Rodriguez G. R. and Brebbia C. A (eds.) WIT Press, Southampton, U. K., p. 107-116.

Oiled rocks and chocolate mousse formation persisting along the coast of Vypeen Island on the third day after an oil spill of unknown origin in April 1998.

GENERAL INTRODUCTION

Modern human civilization has been petroleum driven for more than a century. This naturally occurring substance, which is a complex mixture of hydrocarbons and their derivatives derived from primordial organic deposits, is accumulated in geological reservoirs from whence it is extracted, transported, refined and converted to myriad products such as fuel, plastics and synthetics. Crude oil and natural gas are indigenous components of the natural ecosystem. Living organisms have evolved biological mechanisms to metabolise these complex mixtures within tolerable limits. However, massive influxes of petroleum hydrocarbons into the marine ecosystem occur due to anthropogenic activities with rapid alterations in structure and composition of the refined products introduced, as opposed to the low-level but long term inputs from natural seeps. This often results in the upsetting of the ecological equilibrium of affected habitats. Anthropogenic redistribution of petroleum and its byproducts into the atmosphere, geosphere and hydrosphere takes place through handling losses during every step of petroleum extraction and transportation, discharge of effluents during its processing and combustion or willful disposal of its byproducts such as fuel, plastics and synthetics. "Oil pollution" as a result is a ubiquitous phenomenon. The effects of this form of pollution are evident from its sources of extraction in the marine oil fields to erstwhile pristine habitats like the polar regions.

Visually and physically oil pollution is the most evident of all categories of pollution in the ocean. Aesthetically, this form of pollution in its extreme - as

oil spills - evokes the strongest of reactions, resulting in much media attention and mobilising of public opinion that reflects in mitigative efforts by means of legislation, policies and ocean management programmes.

The effects of tanker and oil rig accidents, spillage, oil well blowouts and sabotage of oil fields as acts of war are immediate, acute and very evident, though locally restricted and strongly dependent on local circulation, prevalent weather and geographical features for dispersal and removal. However, the above account for less than 5% of the petroleum hydrocarbons entering the marine ecosystem. The current estimates are that 65% of oil pollution arises from landbased sources such as dumping, atmospheric fall-out, land run offs, coastal refineries and off shore platforms. Of the remaining 35%, 20% comes from shipping operations such as deballasting and bilge discharge and 10% from intentional discharges. The extent of damage inflicted on biological communities and ecosystems and the effects of low concentrations of petroleum hydrocarbons reaching and persisting in the sea goes virtually unnoticed. Owing to the social factors involved with oil spills, early investigators focused attention on the immediate effects of oil pollution and its effects on mostly non-commercial resources such as marine birds and seals or on mass mortality of adult fishes. This fact coupled with the readily biodegradable nature of oil and the remarkable stability of degraded oil derivatives in the marine ecosystem brought about a trend in scientific thinking that dismissed pollution by petroleum hydrocarbons as one of the lesser evils among marine pollutants. Not much attention was paid to long term impacts such as alterations in the effect of petroleum hydrocarbon toxicity on plankton community structure, subsequent recruitment of juveniles

of exploited fishery resources or epidemiology in afflicted communities. The effects of sublethal levels of oil on ecosystem modification and community structure still remain poorly understood.

The wreck of the oil tanker *Exxon Valdez*, in Prince William Sound, Alaska spilled 11 million tonnes of crude in March 1989. It created unprecedented world opinion against oil pollution, which renewed interest in this field of pollution research. Prior to this two other wrecks, one that of the tanker *Torrey Canyon* off the coast of Lands End, UK (1967) releasing 38.2 million gallons of oil and that of the tanker *Amoco Cadiz* off the coast of Brittany in France (1978) resulted in large-scale oiling of shorelines. The wreck of the *Braer* off the ecologically sensitive Shetland coast involving the discharge of 8 million gallons of oil (1991) was dwarfed by the catastrophe in the Persian Gulf which saw the release of 240 million gallons of oil into the sea during the Gulf War from the Sea Island installations off Kuwait and off Saudi Arabia. Until this event the blow out of *Ixtoc I* oil station was the single most devastating oil pollution incident involving 140 million gallons of crude. Subsequent effects of the Gulf war on the ecosystem are still in controversy as adequate documentation to the situation preceding the war was unavailable. The Persian Gulf, owing to its location rimming the most productive oil fields currently exploited in the world has been subjected to repeated large oil spills. The blow out at platform No.3 at *Nowruz* oil field, Iran involving 80 million gallons of oil (1983), the wreck of the tanker *Pericles GC*; in Persian Gulf, 30 km east-north of Doha spilling 14 million gallons of oil (1983), the tanker *Nova* in 1985 in the Persian Gulf, 140 km south of Kharg Island and innumerable other tanker and platform accidents during the

transport of massive quantities of oil year round lead to constant contamination of the seas in this region. In Indian waters, there have been several large scale spills, including that following the wreck of the Greek tanker *Ampuria* off the coast of Kutcch (1970), the *M. T. Cosmos Pioneer* again in the north western coast of the Arabian sea (1973), the American oil tanker *Transhuron* which ran aground at Kiltan, Lakhshadeep (1974), the blow out of the oil well *Sagar Vikas* at Bombay High (1982), a large spill in the Andaman sea off Nicobar (1992), the bursting of oil pipelines close to Pirotan islands in the Gulf of Kutcch (2000) and numerous minor spills especially in the harbours and coastal areas owing to handling losses and to willful discharge of bilge and ballast waters arising from economic considerations. Three major incidents of spillage were reported from Kochi port itself, (1992, 1993 and 1995) and one off the Vypeen –Cherai coast (1997).

The threat of oil pollution in our seas remains omnipresent though there has been a global reduction in the quantity of oil entering the marine ecosystem through improved oil tanker designs and increasing sophistication of oil extraction equipment and transferring facilities in harbours and dockyards, though the strongest contribution has been from strict law enforcement, especially in the Western hemisphere. Large inputs of oil have mainly arisen from accidents occurring due to inclement weather, mechanical failures and sabotage as an act of war rather than from human error. Shipping lanes and harbours world over show elevated values for petroleum hydrocarbon concentrations in water and sediment with the input source varying from ill maintenance of engines to deliberate discharge of bilge and ballast and bunkering accidents. However, in India there has been a lack of

critical assessment or quantification of oil input into the seas on a national scale. A maritime state like India with an extended coastline of over 8000 km, having its own oil extraction facilities as well as being located along some of the busiest shipping channels in the world, viz. the Arabian sea, has the possibility of facing highly polluted coastal waters in the years to come.

The complexity of effecting legislation over oil pollution results from the involvement of environmental and maritime laws of several countries and agencies simultaneously. The very nature of oil pollution defies national legislation alone for effective containment and requires international co-operation and legislation. The first international convention in oil pollution control was elaborated in 1924, though decisions taken remained ineffective as major oil producing nations were not party to it. The United Kingdom and France paid heavily in terms of ecological damage and revenue as a consequence of the *Torrey Canyon* disaster in 1967 and petitioned the Inter-Governmental Maritime Consultative Organization (IMCO) which responded by creating the IMCO Legal Committee to deal specifically with legislation pertaining to oil pollution at sea. The present framework of legislation is a refinement of legislation drafted from 1967 onwards. The major international legislation existing include laws drafted by the OILPOL- the 1954 International Convention for the Prevention of Pollution of the Sea by Oil, the 1969 Civil Liability Convention for Oil Pollution, the 1971 Convention and the International Oil Pollution Compensation Fund Convention, MARPOL- the 1973 International Convention for the Prevention of Pollution from Ships and its 1978 Protocol, UNCLOS- the 1982 United Nations Convention of the law Of the Sea, international voluntary agreements such as Tanker Owners

Voluntary Agreement concerning Liability for Oil Pollution (TOVALOP) Standing Agreement, the 1984 protocols amending the 1969 CLC and 1971 Conventions and the 1987 modification of the voluntary agreement. The United States has maintained a much stricter legislation policy against oil pollution and has recently effected the 1990 Oil Pollution Act.

Oil input sources other than those arising from extraction and transportation activities are myriad, ranging from automobile exhaust to urban and industrial drainage, even allegedly adulterated fuel oil with high benzene content. The ocean has been considered a vast sump with an infinite capacity for dilution, leading to the release of domestic and industrial effluents into it, generally without treatment or detoxification. Land runoff and precipitation from the atmosphere, particularly in relation to automobile exhaust, also contributes to chronic low level pollution. No reliable estimates are available for these categories of low-level inputs of petroleum hydrocarbons in India. Research on lethal and sublethal toxicity of petroleum hydrocarbons to marine organisms has been taken up by various research institutes and universities.

The history of oil pollution research records deeper insight, with increasing accuracy and reliability of phenomena associated with oil pollution which is reflective of an increase in sophistication of analytical instrumentation and software technology as well as enhanced infrastructure such as satellite imagery and aerial surveys. An understanding of the physical and chemical behaviour and the fate of oil in the water column are essential for evaluating the effects of its presence on the biological components of the marine ecosystems. Physical interactions of oil on the water surface and within the water column have been investigated by physicists and oceanographers in

attempts to understand the processes of evapouration, spreading and dispersion of slicks, chocolate mousse formation, oil droplet entrainment into the water column, sedimentation, sorption onto particulate matter and resuspension of petroleum hydrocarbons from oiled sediments.

Likewise a deeper understanding of the chemical alterations of hydrocarbons or their derivatives entering into the marine ecosystem, their chemical metabolisation, the behaviour and effects of chemical remedies such as surfactants and sorbibants has been gained. Prior to the mid-1970s, the chemical nature of petroleum hydrocarbon mixtures was poorly understood due to a lack of standardised instruments and techniques for analytical purposes. Improvised methods suited to individual situations were evolved which led to poor success in intercaliberations. These anomalies have been resolved to a very large extent over the years with refinement in analytical methodology with bodies such as the International Maritime Organisation providing a protocol of standardised methods for monitoring petroleum hydrocarbons in the environment and the American Petroleum Institute providing standard oil mixtures which could be used as reference oils.

The study of biological effects of petroleum hydrocarbons has shown corresponding changing trends. The focus of research moved from acute toxicity studies of common and commercial organisms in the early seventies to detailing of sublethal toxicology by the mid-eighties. By then the use of controllable environment system within laboratories such as flow-through systems ushered in a period of sublethal toxicity investigations which included classical toxicological, histopathological and physiological experimentation. The effects of various types of oil and refined products on several molluscan,

polychaete, coelenterate and crustacean species were analysed which led to a better understanding of changes in behaviour, physiology and histology of these animals when exposed to petroleum hydrocarbons. A need for coordinating laboratory and field components of oil research was felt in the intervening years of the 1980s, which led to an increased number of transplantation studies examining the complexities of degraded petroleum products in the environment and their intake and metabolisation by marine organisms. By the late eighties and early nineties much attention was being paid to the detoxification mechanisms which proved to exist in organisms exposed to hydrocarbons, especially in natural environments. Attention was also focused on the mutagenicity, behavioural alterations, and synergistic toxicity and on polar and tropical ecosystems and species. Biochemical investigations into the mixed function oxidation detoxification systems, coupled with ultrastructure studies provided much insight into subcellular mechanism of petroleum hydrocarbon metabolism in organisms. The nineties saw an increased demand for justification of research spendings along with the ushuring in of intellectual property regimes which swung research in various biological disciplines, as also oil pollution research in the direction of applications. The "polluters must pay" slogan raised during the Earth Summit (1989) was translated into more effective legislation against offenders and was consequently accompanied by advances in methodology to "fingerprint" sources of petroleum hydrocarbon pollution. The much criticised clean-up operations following the *Exxon Valdez* spill also saw a greater deal of attention being paid to bioremediation and other containment measures which were less damaging to already critically degraded ecosystems. Microbial

degradation is a major clearing mechanism for removal of oil from the environment. The insights gained into the evaluation of the environmental parameters conducive to enhanced rates of biodegradation to rate projection of such processes during the 70s and 80s was further refined and led to the eventual bioengineering of the first patented living organism, a bacterium of the *Pseudomonas* species, used for bioremediation procedures for clearing oil spills. The recent trends in oil research in the West is inclined towards predictions of spill movement and of the impacts of acute, chronic, short-term and long term impacts on the ecosystems which is supported by recent advances in software technology enabling sophisticated simulation methods and advanced level ecosytem modelling. In India such research is rather limited owing to a paucity in infrastructure and the means for repeated trials. The living standards as well as the oil industry in India remains a few paces behind that of developed nations, which does not make such aspects of oil pollution research feasible for application here as yet. However with the liberalisation of markets and subsequent cultural changes, especially in terms of rapid industrialization and a trend in westernisation as far as consumer products and mode of transportation are concerned along with the massive expansion plans of the oil industry, such a change in direction of oil pollution research is also inevitable and imminent.

The effect of chronic low level or sublethal oil pollution manifests in long term shifts in the biological composition of the ecosystem of the affected area with a negative influence on stability and diversity indices and more resistant species dominating. In terms of the fishing industry, deterioration of near shore and inshore water quality plays a crucial role in the movement of

shoals and the health of stocks. The estuarine environment is characterised by short-term, severe abiotic fluctuations which results in a comparatively low diversity with resident species being tolerant of a wide range in fluctuations of physio-chemical parameters. Paradoxically, the impact of oil pollution is comparatively higher in more stable ecosystems such as the littoral communities or near shore pelagic communities due to the higher sensitivity of the component species. However, the estuarine biota cannot be expected to combat stress from various agencies over a threshold limit nor for prolonged periods without serious consequences to physical well being and energy optimisation at the organism level, reproductive success at the community level and stability of species composition at the ecosystem level. By nature estuarine ecosystems are unstable, with drastic changes occurring in population numbers. Anthropogenic activity around estuaries is also high which invariably signifies high inputs in terms of xenobiotics into estuaries. Deteriorating water quality, especially within shallow, partially confined bodies such as estuaries and fjords compounds the increasing fishing pressure on marine resources to the detriment of resource stocks. In almost all estuaries with human colonisation on the shores, chronic low level oil pollution combined with episodic inputs of large quantifies of crude and refined oils in those used as harbours, elevates the levels of petroleum hydrocarbons. It follows that in areas polluted beyond a level of adaptation for the existing system, opportunistic, short-lived, prolific species will dominate with marked fluctuations in abundance through space and time whereas climax species such as large fish will be displaced. The ability of ecosystems to recover from episodic events of oil pollution defines the limits to which such displacement

takes place though in terms of chronic low level pollution the effects are less discernable and require careful and detailed long term monitoring before conclusions can be drawn.

Oil pollution researchers have shown a preference for certain species of marine organisms owing to their commercial importance or their subjectivity to laboratory handling and procedures. These include molluscs such as *Mya arenaris, Mytilus* spp., *Macoma* sp., and crustaceans such as *Cancer* spp., *Crangon* spp., and *Mysidopsis* spp.. *Metapenaeus dobsoni*, the crevatte shrimp, is a penaeid shrimp that is a major component of the penaeid shrimp of both the east and west coast landings with the fishery along the southwest coast being the most important. The fishery for this species is concentrated within inshore limit upto the 80-metre depth zone. The ontogeny of the species includes an estuarine phase with the postlarvae moving into the estuary for feeding and growing into juveniles and migrating back to the sea for maturation and breeding. The vast backwaters of Kerala support a large fishery for *M. dobsoni* with juveniles forming 60-80% of the catch. They are a principal species in traditional prawn filtration farms that impound incoming postlarvae and grow them to marketable size in paddy-cum-prawn culture operations, the largest and most productive farms existing in the Vypeen-Cherai belt, near Kochi. The revenue for the economy of the country from the export of shrimps is substantial in terms of foreign exchange and from the domestic market. *M. dobsoni* was chosen as the test organism as it is commercial important and is a representative of the benthic community, which is most exposed to persistent petroleum hydrocarbons in the subsurface

waters and sediment of the estuary. Bombay High Crude, which is transported to Kochi by sea, was the toxicant utilised to investigate the stress responses of *M. dobsoni* to petroleum hydrocarbons in the present study.

When the stress experienced by an animal exceeds its zone of tolerance or resistance, the altered functional state of the animal affords measurable stress responses that can be investigated through physiological, biochemical, cytological and behavioural studies. These responses may be in alarm at the stimulus or may be an attempt to adapt to or resist the altered state or a failure to do either and finally succumb to the stress due to exhaustion or breakdown of life sustaining metabolic activities. The variations in basal metabolism brought about by these events can be estimated using physiological parameters such as scope for growth, growth efficiency or body condition index in long-term assessments and by measuring the rates of physiological functions such as oxygen consumption, nitrogen excretion or bioaccumulation. Actual damage to the organs and tissues, which is the cumulative effect of stress on metabolic pathways, is done using histopathological techniques. Sophisticated instrumentation techniques such as electron microscopy allow fine structure analysis of individual cells, providing a means for making further insights into and corroborating results obtained through other means of measuring stress responses.

An inference of detoxification mechanisms and identification of biomarkers is possible from the results of stress response investigations. Very often the correlation of laboratory experimentation with that of field results proves impossible due to an absence of ideal or controllable conditions and numerous or unknown variables. However, the laboratory experiments

provide a means for standardisation and the determination of 'worst case scenarios' which are important for predicting and assessing environmental implications of pollution. In the case of correlation between the incidence of diseases and pollution, long term field investigation could provide justifiable inferences whereas a laboratory investigation of incidental pathology using microbiological tests can outline dose response relationships between all parameters concerned without ambiguity.

An attempt was made to understand the effects of petroleum hydrocarbons on the penaeid shrimp *Metapenaeus dobsoni* (Miers) through laboratory based investigation. Its local estuarine habitat, the Cochin backwaters, was monitored for a short period to determine the environmental load of oil contaminants. The methodology employed and the results obtained have been discussed in the ensuing chapters.

Chapter 1:

PHYSIOLOGICAL RESPONSES

1.1 INTRODUCTION

Toxicity studies have been the mainstay of database for toxicological evaluation of biological systems. Examining dose-response relationships is the first protocol performed by toxicological researchers, using acute (lethal), short-term or long-term sublethal toxicity studies. The implications of these can then be correlated with the concentration of the toxicant in the environment of the organism to assess or predict its impacts. The quantitative assessment of the severity and frequency of deleterious effects caused by a xenobiotic provides the first clues for understanding its metabolism by the organism. The progressive effect of xenobiotics on an organism is quantifiable by measuring general stress responses. The conventional approach is to compare the rate of physiological functions. The parameters most often used to trace the effect of PHCs are directly related to the course of their metabolism in animals. Consequently the rate of oxygen consumption, nitrogen excretion, bioaccumulation, and their impact on growth, development, reproduction and alterations in behavioural patterns have been the aspects most investigated.

1. 2 REVIEW OF LITERATURE

Some of the earlier documented scientific evidence of bioassay studies employing petroleum hydrocarbon compounds were provided by Brown *et. al.* (1957), Tarzwell (1969) and La Roche *et al.* (1970). Craddock (1977) extensively reviewed the background, the importance and scope, limitations, bioassay techniques and acute toxicity responses to petroleum along with methods for interpretation of the results of acute toxicity studies using marine organisms. In the mid 1970s the focus of petroleum bioassays shifted from those on specific indicator organisms to tests carried out on a variety of organisms and their various life stages as widespread contamination of whole ecosystems was experienced during this time. Rice *et. al.* (1976) conducted extensive studies on the sensitivity of various Arctic marine species to the WSF (water suspended fraction) of crude and refined oil, allowing comparisons between various phyla and, to a certain extent, among species. Among arthropods, shrimps and crabs were found to be more susceptible to the WSF of crude oil than amphipods, isopods, mysids and barnacles. On the contrary Lee *et. al.* (1977) found amphipods to be more sensitive to crude oil than shrimps and polychaetes and attributed it to their smaller size. Anderson *et al.* (1974) conducted a classic study on total hydrocarbon content of WSF and OWD of several oils and their lethal toxicity to mysid *Mysidopsis almyra*, grass shrimp *Palaemonetes pugio* and the brown shrimp *Penaeus aztecus*. *Mysidopsis bahia* has since then been accepted as a standardised species for short term growth and survival tests. The polyaromatic component of oil has

been held as the principal determinant of toxicity to aquatic animals (Tatem *et al.*, 1978; Neff, 1979; Kulkarni & Masurekar, 1983; Spies 1987; Neff & Stubblefield, 1995; Pelletier *et al.*, 1997). Therefore this concept has been incorporated into oil spill natural resource damage assessments (NRDAs) (French, 1991). However, it was proved that petroleum components other than PAHs namely heterocyclic compounds possess relatively higher water solubility and intrinsic toxicity than PAHs (Connell & Miller, 1981; NRC, 1985). Currently the theory of a narcosis mechanism of toxicity or non specific toxicity holds that all soluble components of oil bioaccumulated in aquatic organisms contributes to toxicity. (Peterson, 1993; Barron *et al.*, 1997). Recently Barron *et. al.* (1999), working *with Mysidopsis bahia*, have demonstrated that oil with low aromatic content can be highly toxic and that PAHs are not the predominant hydrocarbons contributing to the toxicity of weathered middle distillate oils.

Several workers have explored the sublethal effects of PHCs on crustaceans using parameters such as variations in respiratory patterns, osmoregulation, moulting patterns, behavioural changes, growth, development and reproduction. Oxygen consumption patterns in various penaeid species has been investigated by a number of researchers particularly with regard to changes in salinity (Gaudy & Sloane, 1981; Dall,1986; Dalla Via, 1986; Chen & Lin, 1992). Chen and Nan (1994) were able to distinguish penaeids with protein dominated metabolisms from those with lipid dominated metabolisms by comparing their O:N atom ratios.

Variations in the rate of respiration amongst crustaceans exposed to PHCs have not shown any predictable trends in terms of direction or magnitude. An increased oxygen consumption rate was recorded in *Mysidopsis almyra* exposed to water soluble fraction s of No. 2 fuel oil (Anderson *et al.*, 1975), *Penaeus aztecus* exposed to high concentrations of the same (Tatem, 1976) and *Penaeus duorarum* exposed to petroleum wastes (Steed & Copeland, 1967). In the latter study, a decrease in the rate of respiration in *P. aztecus* was noticed. The reverse was noticed in the crab *Carcinus maenas* in which a depression of about 20% in the rate of respiration was noticed at lower concentrations where as it increased by 50% at a ten fold increase in hydrocarbon concentration (Yentsch *et al.*, 1973). Axiak and George (1987) found enhanced rates of oxygen consumption and ammonia excretion in the bivalve *Venus verrucosa* exposed to low concentrations of Kuwait crude oil for a long term exposure of 145 days. Widdows (1985a) reviewed research work done on physiological integration to assess the sublethal biological responses on a variety of marine organisms.

Growth responses to hydrocarbons are also variable with some workers recording no effects as in the case of *P. aztecus* exposed to limited concentrations of the WSF of No.2 Fuel oil to significant reduction as in larval Palaemonetes pugio (Cox and Anderson, 1973). Mecklenberg *et al.* (1977) found that water soluble fractions of crude oil negatively affected moulting and survival in king crab *Paralithoides camtschatica* and the coonstripe shrimp *Pandalus hypsiontus* larvae.

Gravity responses and phototactic behaviour were negatively affected in the late stage larvae of the rock crab *Cancer irroratus* when subjected to water accommodated fractions of No.2 fuel oil (Bigford, 1977). Percy and Mullins (1977) found that exposure to even low concentrations of crude oil significantly impaired the locomotary activity of amphipod *Onismus affinis* which could have an impact on the survival of affected populations in nature. Altered patterns in swimming speed, pause intervals between swimming and allocation in time to active food search were seen in the copepod *Centropages hamatus* exposed to sublethal concentrations of crude oil on copepods (Cowles, 1983).

Crustacean tissues sampled after spills registered paraffins and aromatics bioaccumulated from the contaminated environs: 11.8 (g/g n-paraffins were detected in the barnacle *Mitella polymerus* (Clark et al; 1973), 7-11 (g/g in the crab *Cancer irroratus* and 103-130 (g/g aromatics in the gut, 15-230 (g/g in the stomach, 2-3 (g/g in the claw muscle and 1-4 (g/g in the abdominal muscle of the lobster *Homarus americanus,* making the digestive tract tissues the most prone to bioaccumulation of petroleum hydrocarbons (PHCs) in crustaceans (Scarrat & Zitko, 1972). In their review on research done on metabolism of petroleum hydrocarbons by marine organisms, Varanasi & Malins (1977) stated that oil-in-water dispersion studies are most frequently used in the assessment of rates of uptake and accumulation of petroleum hydrocarbons in marine biota. They also found that the bioaccumulation of hydrocarbons within an organism was directly related to

the molecular weight. Consequently, the heavier molecular weight hydrocarbons being accumulated more than the lighter ones. A maximum number of PHC accumulation studies have been done on molluscs with mussels being chosen as the standard marine organism for PHC pollution assessment studies (Goldberg, 1976; Goldberg et. al., 1978). Menon (1992) found that the rate of depuration of PHC by bivalve *Perna indica* when exposed to realistic concentrations was rather slow from the mantle and other tissues. Stegeman and Teal (1971) put forward a compartmentation theory regarding the depuration of hydrocarbons from organisms stating that accumulated hydrocarbons are stored in different sites and those stored within lipids were not eliminated as rapidly as those stored in nonlipid sites, thus displaying a "biphasic effect". Bioaccumulation of aromatics is considerably higher in shrimps than in clams (Varanasi & Malins, 1977) with the concentrations reaching very high proportions even when the concentrations in the ambient medium are relatively low. Accumulation and depuration of hydrocarbons are rapid in shrimps as compared to molluscs. Anderson (1975) noticed shrimp tissue contained 2.5 ppm naphthalene, a concentration which was two magnitudes higher than that of the medium (.025 ppm) after the exposure of grass shrimp *Palaemonetes* sp. to oil in water dispersion of No.2 fuel oil for 96 hours.

1. 3 MATERIALS AND METHODS

1.3.1 Test Animals

Juveniles and sub-adults of *M. dobsoni* were collected from shrimp farms in different localities of Vypeen, an island off Kochi, using bag nets and transported to the laboratory where they were acclimated for two weeks in seawater, the characteristics of which are mentioned below. Injured and moribund animals were removed during the course of the acclimation. The shrimps were maintained on a diet of boiled clam and shrimp meat, fed ad libitum. Shrimps of varying size ranges were obtained as the season progressed and experiments were scheduled suitably. Shrimps of size ranges upto 25 mm were available only in the post monsoon months whereas those of size ranges between 30 mm and 55 mm were obtained year round. Shrimps of sizes above 60 mm were available only during the premonsoon and early monsoon period. Large size subadult shrimps (80-85 mm) were caught by Chinese net operators at the Kochi barmouth. Shrimps were segregated according to their size into groups of 20-25 mm. 30-35 mm, 40-45 mm, 50-55 mm, 60-65 mm and 80-85 mm total length for the experiments.

1.3.2 Seawater

Seawater for the experiments was collected from an unpolluted area in the Arabian sea off the Kochi coast. The water was stored in dark plastic

carboys for two to three weeks and filtered through a fiberglass filter containing activated charcoal, sand and glass wool. The seawater was diluted to 20-22 ppt with freshwater and aerated to supersaturation levels before being used for experiments. The pH was maintained at 8.2 ± 0.2 at all times. Water temperature ranged between 22-24 °C. Dissolved oxygen remained above 4.5 ppm at all times.

1.3.3 Preparation of Water Accommodated Fraction of Bombay High Crude

100% Water Accommodated Fraction (WAF) was prepared by churning Bombay High Crude (BHC) with 20 ± 2 ppt seawater in the ratio 1:10 in a 10-litre Perspex tank with a bottom outlet for 14 hours with a vortex stirrer. The mixture was allowed to settle and was treated as 100% WAF (Water Accommodated Fraction) of Bombay High Crude. It was then drained out and its hydrocarbon content extracted in a separatory funnel using n-hexane as the solvent. The extraction was performed twice and the total petroleum hydrocarbon content (TPHC) of the extract determined by a fluorescent spectrophotometer (Hitachi F-3010), at 310 nm and 360 nm, using standard methods (IOC, 1984). Fresh WAF was prepared every day for the experiments. The required dilutions were prepared by diluting the WAF with fresh seawater.

1.3.4 Acute toxicity studies

1.3.4.1 96- hour LC_{50} experiments:

Conventional methods were employed to study acute and sublethal responses of the shrimps to PHCs. Intermoult stage juvenile shrimps measuring 20-25 mm, 30-35 mm, 40-45 mm, 50-55mm, 60-65 mm, and sub-adults measuring 80-85 mm total length were subjected to various dilutions of the WAF in 96-hour LC_{50} tests using a semi flow through system. The animals were not fed during the experiments. The LC_{50} was computed using Probit analysis (Finney, 1952).

1.3.4.2 Toxicity tests on freshly moulted shrimps

Juveniles and subadult shrimps were maintained for two weeks in a holding tank until the new moon phase, as moulting is more frequent during the new moon. Freshly moulted shrimp were transferred to a second tank and then subjected to experiments in the semi- flow through system. Lethal concentrations bringing about 50% mortality of the test population were ascertained and the results were subjected to Probit analysis.

1.3.5 Sublethal toxicity studies

1.3.5.1 Estimation of rate of oxygen consumption:

Shrimps exposed to the 1 ppm and 4 ppm of PHCs were subjected to an experiment to measure the rate of oxygen consumption on the 5th, 10th and 15th days. Those maintained in 8 ppm PHCs were tested on the 5th and the 8th day as mortality of shrimps in this dose began from the 9th day onwards. Shrimps maintained under control conditions were also subjected to

the test with each set of experiments. The shrimps were confined individually within a respirometer for a period of 1 hour after removing them from toxicant. The oxygen content of the medium within the respirometer was collected in oxygen bottles and the quantity of oxygen estimated using Winklers method at the commencement of the experiment and at the completion of 1 hour. The animals were then dried and weighed. They were then sacrificed, wrapped in aluminium foil and dried in a hot air oven for 48 hours and weighed again. The rate of oxygen consumption/ hour/ g dry weight was calculated. The experiments were run in triplicate to obtain concordant values.

1.3.5.2 Measurement of tissue load of PHCs:

The Donkin and Evans method (1984) was modified to estimate the PHC content of shrimp tissue. A steam distillation apparatus was used for the purpose. Tissues were sampled on the 5th, 10th and 15th day from the shrimp exposed to 1 and 4 ppm PHC and on the 5th and the 8th day for those exposed to 8 ppm PHC. Shrimp maintained under control conditions were sampled on all days that the dosed shrimp were subjected to analysis. The pooled tissues of five shrimps were homogenised using a tissue homogeniser after rinsing in n-hexane and 3 g of the homogenate was transferred to a 250 ml round bottom flask containing 5 ml (4M) sodium hydroxide solution, 15 ml n-hexane (HPCL grade) and 50 ml distilled water. The total volume was made upto 250 ml. The mixture was saponified for 2 hours at 80(C and the resultant solution neutralised by adding 20 ml of 1M HCl and 10 ml distilled water. The distillation was continued for another 2 hours. The apparatus was cooled to room temperature and the solvent collected in the water estimator transferred

to a clean test tube. The n-hexane extracts were dried over anhydrous sodium sulphate and passed through an activated alumina clean up column. The PHC content of the samples were estimated using the fluorescence spectrophotometer (EX 310 nm , EM 360 nm) and values expressed in chrysene equivalents.

1.3.5.3 Studies on moulting frequency:

Effects of the sublethal concentrations of PHCs and the moulting frequencies in dosed juveniles of the size range 30-35 mm and 50-55 mm total length in the intermoult stage was studied in a semi flow through system for a period of thirty days.

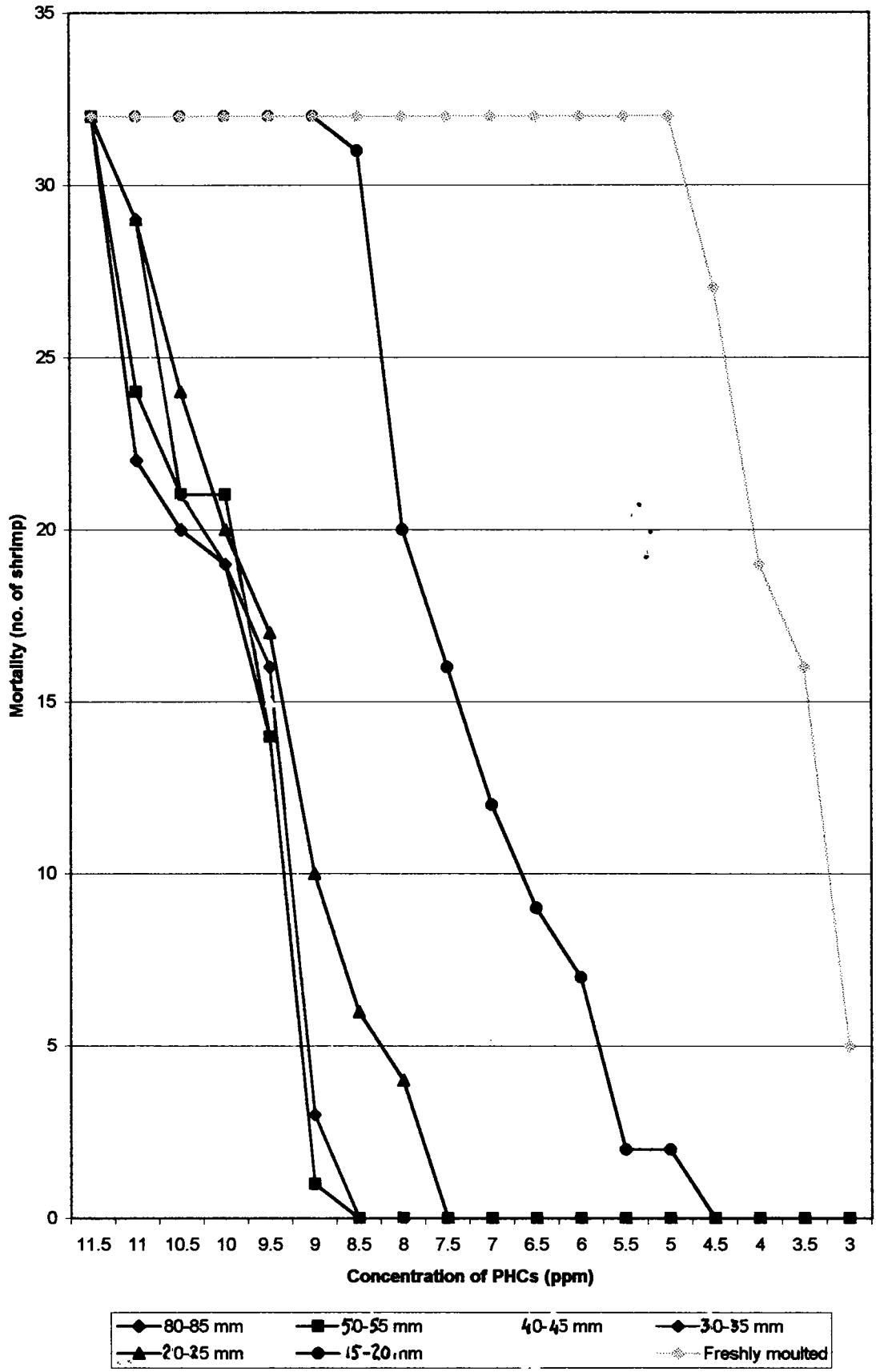
1. 4 RESULTS

1.4.1 Acute toxicity study results

1.4.1.1 96-hour LC_{50} experiments:

Probit analysis of the LC_{50} of the various size groups of shrimps subjected to PHCs showed that the size range 15-20 mm total length was most susceptible to PHCs with an LC_{50} of 6 ppm PHCs. The shrimp of 20 - 25 mm have an LC_{50} of 7.5 ppm PHCs. 30-35 mm, 40-45 mm, 50-55 mm shrimps did not show any significant variation with LC_{50} of 9.2, 9.6 and 9.8 ppm PHCs respectively. Subadults of the size range 80-85 mm recorded an LC_{50} of 10.2 ppm PHCs. There is a marked increase in PHC tolerance between the size range 15-20 mm and 20-25 mm and similarly between 20-25 mm and 30-35 mm. Mortality in most cases occurred within 48 hours.

Fig 1: Pattern of mortality of *M. dobsoni* during the 96- hour LC_{50} study



1.4.1.2 Results of toxicity studies on freshly moulted shrimp:

Freshly moulted shrimps of the size ranges 20-25mm, 30-35 mm, 50-55 mm, 80-85 mm have LC_{50} of 2.5 ppm, 3.5 ppm, 3.5 ppm, 4 ppm and 4 ppm PHCs respectively. Mortality usually occurred within the first twelve hours of moulting and no mortality was seen beyond 36 hours.

Fig. 1 depicts the pattern of mortality of shrimps of various size ranges and moult stages when subjected to petroleum hydrocarbons.

1.4.2 Sublethal toxicity study results

1.4.2.1 Oxygen consumption with varying levels of PHC body burden

The results of oxygen consumption by *M.dobsoni* in comparison with the tissue load and the rate of accumulation of PHCs for the different doses over 15 days are tabulated below:

Table 1: PHC load and rate of PHC accumulation and oxygen consumption in *M. dobsoni* exposed to WAF of Bombay High Crude:

Dose	Days of exposure	Tissue load of PHCs (µg/g)	Rate of accumulation of PHCs	Rate of oxygen consumption (mg/ g/ hr)
Control	0	0	0 (µg/g/hr)	0.57 ± 0.01
	15	0	0	0.58 ± 0.01
1 ppm	5	6.45 ± 0.13	0.05	0.66 ± 0.01
	10	13.05 ± 0.12	0.06	0.67 ± 0.01
	15	20.25 ± 0.12	0.06	0.70 ± 0.02
4 ppm	5	21.9 ± 0.12	0.18	0.72 ± 0.01
	10	42.8 ± 0.13	0.17	0.51 ± 0.02
	15	65.6 ± 0.25	0.19	0.44 ±0.03
8 ppm	5	37.35 ± 0.42	0.31	0.47 ± 0.03
	8	85.95 ± 0.48	0.68	0.38 ± 0.05
		$p < 0.05$		$p < 0.01$

1.4.2.2 Effect of sublethal toxicity on moulting

General observations in the moulting pattern indicated that the shrimps in the size ranges 30-35 mm and 50-55 mm maintained under control conditions moulted between the 11th and the 12th day and the 14th and the 16th day respectively. Under dosed conditions, the delay in moulting resulted when the quantity of PHC in the culture medium increased. The shell of the shrimp formed in dosed conditions was softer than that of the shrimps of the same moult stage maintained under control conditions.

In the course of the 30-day moulting study, 13-15% of shrimps (size 30-35 mm) exposed to 1 ppm and 5-8% of shrimps exposed to 4 ppm PHCs beyond 18 days developed brown to black lesions on the exoskeleton of the abdominal segments and occasionally on the carapace.

1.4.3 Behavioural responses:

The behaviour of *M. dobsoni* maintained under control conditions and those exposed to PHCs were recorded. Shrimps exposed to lethal concentration of WAF (20 ppm) displayed great agitation preceded by rapid twitching of the antennae, jumping and jerking movements (apparently to escape the toxicant), followed by a brief period of erratic swimming. This was followed immediately by swimming in tight circles, very often with the pleopods on one side appearing paralysed and finally the shrimp coming to rest on its side. The pleopods continue to beat for approximately one minute after which the shrimp died. A contraction of chromatophores was noticed during the entire process and this remained for a few minutes after the death

of the shrimp as well. Recovery of the shrimps was possible if they were removed from the toxicant medium at the commencement of swimming in circles and placed in fresh seawater.

The initial response to exposure to PHCs into the test medium with sublethal concentrations of PHCs was a an agitated twitching of the antennae of the shrimp followed by rapid swimming along the edge of the (circular) container and occasionally in a straight line. This behaviour was displayed in all doses beyond the 6th day except in 8 ppm PHCs. The time taken for the shrimp to settle down was inversely proportional to the dose and period of their exposure. Those exposed for shorter durations and lower concentrations of PHCs spend more time swimming. Contraction of chromatophores, especially in the uropods, with a resultant enhancement of colour of the shrimps was noticed during the dosing of shrimps at the first occasion in all concentrations and those maintained in 1 ppm PHCs upto the 10th day. Those maintained under control conditions remained active and responded instantaneously to stimuli such as tapping on the side of the container with jumping or rapid swimming. No impairment of these responses was noticed for the period of the experiment though an initial alertness upon sensing the presence of a human diminished towards the second week. Shrimps would advance towards feed immediately after it was introduced into the test medium and would feed rapidly until satiated. Shrimps exposed to 1 ppm PHCs retained the above responses. Shrimps exposed to 4 ppm PHCs beyond the 7th day and those exposed to 8 ppm PHCs showed a distinct lack of activity and delayed response to feed introduced into the test medium. The

quantity of food intake, assessed by visual quantification, reduced with increase in exposure concentration and time, with shrimps exposed to 8 ppm PHCs beyond 7 days consuming very little feed and extruding faeces soon after feeding.

1. 5 DISCUSSION

Biomarkers provide an early warning system against bioavailable contaminants in the environment and are especially useful in field environment assays (Varanasi, *et al.* 1992; Janssen *et. al.*, 1993). The measurement of any single variable does not suffice to make stress assessments from exposure to complex mixtures of xenobiotics in the environment (Stein *et al.*, 1992) or to assess the likely ecological significance of alterations to physiological activity. A more integrated approach with the measurement of several quantitative biological indices to provide a larger picture of the physiological condition of the organism is required to achieve a more meaningful assessment of stress on aquatic organisms (Axiak & George, 1987).

The most significant contribution of LC_{50} determination is that it is a starting point in studies of sublethal effects of toxicants (Dowden & Bennett, 1965). Small variations in moulting stage, age, sex, and size within a sample population can be smoothed by replication of experiments. The present study indicates that the toxicity response of *M. dobsoni* is size dependent with the LC_{50} increasing with size. The margin between LC_{50} of

large sized juveniles (60-65 mm) and the LC_{50} of subadults (80-85 mm) is narrow as compared to the difference between LC_{50}s of smaller sizes (20-25 mm and 30-35 mm). Mortality in all doses was restricted to the first 48 hours in the 96-hour LC_{50} tests. However, a majority of juveniles (30-35 mm) maintained in the sublethal dose 8 ppm did not survive beyond the 10th day of exposure suggesting that mortality at sublethal doses is more time dependant than in lethal doses. Mortality in sublethal doses may be a cumulative effect of histopathological effects and metabolic disruptions. Capuzzo *et al.* (1984) noted developmental and energy budget abnormalities in lobster larval stages when exposed to PHCs. The LC_{50} of freshly moulted juveniles was much lower than that of intermoult stages of the same size indicating their high sensitivity.

A general assumption is that crustacean larvae are more sensitive to toxicants than adults (Wells and Sprague, 1976; Fisher and Ross, 1993). Exposure to highly variable environmental parameters during the estuarine phase of the lifecycle of some shrimps such as the brown shrimp *Penaeus aztecus* may explain why the larvae are more resistant than adults to PHCs in contrast to the grass shrimp *Palaemonetes pugio* in which the adults are more susceptible and which lacks an estuarine phase in its development (Tatem *et al.*, 1978). This factor may have some bearing to the fact that there is a sharp gradation noticed in the LC50 concentration between the size range 15-20 mm and 20-25 mm and between 20-25 mm and 30-35 mm and beyond upto the sub adult stage (80-85 mm). The difference between the LC_{50} concentration between average sized juveniles and subadults is only 1 ppm

PHCs whereas the juveniles are able to tolerate a difference of almost 3.2 ppm PHCs when growing through to the size of 30-35 mm from 15-20 mm which is essentially the early phase of *M. dobsoni* within the estuary. Further studies on the physiological responses of the various juvenile stages of this species to changes in basic environmental parameters such as temperature, salinity and dissolved oxygen are necessary to corroborate this inference.

The rate of oxygen consumption is an index which can be used to assess compensations on energy brought about by environmental conditions It cannot be treated as an isolated biological response as it is a covariant with other biological functions such as behavioural responses, feeding activity and nutrition (Bayne *et al.*, 1985). It is also suggested that despite conflicting results observed by various researchers in terms of rate of oxygen consumption of animals exposed to PHCs, a definite trend was discernable when behavioural responses and oxygen consumption were measured simultaneously. It is apparent that the direct effect of hydrocarbons is to enhance oxygen consumption and that the observed decrease is respiration is largely due to the suppression of activity, partial closure of or isolation of organisms when exposed to higher concentrations. Oxygen consumption in mussels is influenced by factors such as ventilation volume, the quantity of oxygen in water, the quantity and nature of respiratory pigments and the surface area of respiratory tissue (Prabhudeva and Menon, 1986). Therefore changes in oxygen uptake from the waters by the animal and variations in the amount of water propelled through the gills result in the fluctuations in oxygen consumption which indicates involvement of behaviour as well as

physiological function (Menon and Menon, 1997). In the present study the shrimps which had higher PHC levels respired less quantities of oxygen. This evidently shows that the mechanism involved in oxygen uptake have been hampered with. The rate of oxygen consumption could also have been reduced by inactivity of the animals when exposed to higher levels of PHCs.

The presence of PAH in the tissues of a wide variety of freshwater and marine organisms strongly indicate that these organisms are able to accumulate PAHs present at low concentrations in the ambient media, food or sediments (Varanasi *et. al.*, 1985).

The mode of entry of PHCs into an organism is an important factor in determining the accumulation and distribution within the body as well as its excretion from the body (Varanasi and Malins, 1977). Shrimps spend a maximum amount of time in the sediment water intreface providing maximum exposure to hydrocarbons resuspended into overlying waters from the sediment besides being exposed to that within the surface layers of sediment while burrowing into it. Significant bioaccumulation of petroleum hydrocarbons was found to occur in shrimps and other invertebrates exposed to dredged material samples. (Parrish *et al.*, 1989).

Anderson (1975) found that the grass shrimp Palaemonetes pugio exposed to 30% WSF of No. 2 Fuel oil for 24 hours accumulated a maximum concentration of PHCs after six hours after which the levels declined continuously upto the end of the study at 24 hours suggesting that the shrimp was able to metabolise hydrocabons. Neff and coworkers (1976) found that juvenile brown shrimp exposed to 20% WSF of No.2 fuel oil accumulated a

maximum concentration of naphthalenes within the gills and gut within an hour but the hepatopancreas continued to accumulate upto 70 ppm naphthalene in 20 hours. Ingestion of contaminated sediment and PAH contaminated intestitial water in sediment are major routes of entry for PHCs into the body for benthic animals (Varanasi *et al.*, 1987; Varanasi *et al.*, 1989). The American lobsters were not only able to detect hydrocarbon-tainted feed but also showed a preference for the same (Atema and Stein, 1974). Blackman (1972) found that the shrimp *Crangon crangon* would ingest sunken oil from sediment, which would then remain for long periods in the gastric mill and foregut as globules. Amphipods feed selectively on hydrocarbon contaminants in sediment showing a marked preference for particulates with higher organic content (Harkey *et al.*, 1994).

The moulting sequence of shrimps exposed to PHCs is complex. In *M.dobsoni* juveniles, the increase in moult cycle was directly proportionate with the dose administered indicating dose dependant moulting rates. The thickness of the carapace has a protective function with crustaceans becoming less vulnerable to PHCs as the carapace progressively hardens after moulting (Karnien and Rice, 1974). The energy budget of juveniles maintained in sublethal concentrations may be altered, resulting in diversion of more energy for maintenance. This would lead to the suppression of moulting. In the isopod *Mesidotea entomon*, a significant increase in the duration of moults at higher concentrations of hydrocarbons was seen whereas at lower concentrations the effect was stimulation of the onset of subsequent moult (Percy, 1978). Several indices of stress including body

condition index, scope for growth and O:N ratio have been used to demonstrate reduction in energy availability in stressed animals. Bioenergetics was used to explain why crustacean larvae exposed to heavy metal pollutants showed reduction in somatic growth in comparison to normal larvae although the quantities of energy ingested and assimilated were similar (John and Miller, 1982). Significant increase in body maintenance costs was identified as the causative factor. At the same time juveniles in stages close to moulting were seen to moult prematurely when introduced to even 1 ppm PHCs as also the shrimp maintained under control conditions. The suppression of moulting has been traditionally considered as a general response to environmental stress in crustaceans (Epifanio, 1971). Whether premature moulting of shrimps close to ecdysis a stress response is a matter worth consideration.

A pattern is discernable when the various physiological responses measured are viewed as parts of an integrated biological process during the prolonged exposure of M. dobsoni to PHCs. The responses measured are all dose and time dependant though the rates are variable. When correlated with behavioural responses it is apparent that the enhanced rate of respiration in the low concentrations is in accordance with a low rate of accumulation of PHCs in the body indicating that the animal is expending more energy to metabolise the toxicant. Beyond a threshold level the reduced scope for activity begins to adversely affect routine metabolism with body reserves being expended for standard body maintenance. A sharp decline in the rate of

oxygen consumption corresponds with a sharp increase in PHC load in the tissue and an enhanced rate of accumulation of PHCs, indicating a level beyond which the animal is unable to cope with the enhanced rate of metabolism and is gradually unable to dispose with the toxicant entering the body. The same reduced scope for activity is evident in the moribund behaviour of the animal in the highest dose particularly on the 8th day. Stress induced by partial narcosis of the animal along with reduced intake of nutrients, reduced absorption efficiency and ejection of high calorie faeces could be the most probable reasons for reduced fitness of the shrimps in the higher concentrations especially in 8 ppm of PHCs. Inhibition of feeding may occur because of narcotic effect of hydrocarbons, particularly aromatics which may have direct action upon cilia and muscles of mussel (Johnson 1977). A reduced feeding rate is a well-established stress response. A negative absorption efficiency has been recorded for mussels (Widdows, 1982, 1985b). and shrimps (Manisseri, 1993) exposed to toxicants. Johnson (1977) reported inhibition of feeding in mussel with aromatics directly affecting ciliary and muscular activity. The latest theory of the narcosis mechanism of toxicity (Barron *et al.*., 1997) may hold true in the case of shrimps also as indicated by the behavioral responses that show that the nervous system and chemoreception have a principal role to play in the mechanism of toxicity in shrimps.

REFERENCES

ANDERSON, J. W., NEFF, J. M., COX,B. A., TATEM,H. E. and HIGHTOWER,G. M., 1974. Characteristics of dispersion and water-soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans and fish. *Mar. Biol.* 27:75-80.

ANDERSON, J. W., 1975. Laboratory studies on the effects of oil on marine organisms: an overview. Am. Petrol Inst. Publ. 4249. 1-70.

ATEMA, J. and STEIN, L., 1974. Effects of crude oil on the feeding behaviour of the lobster, *Homarus americanus. Environ. Pollut.* 6: 77-86.

AXIAK, V. and GEORGE,J. J., 1987. Bioenergetic responses of the marine bivalve *Venus verrucosa* on long-term exposure to petroleum hydrocarbons. *Mar. Environ. Res.* 23: 33-47.

BARRON , M. G., ANDERSON, M. J., LIPTON, J. and DIXON, D. G., 1997. Evaluation of critical body residue QSARs for predicting organic chemicals toxicity to aquatic organisms. SAR QSAR. *Environ. Res.* 6: 47-62.

BARRON, M. G., PODRABSKY, T., OGLE, S. and RICKER, R. W., 1999. Are aromatic hydrocarbons the primary determinants of petroleum toxicity to aquatic organisms? *Aquat. Toxicol.* 46: 253-268.

BAYNE, B. L., BROWN, D. A., BURNS, K. A., DIXON, D. R., IVANOVICI, A., LIVINGSTON, D. R., LOWE, D. M., MOORE, M. N., STEBBINGS, A. R. D. AND WIDDOWS, J., 1985. The effects of stress and pollution on marine animals. Praeger Publishers, New York.

BLACKMAN, R. A. A., 1972. Effects of sunken crude oil on the feeding and survival of the brown shrimp, *Crangon crangon.* Int. Counc. Explor. Sea C. M. 1972/K: 13. p 1-6.

BIGFORD, T. E., 1977. Effects of oil on behavioural responses to light, pressure and gravity in larvae of the rock crab *Cancer irroratus. Mar.Biol.* 43:137-148.

CAPUZZO, J. M., LANCASTER, B. A. and SASAKI, G., 1984.The effects of petroleum hydrocarbons on lipid metabolism and energetics of larval development and metamorphosis in the American lobster (*Homarus americanus*). *Mar Environ. Res* , 14 : 201-228.

CLARK, R. C., Jr., FINLEY J. S., PATTEN B. G., STEFANI D. F. and DeNIKE E. E.,1973. Interagency investigations of persistent oil spill on the Washington coast. Animal population studies, hydrocarbon uptake by marine organisms, and algal responses following the grounding of the troopship General M. C. Meigs. In: Proceedings of 1873 Joint Conference on Prevention and Control of Oil Spills, American Petroleum Institute, Washington D. C. p. 793-808.

COX, B. A. and ANDERSON, J. W.,1973. Some effects of No.2 Fuel oil on the brown shrimp Penaeus aztecus. *Am. Zool.* 13: 1308-14.

CHEN, J. C. and LIN, C. Y.,1992. Oxygen consumption and ammonia excretion exposed to ambient ammonia at different salinity levels. *Comp. Biochem. Physiol.* 102 C: 449-452.

CHEN, J. C. and NAN, F. H.,1994. Comparison of oxygen consumption and ammonia- N excretion of five penaeids. *J. Crust. Biol.*, 14 (2): 289-294.

CONNELL, D. W. and MILLER, G. J.,1981. Petroleum hydrocarbons in aquatic ecosystems-behaviour and effects of sublethal concentrations. Part 2. *CRC Critical Rev. Environ. Control* 11, 105-162.

COWLES, T. J.,1983. Effects of exposure to sublethal concentrations of crude oil on the copepod *Centropages hamatus. Mar. Biol.* 78: 53-57.

CRADDOCK, D. R., 1977. Acute toxic effects of petroleum on Arctic and Subarctic marine organisms. In: Effects of Petroleum on Arctic and Subarctic marine environments and Organisms. Vol II Biological Effects. D. C. Malins (ed.) Academic Press Inc., New York.

DALL, W.,1986. Estimation of routine metabolic rate in a penaeid prawn, *Penaeus esculentus* Haswell. *J. Exptl. Mar. Biol Ecol.* 96: 57-74.

DALLA VIA, D. J.,1986. Salinity response of the juvenile penaeid shrimp *Penaeus japonicus*. I. Oxygen consumption and estimation of productivity. *Aquaculture*, 55: 297-306.

DONKIN, P. and EVANS, S. V.,1984 Application of steam distillation in the determination of petroleum hydrocarbons in water and mussels (*Mytilus edulis*) from dosing experiments with crude oil. *Anal Chimi Acta* , 156 : 207-219.

DOWDEN, B. E. and BENNETT, H. J., 1965. Toxicity of selected chemicals to certain organisms. *J. Water Pollut. Contr. Fed.*, 37: 1308-1316.

FINNEY, D. J., Probit Analysis: A Statistical Treatment Of the Sigmoid Response Curve. University Press Cambridge, GBR. , 1952.

FISCHER, W. S. and FOSS, S. S., 1993. A simple test for toxicity of Number 2 Fuel oil and dispersants for embryos of grass shrimp, *Palaemonetes pugio. Mar. Pollut. Bull.* 26: 385-391.

FRENCH, D. P., 1991. Estimation of exposure and resulting mortality of aquatic biota following spills to toxic substances using a numerical model. In: Aquatic toxicity and Risk Assessment Vol. 14. Mayes M.A., Barron M. G. (eds.) American Society for Testing and Materials. Philadelphia. pp. 35-47.

GAUDY, R. and SLOANE, L., 1981. Effect of salinity on oxygen consumption in postlarvae of the penaeid shrimps *Penaeus monodon and P. stylirostris* without and with acclimation. *Mar. Biol.* 65: 297-301.

GOLDBERG, E. D., 1976. Strategies for Marine Pollution Monitoring. Wiley, New York.

GOLDBERG, E. D., BOWEN, V. T., FARRINGTON, J.W., HARVEY, G., MARTIN, J. H., PARKER, P. L., RICEBOROUGH, R. W., ROBERTSON, W., SCHNEIDER, E. and GAMBLE, E., 1978. The Mussel Watch. *Environ. Conserv.*, 5(2): 101-125.

HARKEY, G. A. LANDRUM, P. F. and KLAINE, S. J., 1994. Comparison of whole sediment, eluriate and pore-water exposures for use in assessing sediment-associated organic contaminants in bioassays. *Environ. Toxicol. Chem.* 13: 1315-1329.

JANSSEN, C. R., FERRANDO, M. D. and PERSSOONE, G. , 1993. Ecotoxicological studies with the fresh water rotifer *Brachionus calciflorus*. I. Conceptual framework and applications. *Hydrobiologia*, 255 : 21-32.

JOHNS, D. M. and MILLER, D. C., 1982. The use of bioenergetics to investigate the mechanism of pollutant toxicity in crustacean larvae. In: Physiological mechanisms of marine pollution toxicity. Vernberg, W. B., Calabrese, A., Thurberg, F. P. and Vernberg, F. J. (eds.) Academic Press, New York, p. 261-288.

JOHNSON, F.G., 1977. Sublethal biological effects of petroleum hydrocarbon exposures: Bacteria, algae and invertebrates. In : Effects of petroleum on Arctic and sub arctic marine environments and organisms, Vol. II. Biological effects. Malins, D.C. (ed.), Academic Press, New York, pp 271–318.

KARNIEN, J. F and.RICE. S. D.,1974. Effects of Prudhoe Bay crude oil on molting Tanner crabs, *Chionoecetes bairdi. U.S. Natl. Mar.Fish.Serv.Mar.Fish.Rev.*36: 31-37.

KULKARNI, B. G. and MASUREKAR, V. B.,1983. Acute toxicities of some petroleum hydrocarbons to crab, *Scylla serrata* (Forskal). *Geobios*, 10 (2): 63-67.

LAROCHE, G., EISLER, R. and TARZWELL, C. M., 1970. Bioassay procedures for oil and oil dispersant toxicity evaluation. *J. Water Pollut. Control Fed.* 42: 1982-1989.

LEE, W. Y., WELCH, M. F. and NICOL J. A. C., 1977. Survival of two species of amphipods in aqueous extracts of petroleum oils. *Mar. Pollut, Bull.*8 (4): 92-94.

MANISSERI, M. K., 1993. Investigations on heavy metal stress and energetics in *Metapenaeus dobsoni* (Miers). Ph.D. Thesis, Cochin University of Science and Technology, Cochin, India.

MECKLENBERG, T. A., RICE S. D., and KARNIEN J. F.,1977. Molting and survival of king crab (Paralithoides camstchatica) and coonstripe shrimp (Pandalus hypsinotus) larvae exposed to Cook Inlet crude oil water soluble fraction. In: Fate and effect of petroleum hydrocarbons in marine organisms and ecosystems. D. A. Wolfe (ed.) Permagnon Press Inc., New York. p. 221-228.

MENON, N. R., 1992. Combined toxicity of heavy metals and petroleum hydrocarbons on tropical bivalves. In: Recent advances in Life Sciences. Saxena A. K., Ramamurthi, R., Sreerama Reddy, G. and Saxena V. L. (eds.) Indian Society of Life Sciences, Manu Publications, Kanpur, pp. 51-62.

MENON, N. and MENON, N. R., 1997. The effects of polycyclic aromatic hydrocarbons on marine invertebrates- a review. In: Advances in aquatic biology and fisheries.

MYERS, M. S. 1992. Evaluation of bioindicators of contaminant exposure and in coastal

ecosystems. In Ecological indicators , Vol.I. Proceedings of the International Symposium on Ecological Indicators. D.H.Mc Kenzie, D.E. Hyatt, and V.J. Mc Donald (eds.), 461-498 pp.

NATIONAL RESEARCH COUNCIL (NRC) , 1985. Oil in the sea. Inputs, fates and effects. National Press Academy, Washington D. C, pp 601.

NEFF, J. M., 1979. Polycyclic aromatic hydrocarbons in the aquatic environment :sources, fates and biological effects. Applied Sc. Publ. Ltd., London. pp 262.

NEFF, J. M., DIXIT, D., COX B. A. and ANDERSON, J. W.,1976. Accumulation and release of petroleum derived aromatic hydrocarbons by marine animals. *Mar. Biol. 42: 36-40.*

NEFF, J. M. and STUBBLEFIELD, W. A.,1995. Chemical and toxicological evaluation of water quality following the Exxon Valdez spill. In: Fundamentals of aquatic toxicology Wells, P. G. Butler J. N, Hughes J. S. (eds.) American Society for Testing and Materials. Philadelphia, pp. 141-177.

PARRISH, P. R., MOORE, J. C. and CLARK, J. R.,1989. Dredged materials effects assessment : Single species toxicity/ bioaccumulation and macrobenthos colonization tests. In: Oceans '89 Vol. 2: Ocean Pollution. IEEE Publication No. 89CH2780-5, pp. 611-616.

PELLETIER,M. C, BURGESS,R. M.,HO, K. T.,KUHN,A.,MCKINNEY, R.A. and RYBA,S. A., 1997. Phototoxicity of individual PAHs and petroleum to marine invertebrate larvae and juveniles. *Environ. Toxicol. Chem.* 16: 2190-2199.

PERCY, J. A.,1978. Effects of chronic exposure to petroleum upon the growth and Molting of juveniles of the Arctic marine isopod crustacean Mesidotea entomon. *J. Fish. Res. Board Can.* 35 (5): 650-656.

PERCY, J. A. and MULLINS, T. C.,1977. Effect of crude oil on the locomotary activity of Arctic marine invertebrates. *Mar. Pollut. Bull.* 8 (2): 35-39.

PETERSON, D. R.,1994. Calculating the aquatic toxicity of hydrocarbon mixtures. *Chemosphere* 29: 2493-2506.

RICE, S. D., SHORT, J. W. and KARNIEN,J. F., 1976. Toxicity of Cook Inlet crude oil and

No.2 fuel oil to several Alaskan marine fishes and invertebrates. In: Symposium on sources, effects and sinks of hydrocarbons in the aquatic Environment, AIBS, 9-11 Aug 1976. American University, Washington, D. C., 12p.

SCARRATT, D. J. and ZITKO V., 1972. Bunker C oil in sediments and benthic animals from shallow depths in Chedabucto Bay, N. S. *J. Fish. Res. Board Can.*, 29: 1347-1350.

SPIES, R. B., 1987. The biological effects of petroleum hydrocarbons in the sea: assessments from the field and microscosms. In: Long-Term environmental effects of offshore oil and gas development. Elsevier, Amsterdam.

STEED, D. L. and COPLAND, B. J., 1987. Metabolic responses of some estuarine organisms to a industrial effluent. Publ. Inst. Mar. Sci. Univ. Tex. 12, p. 143-59.

STEGEMAN, J. J. and TEAL, J. M. 1971. Accumulation, release, and retention of petroleum hydrocarbons by the oyster, *Crassostrea virginica. Mar. Biol.* 22: 37-44.

STEIN, J. E. , COLLIER, T. K. , REICHERT, W. L. , CASILLAS, E. , HOM, T. and VARANASI, U. , 1992. Bio indicators of contaminant exposure and sub lethal effects: Studies with benthic fish in Puget Sound, Washington. *Environ. Toxicol. Chem.* , 11: 701-714.

TARZWELL, C. M., 1969. Standard methods for determination of relative toxicity of oil dispersants and mixtures of dispersants and various oils to aquatic organisms. In: Proceedings of 1969 Joint Conference on Prevention and Control of Oil Spills, p. 179-186. American Petroleum Institute and Federal Water Pollution Control Agency, Washington, D.C.

TATEM, H. E., COX, B. A. and ANDERSON, J. W., 1978. The toxicity of oils and petroleum hydrocarbons to estuarine crustaceans. *Estuarine Coastal Mar. Sci.* 6: 365-373.

TATEM, H.E., 1976. Accumulation of naphthalenes by grass shrimp: Effects on respiration, hatching, and larval growth. In: Fate and effects of petroleum hydrocarbon sin marine ecosystems and organisms. D. A. Wolfe (ed.). Permagnon Press Inc. New York. p. 201-209.

VANDERHORST, J. R., GIBSON, C. I. and MOORE, L. J. , 1976. Toxicity of No.2 Fuel oil to coon stripe shrimp. *Mar. Pollut. Bull.* 7: 106-108.

VARANASI, U. and MALINS, D. C., 1977. Metabolism of petroleum hydrocarbons: Accumulation and biotransformation in marine organisms. In: Effects of petroleum hydrocarbons on Arctic and subarctic marine environments and organisms. Vol.II. Biological effects. Malins (ed.) Academic Press, New York, 175-270.

VARANASI, U., REICHERT, W. L., STEIN J. E., BROWN D. W., and SANDBORN, H. R., 1985. Bioavailability and biotransformations of aromatic hydrocarbons in benthic organisms exposed to sediment from an urban estuary. *Environ. Sci. Technol.*, 19:836

VARANASI, U., STEIN,J. E., JOHNSON,L.L., COLLIER, T. K., CASILLAS, E. and MYERS, M. S.,1992 . Evaluation of bioindicators of contaminant exposure and in coastal ecosystems. In Ecological indicators , Vol.I. Proceedings of the International Symposium on Ecological Indicators. D.H.Mc Kenzie, D.E. Hyatt, and V.J.Mc Donald (Eds.), 461-498 pp.

VARANASI, U., STEIN,J. E., and NISHIMOTO, M.,1989. Biotransformation and disposition of PAH in fish. In: Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. Varanasi, U.(Ed.) 93-149 pp. CRC Press, Boca Raton , FL.

VARANASI, U ., STEIN,J.E., NISHIMOTO, M. , REICHERT, W.L. and COLLIER, T. K.,1987. Chemical carcinogenesis in feral fish : Uptake, activation and detoxification of organic xenobiotics. *Environ. Health Perspect.*, 71:155-170

WELLS, P. G. and SPRAGUE,J. B.,1976. Effects of crude oil on American lobster (*Homarus americanus*) larvae in the laboratory. *J. Fish. Res. Board Can.* 33: 1604-1614.

WIDDOWS, J.,1985a. Physiological procedures. In: The effect of stress and pollution on marine animals (Bayne, B. L., Brown, D. A., Burns. K. Dixon, D. R., Ivanovici, A., Livingston, D. R., Lowe, D. M., Moore, M. N., Stebbings, A. R. D. & Widdows, J. (eds.) Praeger Press, New York, 161-78.

WIDDOWS, J., 1985b. Physiological responses to pollution. *Mar. Pollut. Bull.* , 16 : 129.

WIDDOWS, J., BAKKE, T. , BAYNE, B. L. , DONKIN, P. , LIVINGSTONE, D. R. , LOWE, D. M. , MOORE, M. N. , EVANS, S. W. and MOORE, S. L. , 1982. Response of *Mytilus edulis* to the water accomodated fraction of North Sea oil. *Mar. Biol.*, 67: 15 - 31.

YENTSCH, C. S., GILFILLAN, E. S., and SEARS, J. R., 1973. The fate and behaviour of crude oil on marine life. AD-678584. National Technical Information Services, U. S. Dep. Commerce, Springfield, Va., 62 pp.

Chapter 2:

INCIDENTAL PATHOLOGY

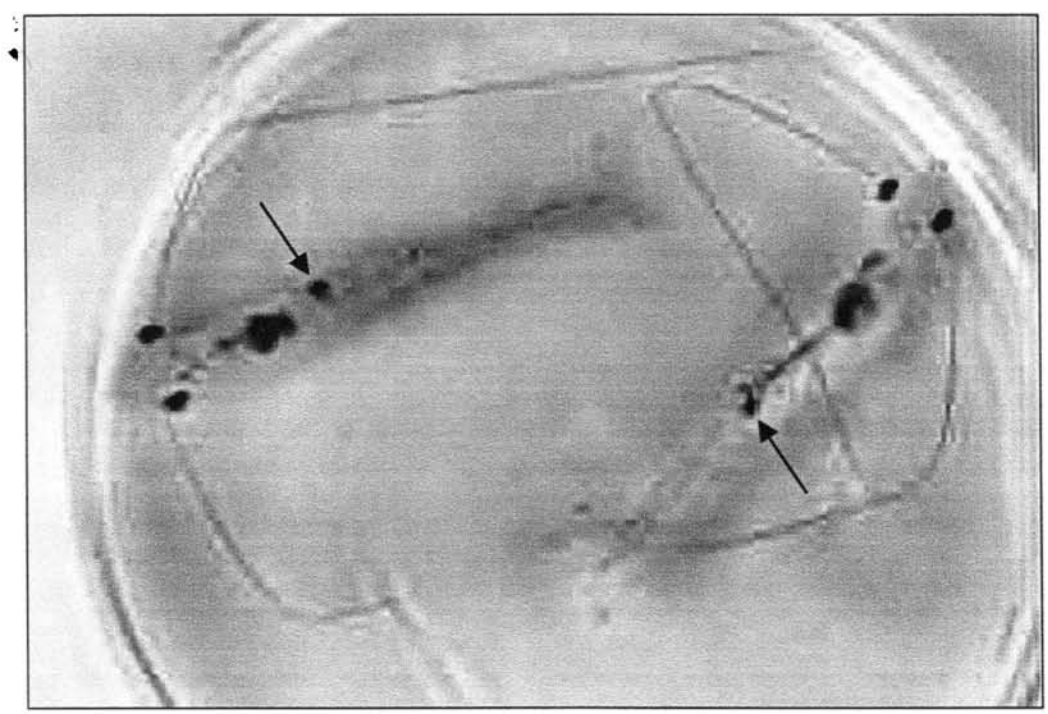


Fig 1. *Metapenaeus dobsoni* Miers: Lesions in the abdominal region (↑) after exposure to 4 ppm PHCs for 19 days.

2.1 INTRODUCTION

In the course of the investigations on the lethal and sublethal toxicity of Bombay High Crude on moulting frequency of *M. dobsoni*, regular incidences of disease were noticed in shrimp exposed to PHCs for more than 18 days, which indicated stress induced pathogenicity. 13-15% of shrimps (size 30-35 mm) exposed to 1 ppm PHCs and 5-8% of shrimps exposed to 4 ppm PHCs beyond 18 days in a 30-days moulting pattern experiment developed symptoms of a pathogenic infestation. The shrimps exposed to 8 ppm PHCs and those maintained under control conditions did not show any such signs. A detailed study was undertaken to identify the causative factor and study its pathogenicity, especially on shrimps dosed with PHCs.

2.2 REVIEW OF LITERATURE

Disease resistance and pathology induced by PHCs has been well documented with a majority of workers concentrating on the effects on fishes. A comprehensive review of earlier works upto the late seventies was written by Hodgins *et al.* (1977). A variety of microorganisms cause diseases in marine animals exposed to toxins (Mix, 1988). Among pathogenic bacteria, members of the genera *Vibrio, Beneckea* and *Psuedomonas* have proved to be chitinoclastic and responsible for shell diseases and syndromes in crustaceans cultured in aquafarms (Cook & Lofton, 1973). Of the twenty species of *Vibrio* (Kriez & Holt, 1984) several have been identified as

pathogens of marine animals. Colewell and Grimes (1984) described eight pathogenic *Vibrio* spp. as from fish. Mortality in wild populations of blue crab (Krantz, 1969), brown shrimp in the Gulf of Mexico (Vanderzant, 1970) and from cultured tiger prawns in hatcheries (Pitago, 1988; Song *et al.* 1993; Jiravanichpaisal *et. al*, 1994; Karunasagar *et. al,* 1994;) due to vibriosis has been reported. Several workers have recorded the progression of vibriosis and the levels of pathogenicity of *Vibrio* spp. in eels (Austin & Austin, 1987) and shrimps (Lewis, 1973; Lightner & Lewis, 1975; Pillai, 1982; Takahashi *et al.*, 1984: Takahashi *et al.*1985, Abraham *et. al.*, 1997).

Tubiash *et. al.* (1965) were among the first to isolate gram–negative bacteria from wild populations of the hard clam (*M. mercenaria*) and experimentally produce mass mortality of clam larvae using pure cultures of the same. Alvarez *et al.* (1998) detailed the isolation, characterisation and pathogenicity of *Vibrio harveyi* infecting penaeid shrimps and fish.

A majority of bacterial diseases seen in crustaceans are of secondary etiology (Lightner, 1977). Several species of *Vibrio,* which otherwise form the normal microbial flora of marine organisms, have been demonstrated in laboratories as the causative organisms of vibriosis (Vanderzant *et al.*, 1971). External symptoms of vibriosis described by Takahashi *et al.* (1984) are cloudiness of the muscles in the affected region and brown spots on the gills and lymphoid organs.

2.3 MATERIALS AND METHODS

Kochs Postulates were tested in four steps to determine the causative pathogen and to identify it upto the generic level. Thereafter the shrimps were challenged with various combinations of PHCs and pathogen concentrations to determine the effect of PHCs on susceptibility to disease.

2.3.1 Determination of pathogen through Kochs Postulates

2.3.1.1 Isolation of pathogen

Samples of exoskeleton and muscle tissue with lesions were aseptically excised and impression smears were made on nutrient agar plates supplemented with colloidal chitin (peptone. 5 gm, beef extract 0.3 gm, colloidal chitin 5 gm, sea water 100 ml, pH 7). The plates were incubated at 28 ° C in a bacteriological incubator. Chitinoclastic colonies (with a halo zone) were isolated onto chitin agar slants (nutrient agar + colloidal chitin). These cultures were streaked onto chitin agar plates for purification and the pure colonies were stocked in chitin agar vials overlaid with sterile liquid paraffin.

2.3.1.2 Identification of pathogen

Standard methods for identification of the pathogen (Baumann *et. al,* 1984) such as Gram staining, spore staining, mannitol motility tests, marine oxidation- fermentation tests, oxidase test and IMVC test were performed and the pathogen identified to the generic level.

2.3.1.3 Inoculation of shrimps with isolate

Suspension of the isolate in sterile saline was prepared by harvesting an eighteen-hour-old slant culture with physiological saline. Optical Density of

the suspension was measured at 600 nm in a Hitachi model 200-20 UV-Visible Spectrophotometer and the corresponding cell number was estimated by direct counting of the gram stained smear preparation using a Nikon Optiphot -2 Research Microscope. Dilutions ranging from 1x 10^7 to 1x 10 cells per ml were prepared using sterile saline and injected subcutaneously in last abdominal segment of shrimps of the 30-35 mm size range maintained under control conditions in a semi flow through system. Three replicates of each dose were conducted. The external indication of disease manifestation was followed.

2.3.1.4 Reisolation of and identification of the pathogen

Isolation of the pathogen from diseased was performed using the technique mentioned above. The pathogen isolated was identified using the standard tests mentioned above and compared with the original isolate of the pathogen.

2.3.2 PHC stress related pathological assay

30-35 mm-sized shrimps were maintained in 8 ppm, 4 ppm and 1 ppm concentrations of PHC for one week. Dilutions containing the pathogen at concentrations from 1x 10^7 to 1x 10^2 cells per ml were prepared using sterile saline and injected into shrimps maintained under control and dosed conditions, replicating the experiment thrice for each dose. The manifestation of disease, moulting pattern and mortality in the shrimps challenged with the pathogen were monitored. The results obtained were compared by statistical analysis using the test for proportions.

2.4 RESULTS

The pathogen isolated from the infected shrimps formed cream coloured colonies of the spreading type. Morphologically and biochemically, all the three isolates obtained were identical for the morphological and biochemical characteristics tested. The isolates were nonspore forming gram-negative short rods and were fermentative, oxidase positive, motile, catalase test positive. They were capable of indole production and citrate utilization. They also produced negative results for the Voges-Proskaeur test. All the strains were found to be highly chitinoclastic in nature. Based on these characters they were identified as a species of *Vibrio*.

The results of the pathological assay are presented below:

Table 1: Effect of PHC stress on susceptibility of *M. dobsoni* to vibriosis.

Response	**Treatment**	**100000 cells**	**10000 cells**	**5000 cells**	**1000 cells**	**100 cells**
Mortality	Control	100	35	10	0	0
	4 ppm PHC	-	100	100	45	0
	1 ppm PHC	-	100	100	20	5*
Manifestation	Control	-	90	65	20	0
	4 ppm PHC	-	-	-	60	45
	1 ppm PHC	-	-	-	80	60
Moult	Control	-	0	30	80	100
	4 ppm PHC	-	-	0	10	20
	1 ppm PHC	-	-	-	20	15

$p < 0.01$ *not significant at 1% level

2.5 DISCUSSION

Pollution has been strongly linked with the incidence of diseases in aquatic animals (Sindermann, 1982, 1989; Hillman, 1992; Anderson 1993). Opportunistic pathogens amongst the normal bacterial flora of marine invertebrates can cause infections when the animal is stressed. The disease so caused can be termed as environmentally induced (Guillard, 1959; Tubiash *et al.* 1965). *Vibrio* spp. have been isolated from the water samples from hatcheries where incidence of diseased prawn larvae were reported (Jiravanichpaisal *et al*; 1994; Karunasagar *et al;* 1994).

The carapace of crustaceans has a protective function as it progressively hardens after moulting (Karnien & Rice, 1974). This offers a protective function to the toxic effect of toxicants such as PHCs. Juveniles were seen to moult prematurely when introduced to even 1 ppm PHCs as was the case with the shrimp maintained under control conditions which were injected with *Vibrio* spp. This suggests that premature moulting could be a stress response dependent on energy availability. The energy budget of juveniles (Capuzzo *et al.*, 1984) maintained in sublethal concentrations of PHCs may be altered to allocate more energy for body maintenance than for moulting associated processes, leading to the suppression of the latter. In the isopod *Mesidotea entomon*, a significant increase in the duration of moults at higher concentrations of hydrocarbons was seen whereas at lower concentrations the effect was the induction of moulting (Percy, 1978).

Vibrios are known aetiological agents responsible for mass mortality of

shrimps under cultured and wild conditions (Nash, 1992; Jiravanichpaisal *et al*; 1994; Karunasagar *et al*, 1994). Stress caused by the inability of dosed shrimp to moult may be a factor in the *Vibrio* infection. Suppression of moulting in combination with other histopathological and physiological stress responses results in poor body condition, in addition to aging or deterioration of the carapace. In crustaceans the carapace may be considered a vital disease resisting barrier and its damage or deterioration provides a route of entry to pathogens (Orihel, 1975). The normal bacterial flora of shrimp includes chitinoclastic bacteria that can become opportunistic pathogens when shrimps are subjected to toxicant stress (Nimmo *et al.* 1977). Moulting also appears to be a mechanism for elimination of pathogens in shrimps injected with *Vibrio* sp.. A corollary may be that persistent external manifestation of the pathogen takes place in shrimps that are unable to moult and rid themselves of the pathogen creating lesions in the exoskeleton. However no simplistic explanations can be given to susceptibility and immunity of the shrimps, as a host of factors from disruptions in hormonal control to induced cellular and humoral bactericidal activity (Hodgkin *et.al.*, 1977) could be responsible for disease manifestation.

The lack of disease manifestation in shrimps maintained under control conditions and in 8 ppm PHCs in the trial round of injections with the *Vibrio* spp. and lower number of manifestations in the lower ranges of pathogen dilutions in 4 ppm PHC dosed shrimps as compared to the 1ppm PHC dosed shrimps suggests the possibility of the pathogenicity of the *Vibrio* itself being limited by the PHCs. Walker *et al.* (1974) found that PHCs limit the total viable

number and population of chitinolytic bacteria.

The relevance of this study lies in the evidence that stress caused by low sublethal levels of PHC contamination provides an environment conducive for opportunistic pathogens present in as less as 100 cells/ml to infect juvenile shrimps. This advocates the need for monitoring and controlling low inputs of petroleum hydrocarbons into the estuarine ecosystem.

REFERENCES

ABRAHAM, T. J., MANLEY, R., PALANIAPPAN, R. and DHEVENDARAN, K., 1997. Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. *J. Aqua. Trop.*, 12 (1): 1-8.

AIVAREZ,J. D., AUSTIN B., ALVAREZ A. M. and REYES H.,1998. *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. *J. Fish. Dis.* 21: 313-316.

ANDERSON, R. S., 1993. Modulation of nonspecific immunity by environmental stressors. In: Advances in Fisheries Science: Pathobiology of marine and estuarine organisms. Couch J. A. and Fournie J. W. (eds.). CRC Press, ondon, pp.483-510.

AUSTIN ,B. and AUSTIN D. A., 1987. Bacrterial fish pathogens. Ellis Horwood Ltd. Publ. Chicester, 364 pp.

BAUMANN,P., FURNISS,A. L. and LEE,J. V.,1984. Genus Vibrio (Pacini 1854) In: Bergeyis Manual of Systematic Bacteriology. Vol. 1 (Krieg N. R. & Holt J.G. eds.) pp. 518-538. Williams and Wilkins, Baltimore.

CAPPUZO, J. M., LANCASTER, B. A. and SASAKI,G. C., 1984. The effects of petroleum hydrocarbons on lipid metabolism and energetics of larval development and metamorphosis in the American lobster (*Homarus americanus* (Milne Edwards). *Mar. Environ. Res.* 14: 201-228.

COLEWELL, R.R. and GRIMES,D.J., 1984. Vibrio Diseases of Marine Fish Populations.

Helgolander Meeresunters, 37:265-287.

COOK, D.W and LOFTON S. R., 1973. Chitinoclastic bacteria associated with shell diseases in *Penaeus* shrimp and the blue crab (*Callinectes sapidus*). *J. Wildl. Dis.*, 19: 158-159.

FINNEY, D. J., 1952. Probit Analysis: A Statistical Treatment Of the Sigmoid Response Curve. University Press Cambridge, GBR.

GUILLARD, R. R. L.,1959. Further evidence of the destruction of bivalve larvae by bacteria. *Biol. Bull.* (Woods Hole) 117: 258-266.

HILLMAN, R. E., LORDO, R. A., MENTON, R. G., PEVEN, C. S., UHLER, A. D., CRECELIUS, E. and STEINHAUER, W. G., 1992. Relationship of environmental contaminants to occurrence of neoplasia in mussels (*Mytilus edulis*) from East and West coast Mussel Watch sites. In: Global Ocean Partnership Proc. Mar. Technol. Soc. pp. 230-239.

HODGKINS, H. O., McCAIN, B. B., and HAWKES, J. W., 1977. Marine Fish and Invertebrate diseases, host disease resistance, and pathological effects of petroleum. In: Effects of petroleum hydrocarbons on Arctic and subarctic marine environments and organisms. Vol.II. Biological effects. Malins (ed.) Academic Press, New York, 95-173.

JIRAVANICHPAISAL, P., MYAZAKI, T. and LIMSUWAN, C., 1994. Histopathology, biochemistry and pathogenicity of Vibrio harveyi infecting black tiger prawn *Penaeus monodon*. *J. Aquat. Ani. Health* 6, 27-35.

KARNIEN, J. F and RICE S. D., 1974. Effects of Prudhoe Bay crude oil on molting Tanner crab, *Chionoecetes bairdi*. *U.S. Natl. Mar.Fish.Serv.Mar.Fish.Rev.*36: 31-37.

KARUNASAGAR, S., PAI, R. and MALATHI, G. H., 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture* 128, 203-209.

KRANTZ, G. E., COLWELL, R. R. and LOVELACE, E., 1969. *Vibrio parahaemolyticus* from the blue crab *Callinectus sapidus* in Chesapeake Bay. Science 164: 1286-7.

KRIEZ, N. R. and HOLT, J. G., 1984. Bergeys's Manual of Systematic Bacteriology. I. Williams

and Williams Co., Baltimore, pp. 964.

LEWIS, D.H.,1973. Responses of brown shrimp to infection with *Vibrio* spp. *Proc. World Mariculture Soc.* 4: 333-338.

LIGHTNER, D.V.,1977. Shrimp diseases. In: Disease diagnosis and Control in North American Marine Aquaculture Development in Aquaculture and Fisheries Sciences Vol. 6. C. J. Sindermann (eds.) Elsevier, New York, 10-77

LEWIS
LIGHTNER, D. V. and D. H., 1975. A septicemic bacterial disease syndrome of penaeid shrimp. *Mar. Fish. Rev.*37: 25-28.

MIX, M. C., 1988. Shellfish diseases in relation to toxic chemicals. *Aquatic. Toxicol.* 11: 29-42.

NASH, G., NITHMITHACHOKE. C., TUNGMANDI, C., ARKARIAMOMn, A., PRATHANIPATH, P. and RUAMTHAVEESAB, P., 1992Vibriosis and its control in pond reared *Penaeus monodon* in Thailand. In: Diseases in Asian Aquaculture I M Shariff, R. P. Subassinghe and J. R. Arthur, (eds) 143 –145. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

NIMMO, D.W.R., LIGHTNER, D. V. and BAHNER, L. H., 1977. Effects of cadmium on the shrimps, *Penaeus duorarum, Palaemonetes pugio* and *Palaemonetes vulgaris*. In: Physiological Responses of Marine Biota to Pollutants Vernberg F.J., Calabrese, A., Thurberg, F. P. and Vernberg, W.B. (eds.), Academic Press, Inc., New York, pp.131-183.

ORIHEL, T.C,, 1975. The peritrophic membrane: Its role as a barrier to infection of the arthropod host. In: Invertebrate Immunity (Maramorsch K. and Shope R.E Eds.), pp.65-73. Academic Press New York.

PERCY, J. A., 1978. Effects of chronic exposure to petroleum upon the growth and Molting of juveniles of the Arctic marine isopod crustacean *Mesidotea entomon. J. Fish. Res. Board Can.* 35: 650-656.

PITAGO, C. L., 1988. Isolation and identification of luminous bacteria causing mortality in *Penaeus monodon* hatcheries in Panay, SEAFDEC *Asian Aquaculture*:10 (1): 9.

SINDERMANN, C. J., 1982. Implications of oil pollution in production of disease in marine

organisms. Philos. Trans. R. Soc. London B 297, 385- 399.

SINDERMANN, C. J., 1989. Pollution associated diseased conditions in estuarine/coastal fish and shell fish: a status report and perspective for the 1990s'. ICES, Marine Environmental Quality Commission, Copenhagen, Denmark, 32 pp.

SONG Y.-L., CHENG, W. and WANG, C.-H., 1993. Isolation and characterization of *Vibrio damsela* infections for cultured shrimp in Taiwan . *J. Invert. Pathol.* 61: 24-31.

TAKAHASHI, Y., NAGOYA, H. and MOMOYAMA, K., 1984. Pathogenicity and characteristics of Vibrio spp. isolated from diseased post larvae of Kuruma prawn, *P. japonicus* Bate. *J. Shimonoseki Univ. Fish.* 32 (!-2) : 23-31.

TAKAHASHI, Y., SHIMOYAMA, Y. and MOMOYAMA, K., 1985. Pathogenicity and characteristics of *Vibrio* spp. isolated from diseased post larvae of Kuruma prawn, *P.japonicus* Bate. *Bull. Jap. Soc. Sci. Fish*, 5 (15) : 721-730.

TUBAISH, H. S., CHANLEY, P.E., and LEIFSON, E., 1965. Bacillary necrosis, a diesease of larval and juvenile bivalve mollusks. I. Etiology and epizootiology. *J. Bacteriol.* 90: 1036-1044.

VANDERZANT, C; NICKELSON, R. and PAULIER, J. C., 1970. Isolation *of Vibrio parahaemolyticus* from Gulf coast Shrimp. *J. Milk Food Technol.* 33: 161-162.

VANDERZANT, C; NICKELSON, R. and JUDKINS, P. W., 1971. Microbial flora of pond reared brown shrimp (*Penaeus aztecus*). *Appl. Microbiol.*, 21: 916-921.

WALKER, J. D., SEESMAN, P. A., and COLWELL, R. R., 1974. Effects of petroleum on estuarine bacteria. *Mar. Pollut. Bull.* 5 (12): 186-188.

Chapter 3:

HISTOPATHOLOGY

3.1 INTRODUCTION

Histopathological techniques are a rapid, sensitive, reliable and comparatively inexpensive tool for the assessment of stress response to xenobiotics. Cytological and histopathological alterations provide a direct record of stress effects. Cell damage is a result of persistent or irreversible biochemical and subcellular dysfunction caused by stress. Often stressed cells undergo irreversible structural and biochemical changes, which result in alterations in the physiology of the animal. Assessment of histopathological manifestations provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism. The route that the toxicant takes during its metabolisation often dictates the choice of organs for examining the effect of a xenobiotic. A means to study both local and systemic effects in crustaceans leads most researchers to choose the gills for the former and the hepatopancreas for the latter leading to what is described as the "gill-gut syndrome"! Cells, particularly in organs that are involved in detoxification, such as the hepatopancreas and gills of penaeids, show general and specific responses to specific stressors.

3.2 REVIEW OF LITERATURE

Bell (1948) authored the first handbook of penaeid histology which gave pictorial descriptions of the various organs and tissues. The pictorial album prepared by Bell and Lightner (1988) employed advanced photographic techniques to provide detailed photographs on penaeid histology including gills and hepatopancreas. The reviews compiled by Gibson and Barker (1979) give a complete description of work carried out on the penaeid hepatopancreas. Dall and Moriarty (1983) subsequently reviewed research on crustacean hepatopancreas in terms of genesis, morphology and physiology. Some of the earliest such works include that of Hirsch and Jacobs (1928, 1930) who first described the formation, differentiation and migration of new cells from the apical region of each hepatopancreatic diverticulum. The penaeid hepatopancreas is a large, compact, paired glandular organ occupying a substantial portion of the cephalothoracic cavity. A thin membrane of connective tissue, the *tunica propria*, envelops it. It consists of several blindly ending tubules, held together loosely by basophilic connective tissue strands, which provide an increased surface area for digestion and absorption. Wandering haemocytes are present in the connective tissue and in the blood sinuses between the tubules. The tubules open into secondary ducts that further open into the primary duct of each side. The primary duct joins the alimentary canal in the region between the pyloric stomach and the midgut. The tubule has a lumen

in the centre, which is lined by an epithelium of one cell thickness except in the distal blind end (Gibson & Barker, 1979). Five types of cells have been identified in the hepatopancreatic tubule epithelium that take part in the digestive process which has a twenty four hour cycle in penaeids (Al-Mohanna, 1983). The E-(embryonic or Embryozellen) cells, F-(fibrillar) cells, B-(blister-like or Blasenzellen) cells (Jacobs, 1928), R-(resorptive/absorptive or Restzellen) cells and the more recently discovered M-(midget) cells (Al-Mohanna *et. al.*,1984) play their individual roles in digestion, absorption and excretion in the hepatopancreas. The undifferentiated E-cells are found at the distal tips of each tubule with proximal nuclei and conspicuous nucleolar bodies. They lack a brush border along the luminal margins. The R-cells are the most abundant cell type, occurring throughout the hepatopancreas. They are multivacuolate and have absorptive and storage functions and are surrounded by a network of myoepithelial cells with prominent nuclei and associated contractile fibres. B-cells have smaller nuclei and nucleolii, displaced to the periphery by a single large vacuole that forms by the aggregation of smaller vacuoles as digestion progresses and are secretory in nature. They have exaggeratedly convex luminal surfaces and are not usually seen in the distal regions of the tubules. F-cells are basophilic and are interspersed between B- and R-cells in the middle regions of tubules (Johnson, 1980; Al Mohanna *et. al.*, 1985; Al-Mohanna & Nott, 1987).

Young (1959) identified the surfaces for respiratory exchange in

penaeids to be the gills, inner branchiostegites and the cuticle, which is very thin with an underlying system of capillaries and described the gross morphology of the gills of *Penaeus setiferus*. Penaeids have dendrobranchiate gills. Blood is carried to the paired branches of the gill lamellae by secondary vessels and from there to individual filaments. Some of it passes round the tip of the gills where a part of it diffuses while the rest diffuses through the microlacunae. Rapid diffusion is facilitated in this region by the layers of the cuticle, epithelium and basal lamina, which forms the water –blood barrier, as they are only 1.0-1.5 μm thick (Bliss & Mantle, 1983). Dall *et al.* (1990) reviewed the work of several authors in their classic work on the biology of Penaeoids. Penaeids are adapted to breathing both in the water column and while lying buried in the substratum. This is facilitated by water being pumped backwards over the gills by the scaphognathite, washing over the gills and escaping out of the edges of the branchiostegite. When the shrimp lies buried, the antennules fit closely together to form the roof of respiratory tube, while the scaphocerites of the antennae form the floor. The canal for influx of water bifurcates around the labrum, the floor being continued by mandibular palps and exits of the first maxillipeds. Along with respiration, penaeid gills also perform the function of excretion of nitrogen, in the form of ammonia, by diffusion (Lucu, 1990). The gill of a penaeid shrimp has a central axis that attaches to the cephalothoracic wall by a tubular structure, close to the ventral end of the central axis. Primary filaments branch from this central axis, each further

dividing into secondary filaments. In each division of the gill, efferent and afferent blood vessels are present. The secondary filaments are of two types- branching and non-branching, the difference being the bifurcated pillar cell processes in the branching secondary filaments and a larger size. In the non-branching filaments, the basally located afferent and efferent vessels are separated by a thin septum. The epithelial pillar cell processes span the distance between opposite cuticular walls. Interconnecting spaces called lacunae are present between the pillar cells through which the haemolymph with circulating haemocytes moves (Bell & Lightner, 1988).

Histopathological studies on crustaceans with regard to xenobiotic damage are few in comparison to those on molluscs. Accumulation of heavy metals in the hepatopancreas was reported by Dall and Moriarty (1983), Al-Mohanna and Nott (1987) and Manisseri and Menon (1995). Histopathological effects of different concentrations of Bombay High crude oil in crabs *Charybdis lucifera* and *Scylla serrata* were described by Chandy and Kolwalkar (1984) who found the degree of damage to correlate well with percentage mortality. Starvation is a stress factor that has been thoroughly investigated using histopathological methods in crustaceans, especially in aquaculture systems. Clifford and Brick (1983) and Dall and Smith (1986) reported progressive suppression of metabolism in starved *Penaeus japonicus*.

Perhaps due to their high lipid content, the liver in marine fish and the hepatopancreas of several invertebrates are sites of hydrocarbon storage

(Lee *et. al.* 1972a, b). Lee and coworkers (1976) found that the blue crab *Callinectes sapidus* accumulated benzo (a) pyrene in much larger quantities in the hepatopancreas and the gills than in the stomach and muscle. Landrum (1988) found that lipids were important in storage of organic xenobiotics gathered by bioaccumulation in the amphipod *Pontoporeia* sp. and that the elimination rate constant of these xenobiotics is inversely proportional to the lipid content of *Diporeia* sp. Kukkonen and Landrum (1998) who concluded that though the lipid content of *Diporeia* sp. does not directly affect the uptake of benzo (a) pyrene, and that it affects the elimination rate which in turn affects the overall tissue concentration of B(a)P that the amphipod accumulates.

Gills are the primary target and uptake site for many xenobiotics in water (Evans, 1987). Recently rainbow trout gill epithelial cells have been identified as suitable cell models for toxicity screening purposes (Lilius *et al.*, 1995), focusing on the use of such cells in suspension in a test battery for toxicants (Sandbacka *et al.*,1999). Widdows *et. al.*(1985) found that the gill tissue in *Mytilus edulis* recovered from crude oil exposure at a higher rate than other tissues from chronic oil exposure.

Statistical analysis of cellular damage have proved to be an important method in describing histological alterations due to pollution effect (Widdows *et al.*, 1982; Cajaraville *et al.* 1992). Methods such as assessing the correlation coefficient has been in favour with biologists since the beginning of the century. Correlation coefficient is a measure of association between

variables. It however does not measure the dependence of one variable over the other but is a measure of the symmetrical association between variables. One such criteria for ranking variables was devised by Spearman (1904).

3.3 MATERIALS AND METHODS

3.3.1 Test animals:

Shrimps of the size group 30-35 mm size were procured from shrimp farms in Vypeen and acclimated in the laboratory and were exposed to 1 ppm, 4 ppm and 8 ppm of PHCs using the semi-flow through system and WAF of Bombay High Crude as mentioned in Chapter 2. A few shrimps were starved, each in a separate tub to avoid cannibalism, to study the effect of starvation. One set of shrimps was also maintained as control animals.

3.3.2 Sampling and fixation of tissues:

Samples of hepatopancreas and gills taken from shrimp sacrificed on the 5^{th}. 10^{th} and 15^{th} days in the case of those in the 1 ppm and 4 ppm doses and the 5^{th} and the 8^{th} day in the case of 8 ppm were preserved in Bouin's (Luna, 1968) and Davidson's (Humason, 1972) fixatives respectively.

3.3.3 Post-fixation and staining of tissues:

The hepatopancreas sections were washed in running water and transferred to 70% alcohol for post fixation treatment. They were cleared in a

saturated solution of lithium carbonate in 70% alcohol to remove to remove the yellow colour of picric acid. The tissues were then transferred to 90% alcohol for two hours, then transferred to two changes of 95% alcohol lasting one hour each following which they were transferred to two changes of absolute alcohol for one hour each. They were then transferred to a 1:1 solution of absolute alcohol and methyl benzoate for one hour until the tissues became translucent after which they were transferred to benzene for fifteen minutes and then to benzene saturated with paraffin wax for six hours. The tissues were then infiltrated with two changes of paraffin wax at 58-60 °C in a hot air oven for one hour each. The tissues were embedded in paraffin wax at 60-62 °C. The wax blocks with embedded tissue were sectioned using a rotary microtome at 4-5 μm thickness, heat fixed onto albumin coated micro-slides, deparaffinised in xylene, hydrated by passing through a descending series (absolute, 90%, 70%, 50%, and 30%) of alcohol and distilled water. A saturated solution of iodine was used as a mordant after which the tissues were cleared in sodium metabisulphate, dipped in distilled water and stained using Mallory's triple stain. The stained tissues were then dehydrated by passing them through an ascending series (70%, 90%, 95% and absolute) of alcohol and finally cleared in xylene and mounted in DPX.

The gill sections were directly embedded in paraffin wax after fixation and infiltration with wax and sectioned at 5 μm using a rotary microtome and heat fixed onto albumin coated glass micro-slides. They were hydrated in a

descending series of alcohol as in the case of hepatopancreas tissues, rinsed in distilled water and stained with Delfields haematoxylin stain for 4-6 minutes, washed in distilled water, stained for two minutes in Phloxin/eosin, subjected to an ascending series of alcohol (70%, 90%, 95% and absolute), cleared in xylene and mounted in DPX.

The sections were viewed and photographed under a light microscope at 20X, 40X and 80X magnifications.

3.3.4 Statistical analysis of damage to tissues:

An ocular and stage micrometer (Erma) was used for counting the number of damaged cells and other characteristics within fields. Twelve parameters were identified for quantifying the changes occurring in shrimp hepatopancreas due to exposure to PHCs in comparison to normal structure and changes occurring due to starvation. A total of fifty fields were observed in several replicate sections of hepatopancreas for every parameter for the different treatments with various PHC concentrations in combination with time of exposure. Non parametric methods were employed namely, the Wilcoxon sign ranked test in combination with Spearman's rank correlation method, to arrive at the statistical significance of the changes observed. Fifty fields were observed for each character from microslides of hepatopancreas of shrimps exposed to each dose on various days. The null hypothesis postulated was that there is no difference in the various parameters before and after exposure to PHCs. The alternative hypotheses were that each

parameter varied from that of the control after exposure to PHCs. WRST for paired observations and Spearmans Rank correlation test was performed and the r value for each pair of observations was obtained. The 'r' value is the sum of the rank of negative differences. If the hypothesis of no difference in the observations is true, then the sum of positive ranks and negative ranks could be roughly equal. On the other hand if the alternative hypothesis is true, the sum of one of these ranks would be larger than the other. Thus in this statistical analysis the sum of negative ranks 'T' is taken and the hypothesis of equal averages is rejected if the observed 'T' value is less than or equal to 5 at 5% level of significance. The unlikeness of observing an increase in degeneration in the absence of pollutants implies a positive correlation between the detailed ranks tested. The hypotheses considered were (1) that there is no tendency for the PHC dosed tissues to undergo degeneration against the alternative (2) that there is some tendency to increase the degree of degeneration that is, increase the positive correlation.

The results obtained through Wilcoxon signed rank test were interpreted according to the degree of damage observed in the tissues. 1: nil damage; 2: mild damage; 3: medium damage; 4: severe damage.

Positive correlation between detailed ranks was calculated through Spearman's correlation coefficient, using the formula:

$$r = \frac{1 - 6 \Sigma d^2}{n (n^2 - 1)}$$

where n= number of observations, d = difference in rank

An analysis of variance was performed on the ranks obtained through the Wilcoxon signed rank test to test the differences in means for the various parameters for statistical significance. The ANOVA is performed by partitioning the total variance into the component that is due to true random error (that is the within-group variability SS) and the components that are due to differences between the means. Significant differences between the effect of PHCs over variation in dose and period of exposure were appraised through this test. The various parameters were then ranked using discriminant function analysis that is used to determine the variables which discrimnate between groups. The ranking obtained was used to determine which parameter(s) were most representative of PHC induced damage.

3.4 RESULTS

Exposure to petroleum hydrocarbons produced discernable changes in the structure hepatopancreas and gills of *M. dobsoni.*

3.4.1 Histopathology of hepatopancreas:

External examination of the hepatopancreas with the naked eye showed a progressive shrinkage and discolouration of the hepatopancreas

with increased dosage and time of exposure. Hepatopancreas of shrimps dosed at 4 ppm beyond five days and 8 ppm showed increased textural fragility. Those of shrimps dosed with 4 ppm PHCs for 15 days and 8 ppm PHCs for 5 as well as 8 days were spongy in texture and that of shrimps starved for eight days appeared shrunken and was cream in hue. In contrast, the hepatopancreas of shrimps under controlled condition were turgid and brown in colour. Shrimps under controlled condition fed actively twice a day whereas those dosed with 4 ppm PHCs beyond eleven days and those dosed with 8 ppm PHCs beyond three days showed reduced intake of food.

3.4.1.1 Visual appraisal under the light microscope:

a) Control specimen:

Longitudinal and transverse sections of the hepatopancreas of shrimps maintained under controlled conditions sampled on the first day and the fifteenth day did not show any differences in the number or structure of various digestive cell types or tubular structure and arrangement. The general structure of the hepatopancreatic tubules corresponded with that cited in literature for normal structure. Numerous young B-cells were present in the areas bordering the proximal region. The proximal region was predominated by mature B-cells with a single large vacuole (Fig 1 a). Often these were observed in the process of being extruded into the lumen. The myoepithelium providing support to the tubule epithelium appeared intact with haemal sinuses containing a few haemocytes. R-cells, which stain darker than other cells and have a large prominent nucleus with a clear

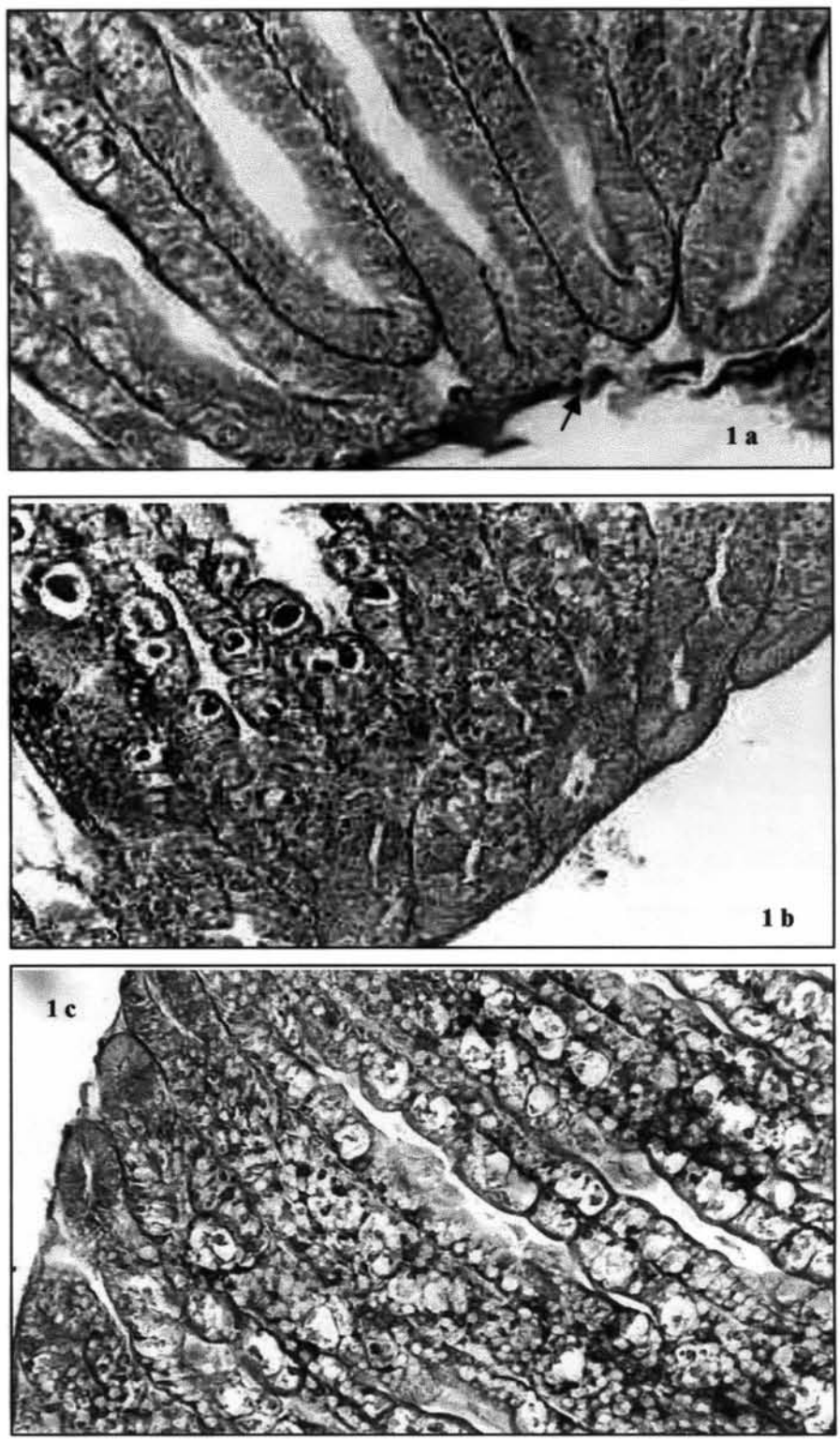
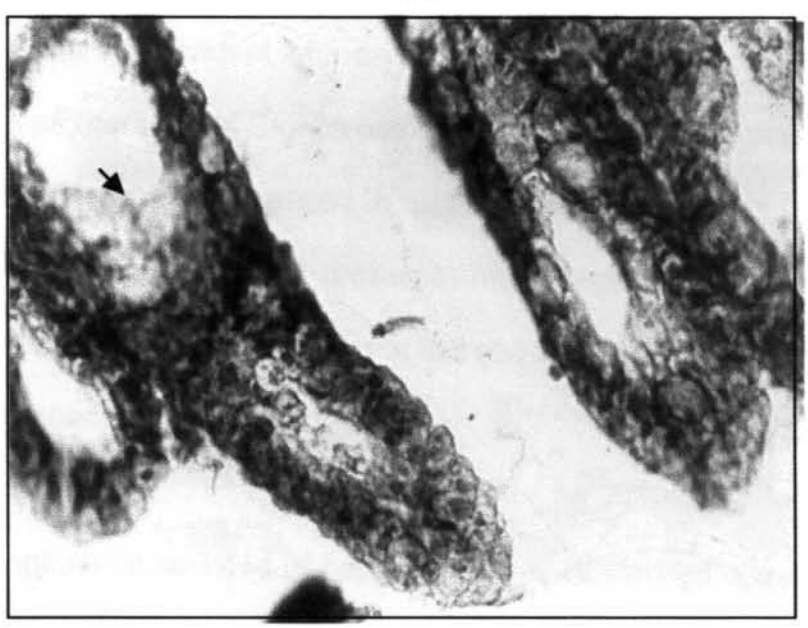


Fig. 1. *Metapenaeus dobsoni* Miers: L. S of hepatopancreas:

a. Tubules showing E-, F- and B- cells, *tunica propria* (↑) and "cellophane like" digested food materials and secretions (control conditions X 240)
b. Tubules showing increased number of B- and F-cells. (1 ppm, 5 days, X 240)
c. Tubules showing proliferation of B- and F-cells and increased number of darkly staining R-cells (1 ppm, 15 days, X 240)

2 a



2 b

Fig. 2: *Metapenaeus dobsoni* Miers: L. S. of hepatopancreas:

a. Presence of mature B-cells along the length of the tubules and extrusion of cells into the lumen (▲)(20 ppm, 15 minutes, X 240)
.b. Atrophy of cells, accumulation of cell debris in the lumen (▲) decrease in the height of tubular epithelium, separation of tubules and rupture of the *tunica propria* (starved, 5 days, X 480).

nucleolus, were frequently present in the middle and proximal regions of the tubule (Fig 3 a & b). The distal region of the hepatopancreatic tubule had intact embryonic or E-cells (Fig 4 a). The *tunica propria* covering the hepatopancreas was intact.

b) Starved specimen:

At the end of eight days of starvation, the hepatopancreatic tubules showed an increased spacing between tubules due to shrinkage of tubules and an increased lumen within the tubules due to reduction in the height of the epithelium. The structure of the tubule maintained its shape but atrophy of the epithelium was evident with a reduction of B-cells even in the proximal regions. A proliferation of R-cells was seen in the middle and proximal regions but these were though increased in numbers, reduced in size (Fig 3 e). The *tunica propria* appeared to be broken in many regions indicating that it may have been in a fragile condition in the starved shrimp.

c) Dosed specimen:

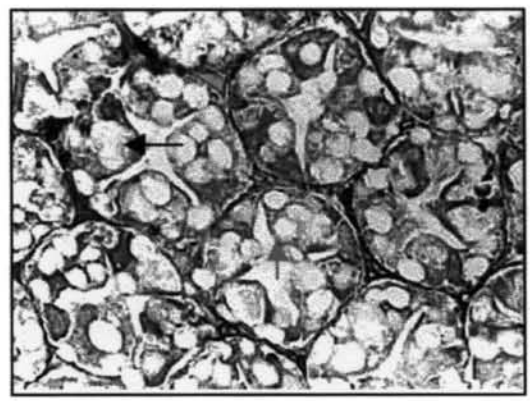
i)1 ppm PHC dose:

Longitudinal sections of hepatopancreas of shrimps dosed with 1 ppm were not conspicuously different from that of the control specimen after five days of dosing. However by the fifteenth day an increase in the number of vacuolated B-cells towards the distal end of tubules with the presence of a number of F- and R-cells in the middle regions was visible (Fig 1 c). Vacuoles were more numerous in the E-cell region and only a single layer of E-cells was seen at the end of tubules. These too appeared reduced in size

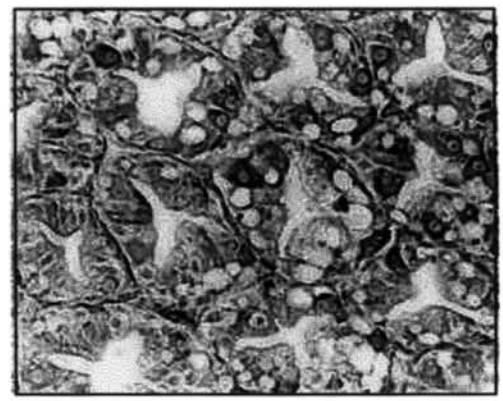
(Fig 4 b). A larger number of B-cells were noticed in the middle regions of the tubules. Corresponding regions where F-cells predominate in control specimen had more B-cells. In the proximal region there was a predominance of B-cells with excretory/secretory material inside the vacuoles. A proliferation of R-cells was also seen. The myoepithelium remained largely intact though in several areas the tubules did not remain as tightly packed in comparison to control specimen. A slight distortion of shape of the tubules was also noticed.

ii) 4 ppm PHC dose:

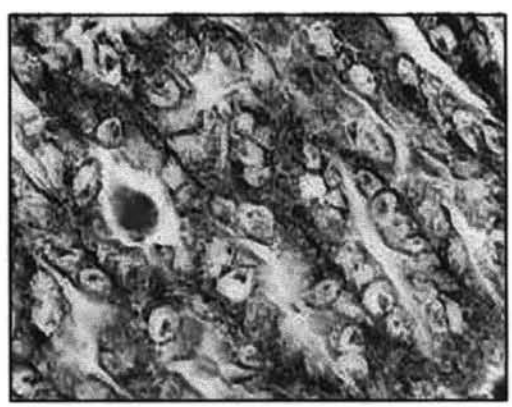
Longitudinal sections through hepatopancreas of *M. dobsoni* exposed to 4 ppm of petroleum hydrocarbons for 5 days showed an increased proliferation of F- and R-cells in the middle regions of tubules, indicating enhanced secretion and storage in the tubules similar to that seen in 1 ppm PHCs 15 days (Fig 1 c). Transverse sections revealed that interspersed between these are areas where cells have disintegrated, shrunk or eroded (Fig 3 c). Mature B-cells predominated in regions where there is a normal predominance of F-cells and immature B-cells. In *M. dobsoni* dosed with 4 ppm PHCs for 15 days, only a single row of E-cells is seen indicating the formation of fewer cells (Fig 4 c). Increased vacuolation, secretion in the lumens and breakage of tubule connective tissue lining is seen. In the proximal regions of tubules sloughing off of the tubule epithelium appeared to have set in by the fifteenth day.



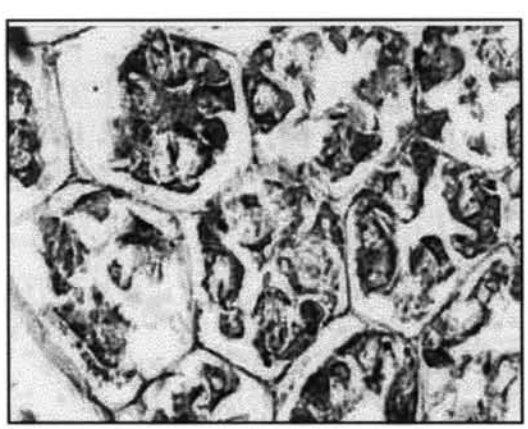
3 a



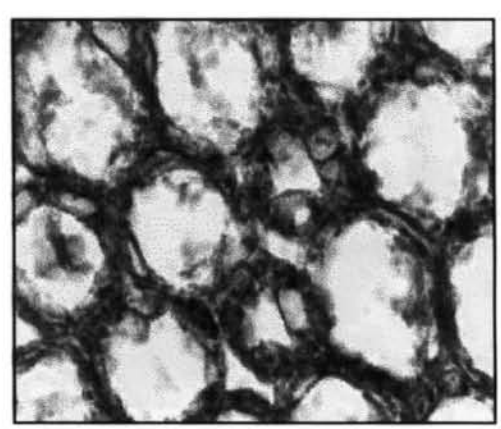
3 b



3 c



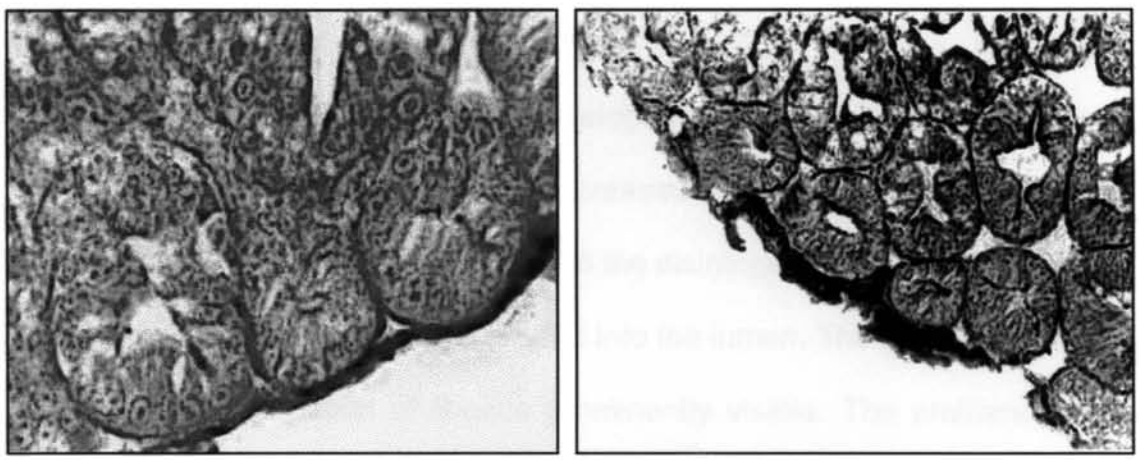
3 d



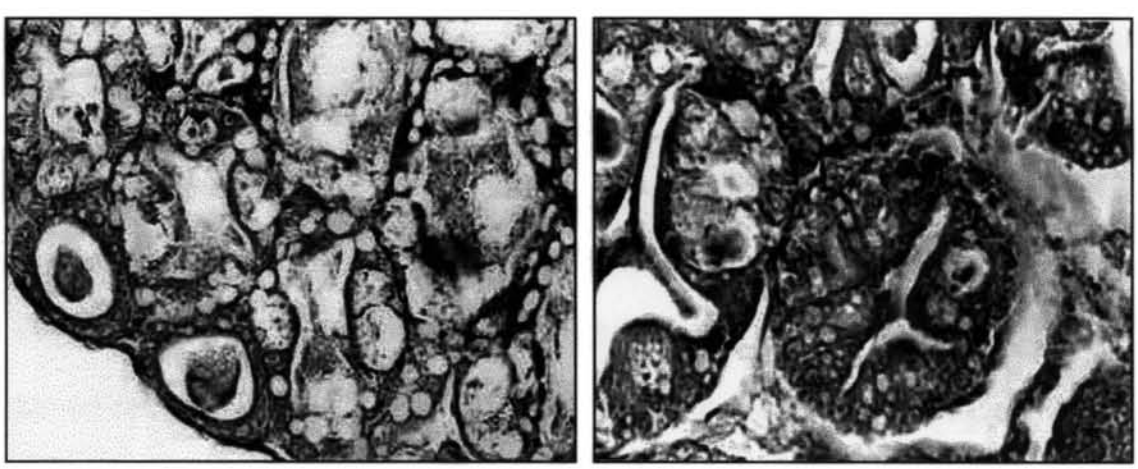
3 e

Fig 3: *Metapenaeus dobsoni* Miers: C. S. of hepatopancreatic tubules:

a. Proximal region of tubule: B-cells with single vacuole (↑), F-cells with numerous smaller vacuoles (↑), and darkly staining R-cells (↑) (control conditions, X 200)
b. Mid region of the tubule: F- and R-cells predominate (control conditions, X 200)
c. Mid-region of the tubule: Increased number of mature B- and R-cells (4 ppm, 5 days, X 200)
d. Proximal region of tubule: Atrophy and delamination of cells and shriveling of the myothelium (8 ppm, 8 days, X 200)
e. Proximal region of tubule: Severe atrophy of cells and enlargement of lumen (Starved, 8 days, X 200)



4 a 4 b



4 c 4 d

Fig 4: *Metapenaeus dobsoni Miers*: C. S. of hepatopancreas:

a. Distal region of tubule: Intact E-cells and *tunica propria* (control conditions, X 320)
b. Distal region of tubule: Mild vacuolation in E-cell layers (1 ppm 15 days, X 200)
c. Distal region of tubule: Reduction in E-cell layer thickness and increased vacuolation (4 ppm 15 days, X 320)
d. Distal region of the tubule: Extensive vacoulation of E-cells, presence of a few B-cells and shrinkage of tubules (8 ppm, 5 days X 320)

iii) 8 ppm PHC dose:

Longitudinal section through hepatopancreas of *M. dobsoni* exposed to 8 ppm PHCs for 5 days showed increased formation of mature B-cells towards the distal end of the tubules and the disintegration of tubule structure. A number of B-cells were seen extruded into the lumen. The tubule epithelium was reduced with nuclei of R-cells prominently visible. The proliferation of vacuolated B-cells almost upto the distal end of the tubules and reduction in the number of E-cells were noticed by the eighth day of exposure. The tubules also appeared shrunk and disintegrated (Fig. 3 d). In some areas the *tunica proporia* lay detached from the tubules (Fig 4 d). Collapse of structural integrity of the tubule is evident with heavily vacuolated cells and E-cells piled up in the lumen. In the proximal region, cell debris was seen in the lumen of the tubules. Shrinkage and breakage of tubules and enlargement of tubule lumen resulted in structurally void areas in the hepatopancreas. The tubules appear shortened and the middle region of the hepatopancreas almost devoid of any distinct and defined morphological features.

iv) 20 ppm PHC dose:

Shrimps exposed to a lethal dose of 20 ppm for 15 minutes showed an increased number of B-cell vacuoles present right upto the distal region of the tubules and an extremely reduced number of F-cells. A number of cells were found extruded into the lumen in the proximal region. However no shortening or distortion of tubules was noticed (Fig 2 b).

3.4.1.2 Statistical analysis:

Twelve types of histopathological changes noted in the hepatopancreas were selected for performing statistical analyses. These were cytorrhexis in the proximal tubules, proliferation of R-cells, proliferation of haemocytes, reduction in tubule width due to shrinkage of the tubules, detachment of the *tunica propria*, obliteration of the tubule lumen by cellular debris and delaminated cells, reduction in epithelial height due to atrophy of cells, increase in epithelial height due to vacuolation and storage of lipid, proliferation of B-cells, vacoulation of E-cells, reduction in the number of E-cells and proliferation of connective tissue. The average ranks ascribed to the variations of these parameters in the different treatments through Wilcoxons Signed Rank test are displayed in Table 1.

a) Results of analysis of variance (ANOVA):

The ANOVA performed on the data showed that the average effects of all the twelve treatments, in general, are significantly different from each other, since $F_{11,48}$ obtained for all the treatments were significant at 1% level. Comparisons of the mean levels obtained in respect of the treatment control (0 days) data relating to this experiment, wherein the different levels of toxicant have been administered for different periods of time, have been presented in Table 2 and 3.

i) **Cytorrhexis in proximal tubules:** Significantly low damage was observed in treatments starved (5 days), starved (8 days) , 1 ppm (5 days) , 1 ppm (10 days) and were comparable with the controls. The

Table 1: Average ranks obtained through Wilcoxons Signed Rank Test for the histopathological parameters observed in the hepatopancreas of *M. dobsoni* (Miers) when subjected to various treatments with PHCs:

	TI	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
A	1	1	1	3	4	1	2	2	1	1	2	1
B	1	1	1	1	2	4	2	2	2	2	3	1
C	1	3	4	2	2	2	2	2	2	2	2	1
D	1	3	4	1	1	1	1	1	1	1	1	1
E	1	1	2	2	3	2	2	2	1	1	1	1
F	1	1	1	3	4	2	2	2	2	2	3	1
G	1	1	1	3	3	2	3	4	2	2	4	1
H	1	1	2	3	3	2	3	4	2	2	3	1
I	1	2	2	2	2	2	3	4	1	2	3	1
J	1	2	2	2	3	2	2	3	2	2	2	1
K	1	1	1	1	1	2	3	3	2	2	2	1
L	1	1	1	4	4	2	3	3	2	2	2	1

Legend:

	Parameter		Parameter		Treatment		Treatment
A	Cytorrhexis in proximal tubules	G	Reduction in height in epithelial cells	T1	Control (0 day)	T7	4 ppm (10 days)
B	Proliferation of R-cells	H	Obliteration of lumen	T2	Starved (5 days)	T8	4 ppm (15 days)
C	Proliferation of haemocytes	I	Vacuolation of E-cells	T3	Starved (8 days)	T9	1 ppm (5 days)
D	Reduction in tubule width	J	Reduction in E-cells	T4	8 ppm (5 days)	T10	1 ppm (10 days)
E	Detachment/rupture of *tunica propria*	K	Proliferation of connective tissue	T5	8 ppm (8 days)	T11	1 ppm (15 days)
F	Presence of B-cells in distal region	L	Increase in height of epithelium	T6	4 ppm (5 days)	T12	Control (15 days)

Table 2: Analysis of variance of the effect of PHCs on various histopathological parameters observed in the hepatopancreas of *M. dobsoni* (Miers):

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
T1	1.00 (1.00)	1.08 (1.01)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
T2	1.00 (1.00)	1.00 (1.00)	2.64 (1.62)	2.60 (1.61)	1.12 (1.06)	1.00 (1.00)	1.32 (1.15)	1.00 (1.00)	1.78 (1.32)	1.88 (1.37)	1.00 (1.00)	1.00 (1.00)
T3	1.00 (1.00)	1.00 (1.00)	3.50 (1.87)	3.74 (1.93)	2.12 (1.45)	1.00 (1.00)	1.00 (1.00)	1.92 (1.38)	2.00 (1.41)	1.78 (1.33)	1.00 (1.00)	1.00 (1.00)
T4	2.70 (1.64)	1.10 (1.05)	1.62 (1.26)	1.10 (1.05)	1.42 (1.19)	2.60 (1.61)	2.68 (1.63)	2.64 (1.61)	2.30 (1.51)	1.82 (1.35)	1.00 (1.00)	3.60 (1.89)
T5	3.60 (1.89)	1.00 (1.00)	1.66 (1.28)	1.00 (1.00)	2.96 (1.71)	2.66 (1.63)	2.66 (1.63)	2.80 (1.67)	2.04 (1.41)	2.58 (1.60)	1.00 (1.00)	3.80 (1.95)
T6	1.00 (1.00)	3.56 (1.89)	1.68 (1.29)	1.12 (1.06)	1.06 (1.02)	1.8 (1.32)	2.06 (1.43)	2.04 (1.41)	2.86 (1.69)	1.78 (1.33)	1.88 (1.36)	1.62 (1.27)
T7	1.78 (1.33)	1.94 (1.39)	1.56 (1.24)	1.18 (1.05)	1.34 (1.16)	1.58 (1.25)	2.70 (1.64)	2.86 (1.69)	3.72 (1.93)	1.80 (1.34)	2.54 (1.59)	2.54 (1.59)
T8	2.06 (1.43)	1.78 (1.33)	1.78 (1.35)	1.30 (1.14)	1.86 (1.35)	1.58 (1.25)	3.66 (1.91)	3.72 (1.93)	1.86 (1.36)	2.80 (1.67)	2.70 (1.64)	2.94 (1.71)
T9	1.00 (1.00)	1.74 (1.31)	1.50 (1.22)	1.00 (1.00)	1.16 (1.07)	1.16 (1.07)	2.34 (1.52)	1.86 (1.36)	1.00 (1.00)	1.98 (1.41)	1.62 (1.26)	1.62 (1.26)
T10	1.00 (1.00)	1.66 (1.28)	1.00 (1.00)	1.10 (1.05)	1.00 (1.00)	1.42 (1.18)	2.00 (1.39)	2.00 (1.39)	2.10 (1.45)	1.92 (1.38)	1.72 (1.31)	1.78 (1.33)
T11	1.66 (1.03)	2.68 (1.63)	1.00 (1.00)	1.18 (1.09)	1.00 (1.00)	2.35 (1.54)	3.54 (1.88)	2.92 (1.71)	2.65 (1.64)	2.08 (1.44)	2.18 (1.47)	1.98 (1.41)
T12	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
F11,48	574.06**	37.40**	46.95**	129.72**	31.35**	20.18**	26.11**	32.46**	80.99**	64.76**	34.41**	102.72
SE	0.01	0.05	0.07	0.02	0.04	0.05	0.06	0.05	0.03	0.02	0.04	0.03
CD	0.037	0.134	0.200	0.073	0.120	0.150	0.183	0.150	0.094	0.069	0.122	0.098

** $p > 0.05$

Note:- Values within paranthesis are the respective values transformed to their square roots

Table 3: Ranking order of the various treatments with PHCs in relation to histopathological changes effected in the hepatopancreas of *M. dobsoni* (Miers):

	1	2	3	4	5	6	7	8	9	10	11	12
P1	T5	T4	T8	T7	T11	T6	T2	T3	T9	T10	T1	T12
P2	T6	T11	T7	T8	T9	T10	T4	T1	T5	T3	T2	T12
P3	T3	T2	T8	T6	T5	T4	T7	T9	T1	T11	T10	T12
P4	T3	T2	T8	T7	T11	T6	T4	T10	T9	T11	T5	T12
P5	T5	T3	T8	T4	T7	T9	T2	T6	T1	T10	T11	T12
P6	T5	T4	T11	T6	T7	T8	T10	T9	T2	T1	T3	T12
P7	T8	T11	T5	T7	T4	T9	T6	T10	T2	T1	T3	T12
P8	T8	T11	T7	T5	T4	T6	T10	T3	T9	T1	T2	T12
P9	T8	T7	T11	T5	T10	T6	T4	T3	T2	T9	T1	T12
P10	T8	T5	T11	T9	T10	T2	T4	T7	T6	T3	T1	T12
P11	T8	T7	T11	T6	T10	T9	T2	T1	T4	T5	T3	T12
P12	T5	T4	T8	T7	T11	T10	T6	T9	T3	T2	T1	T12

Legend:

	Parameter		Parameter		Treatment		Treatment
P1	Cytorrhexis in proximal tubules	P7	Reduction in height in epithelial cells	T1	Control (0 day)	T7	4 ppm (10 days)
P2	Proliferation of F-cells	P8	Obliteration of lumen	T2	Starved (5 days)	T8	4 ppm (15 days)
P3	Proliferation of haemocytes	P9	Vacuolation of E-cells	T3	Starved (8 days)	T9	1 ppm (5 days)
P4	Reduction in tubule width	P10	Reduction in E-cells	T4	8 ppm (5 days)	T10	1 ppm (10 days)
P5	Detachment/rupture of *tunica propria*	P11	Proliferation of connective tissue	T5	8 ppm (8 days)	T11	1 ppm (15 days)
P6	Presence of B-cells in distal region	P12	Increase in height of epithelium	T6	4 ppm (5 days)	T12	Control (15 days)

highest damage was observed in 8 ppm (8 days) followed by 8 ppm (5 days), 4 ppm (15 days), 4 ppm (10 days) and 1 ppm 15 days in a decreasing order.

ii) Proliferation of R-cells: Significantly low proliferation of F-cells was observed in treatments starved (5 days), starved (8 days), 8 ppm (8 days) and control (15 days). Maximum proliferation was noticed in the 4 ppm (5 days) treatment, followed by 1 ppm 15 days, 4 ppm 10 days, 4 ppm 15 days, 1 ppm 5 days and 1 ppm 10 days.

iii) Proliferation of haemocytes: Very low values are observed for this parameter in the controls whereas in starved (8 days) the same was extremely high. The values were substantially high in the treatments starved (5 days) and 4 ppm 15 days. In all other treatments, the proliferation could be termed only as intermediate.

iv) Reduction in tubule width: No reduction in tubule width was seen in 8 ppm (8 days) and 1 ppm (5 days). Severe reduction in tubule width was noticed in starved (8 days) and substantial reduction in starved (5 days). In the rest only mild reduction in width of tubules was noticed.

v) Detachment of *tunica propria*: No detachment of *tunica propria* was discerned from the values obtained for the controls as well as for 1 ppm (10 days) and 1 ppm (15 days). Intermediate to substantial damage was noticed in 8 ppm (8 days), starved (8 days) and 4 ppm (15 days). Only mild damage was seen in the rest of the treatments.

vi) Obliteration of lumen: This parameter showed very low values for the

control and starvation treatments. Moderate to substantial damage was observed in 8 ppm (8 days), 8 ppm (5 days) and 1 ppm (15 days). Only mild obliteration of the lumen was observed in all other treatments was discernable.

vii) Reduction in epithelial height: Significantly low reduction in the height of epithelial cells was observed in the controls. Severe damage was observed in starved specimen (8 days), 4 ppm (15 days), 1 ppm (15 days), 8 ppm (8 days), 4 ppm (10 days) and 8 ppm (5 days). In other treatments, the damage was only mild.

viii) Proliferation of B- cells: Significantly low proliferation of B-cells in the proximal tubules has been caused in the control and starved (5 days) conditions. Severe damage was seen in 4 ppm (15 days) and 1 ppm (15 days) and moderate to substantial damage seen in 8 ppm (8 days) and 4 ppm (10 days). In the rest of the treatments, the damage appeared to be mild.

ix) Vacuolation of E-cells: This parameter was low in the controls and 1 ppm (5 days). Severe vacuolation of E-cells was seen in 4 ppm (15 days) and 4 ppm (10 days). In the rest only mild damage was seen.

x) Reduction in number of E-cells: Values for this parameter were low in the controls whereas moderate to substantial reduction in the number of E-cells was seen in 4 ppm (15 days), 8 ppm (8 days) and 1 ppm (15 days).

xi) Proliferation of connective tissue: Significantly less proliferation of

connective tissue was seen the controls, starved conditions and 8 ppm (5 days). Moderate damage was seen in 4 ppm (15 days), 4 ppm (10 days) and 1 ppm (15 days). Only mild damage was seen in the rest.

xii) Increase in height of epithelium: Significantly less increase in the height of epithelium was seen in the control and starved conditions. High increase in epithelial height was seen in 8 ppm (8 days), 8 ppm (5 days), 4 ppm (15 days) and 4 ppm (10 days) in the order of decreasing severity. In the rest of the treatments, only mild damage was seen.

Summarising the findings, the results significantly indicate the uniformity of least damage in the controls. However, on an overall basis, the addition of increasing concentrations of PHCs for increased durations produced mixed effects on the various parameters discussed.

The dose 4 ppm PHCs (15 days) significantly affects the reduction in the height of tubular epithelium, proliferation of B-cells in the distal regions of the tubules, vacuolation of E-cells and reduction in the number of E-cells. The dose 8 ppm PHCs (8 days) can also be ranked first for maximum effect on the parameters cytorrhexis of tubular epithelium, disruption or detachment of *tunica propria* and obliteration of the tubular lumen. Starvation for eight days brings about only proliferation in haemocyte numbers and reduction in tubule width but its severity was highest in these parameters. Similarly, the effect of the dose 4 ppm PHCs (5 days) affects proliferation of R-cells most severely.

Among the parameters observed, reduction in the number of E-cells

and the proliferation of connective tissue appear to be least affected by the various doses and can thus be taken as least indicative of the extent of toxicant effects.

b) Results of the Spearmans Rank Correlation test:

The correlation matrix of histopathological changes noted within the hepatopancreas of shrimps maintained under control conditions and exposed to the stress of various levels and duration of toxicant exposure or starvation are presented in Table 4. The correlation between the occurrence of various histopathological changes from A $\rightarrow$ L were all found to be significantly associated, a majority of them positively and a few negatively.

Cytorrhexis in the proximal tubules was found to be significantly associated at 1% level with the detachment/rupture of *tunica propria* (r=0.691), obliteration of the lumen (r= 0.669), reduction in the height of epithelial cells (r= 0.437), proliferation of B-cells in the distal region of the tubules, (r=0.548), vacuolation of E-cells (r= 0.386), reduction in the number of E-cells (r=0.521) and increase in the height of tubule epithelium (r= 0.902). Infact cytorrhexis in the proximal tubule is very highly correlated with increase in the height of tubule epithelium as revealed by the 'r' value. However negative correlation was observed at 5% level significance with the proliferation of R-cells (r=-0.252) and with reduction in tubule width (r=-0.256).

Proliferation of R-cells was found to be significantly correlated at 1% level with reduction in height of epithelial cells (r= 0.378) and proliferation of connective tissue (r=0.595) and at 5% level of significance, with proliferation

Table 4: Correlation matrix for histopathological changes occurring in the hepatopancreas on exposure to various concentrations of WAF of BHC for varying lengths of time and other treatments:

	A	B	C	D	E	F	G	H	I	J	K	L
A	1.000											
B	-0.256*	1.000										
C	0.091	-0.168	1.000									
D	-0.256*	-0.281*	0.759**	1.000								
E	0.661**	-0.285*	0.419**	0.225	1.000							
F	0.669**	0.222	-0.089	-0.357**	0.337**	1.000						
G	0.437**	0.378**	-0.105	-0.388**	0.126	0.579**	1.000					
H	0.548**	0.296**	0.065	-0.186	0.384**	0.581**	0.821**	1.000				
I	0.386**	0.295**	0.184	0.022	0.311*	0.367**	0.671**	0.792**	1.000			
J	0.521**	0.168	0.294*	0.012	0.575**	0.466**	0.670**	0.714**	0.691**	1.000		
K	-0.059	0.595**	-0.153	-0.267*	-0.126	0.143	0.620**	0.560**	0.684**	0.446**	1.000	
L	0.902**	0.007	-0.027	-0.391**	0.540**	0.777**	0.644**	0.732**	0.550**	0.635**	0.224	1.000

r** > 0.332 (Significant at 1% level)

r**= 0.255 to 0.332 (Significant at 5% level)

Legend:

A	Cytorrhexis in proximal tubules	G	Reduction in height in epithelial cells
B	Proliferation of F-cells	H	Obliteration of lumen
C	Proliferation of haemocytes	I	Vacuolation of E-cells
D	Reduction in tubule width	J	Reduction in E-cells
E	Detachment/rupture of *tunica propria*	K	Proliferation of connective tissue
F	Presence of B-cells in distal region	L	Increase in height of epithelium

Table 5: Index of efficiency of the different treatments over the twelve parameters of histopathological change in the hepatopancreas of *M. dobsoni* (Miers) exposed to PHC and other stress factors:

Exposure level and duration	**Score**	**Ranking order**
Control (0 day)	60.094	11
Starved (5 days)	89.018	9
Starved (8 days)	103.517	7
8 ppm (5 days)	121.044	4
8 ppm (8 days)	139.987	2
4 ppm (5 days)	107.172	6
4 ppm (10 days)	124.696	3
4 ppm (15 days)	149.773	1
1 ppm (5days)	88.581	10
1 ppm (10 days)	91.402	8
1 ppm (15 days)	118.185	5
Control (15 days)	56.820	12

of B-cells in the distal region of the tubules (r= 0.296) and vacuolation of E-cells (r=0.295). However in a significant contrast, proliferation of R-cells were observed to be negatively and significantly correlated at the 5% level with reduction in tubule width (r=-0.281) and detachment/rupture of *tunica propria* (r=-0.285).

Proliferation of haemocytes was observed to be highly correlated at 1% level with reduction in tubule width (r=0.759) and detachment or rupture of the *tunica propria* (r= 0.419) and at 5% level of significance, with reduction in the number of E-cells. (r=0.294). Reduction in tubule width was observed to be negatively but significantly correlated at 1% level with the obliteration of the lumen (r=-0.357) with reduction in height of epithelial cells (r=-0.388), with increase in the height of tubule epithelium (r=-0.391) and at 5% level only with proliferation in connective tissue (r=-0.267).

Detachment/rupture of *tunica propria* is significantly correlated at 1% level with the obliteration of the lumen (r= 0.337), proliferation of B-cells in the distal regions of the tubules(r= 0.384), reduction in the number of E-cells (r= 0.575) and increase in the height of tubule epithelium (r= 0.540). At the 5% level it was significantly correlated only with the vacuolation of E-cells (r= 0.311).

The obliteration of lumen is significantly correlated with increase in the height of tubule epithelium (r= 0.777), reduction in the number of cells (r= 0.466) and vacuolation of E-cells (r= 0.367) due to the effect of various toxicant exposures.

Reduction in the height of epithelial cell of the tubules is significantly correlated at 1% level with the proliferation of B-cells in the distal regions of the tubules (r= 0.821), vacuolation of E-cells (r=0.671), reduction in number of E-cells (r= 0.670) and proliferation of connective tissue (r= 0.620). The high degree of correlation evidence indicates that the reduction in the height of epithelial cells is highly associated with the other types of degenerative changes mentioned here.

The proliferation of B-cells in the distal regions of the tubules is directly related to the vacuolation of E-cells (r= 0.792), the increase in the height of the tubule epithelium (r= 0.732), the reduction in the number of E-cells (r= 0.714) and the proliferation of connective tissue (r=0.568).

Vacuolation of E-cells is significantly related with reduction in the number of E-cells (r= 0.691), proliferation of connective tissue (r= 0.684) and increase in the height of tubule epithelium (r= 0.550).

The reduction in the number of E-cells is significantly associated at 1% level with proliferation of connective tissue (r= 0.446) and the increase in the height of tubule epithelium (r= 0.635).

c) Results of the discriminant function test:

The discriminant function index varied from 56.82 to 199.773. As per this score, maximum damage was caused by the administration of PHCs at 4 ppm for 15 days, followed by 8 ppm for 8 days, 4 ppm for 10 days, 8 ppm for 5 days, 1 ppm for 15 days, 4 ppm for 5 days, starvation for 8 days, 1 ppm

for 10 days, starvation for 5 days, and 1 ppm for 5 days. The control of 0 day and that of 15 days recorded the least score. The ranking is presented in Table 5.

3.4.2 Histopathology of the gills:

3.4.2.1 Visual appraisal under the light microscope:

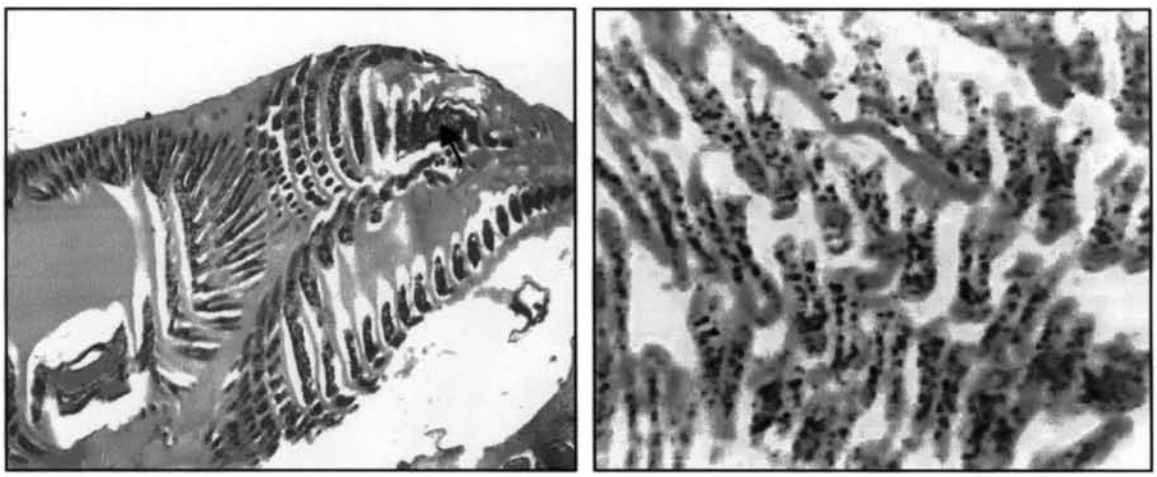
a) Control specimen:

Each gill consisting of an axis with a series of paired branches along its length. Intact vertical filaments of each branch are provided with bifurcating secondary filaments. Circulating haemocytes which carry oxygenated blood through the afferent system around the tip of the filament and out through the efferent system were present in the filaments and the axis in the primary afferent vessel and primary efferent vessel. No congestion or proliferation of haemocytes was noticed. The epithelial pillar cells and the vesicular ends of the primary and secondary filaments appear to be of characteristic shape.

b) Dosed specimen:

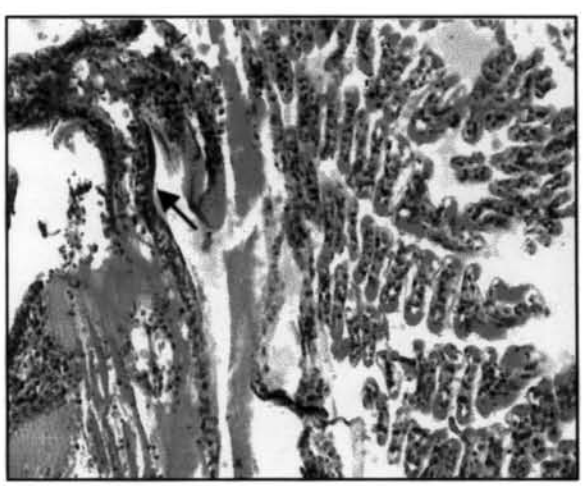
i) 1 ppm PHC dose:

A slight increase in numbers of circulating haemocytes was the only discernable change noted in all gills at 5 days of exposure to PHCs and in a majority of the filaments at 10 days. At 15 days congestion of haemocytes in the distal regions of some gills were noticed which gave these regions a blackened effect. However, several gills did not show this kind of lesion. A

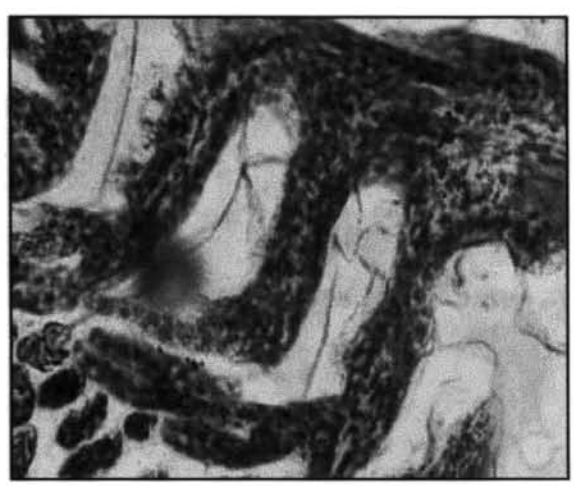


5 a

5 b



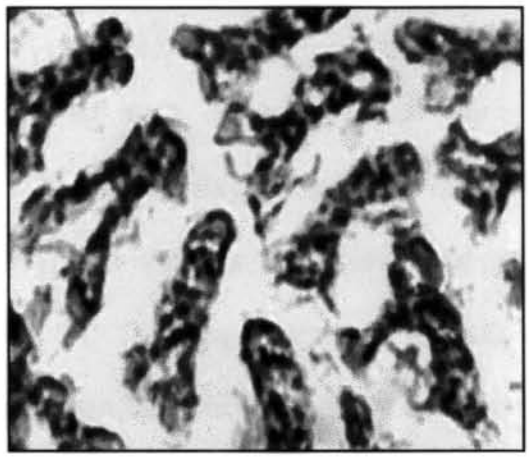
5 c



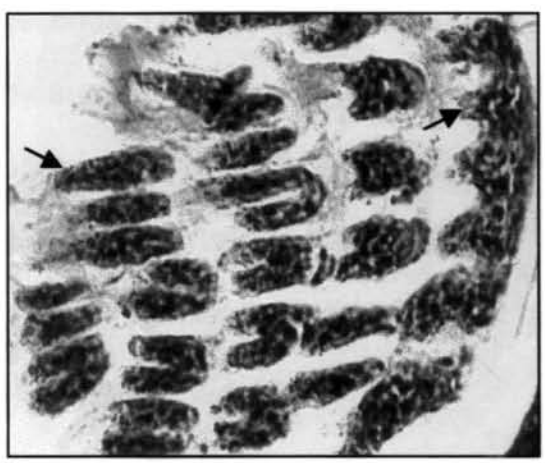
5 d

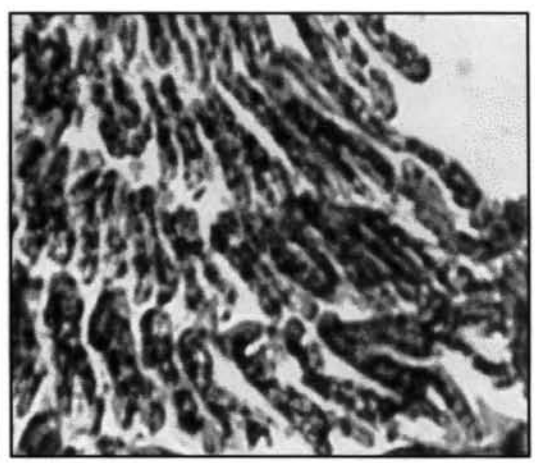
Fig 5: *Metapenaeus dobsoni* Miers: L. S. of gills:

a. Complete gill filament showing intact structures. Sloughing off of haemocytes (▲) restricted to the tip of the filament (control conditions, X 60).
b. Mild proliferation in the number of haemocytes in the gill filaments (1 ppm, 10 days, X 120)
c. Bands of necrotic haemocytes (▲) in the rachis and swelling of the tips of the secondary filaments (4 ppm, 10 days, X 120)
d. Congestion of necrotic haemocytes in the filaments (4 ppm, 15 days, X 240)



6 a





6 b

6 c

Fig. 6. *Metapenaeus dobsoni* Miers: L. S. of gills:

a. Shrinkage of pillar cells of filaments and necrosis of haemocytes (8 ppm , 5 days, X 320)
b. Necrotised stumps (↑) of filaments which have sloughed off, proliferation and necrosis of haemocytes (8 ppm, 5 days, X 240)
c. Shrinkage, atrophy and necrosis of secondary and secondary branching filaments (8 ppm, 8 days, X 120)

number of filaments appeared to have enlarged slightly in all days.

ii) 4 ppm PHC dose:

The congestion of axis, lamellae and filaments by haemocytes increased from the 5th to the 15th day of exposure resulting in gross distention of the filaments. The haemocytes themselves appeared blackened and necrotic especially on the 10th day. Bands of blackened haemocytes often formed boundaries between necrotic and viable areas in the same lamellae or rachis in the 10th and 15th days. The apical portions of the main rachis and secondary branching filaments were seen to be sloughing off in several gills by the 15th day with newly forming epidermis migrating inwards to the area where cells were being sloughed off. The areas sloughed off were heavily choked with necrotic haemocytes. A few secondary filaments showed a peculiar shrinking of epithelial pillar cells.

iii) 8 ppm PHC dose:

A proliferation in the numbers of shrunken pillar cells was noticed in secondary filaments examined on the fifth day. A large number of gills showed necrotic stumps, which were remnants of secondary gill filaments with sloughed off distal portions. The 8th day of exposure to 8 ppm evidenced the total atrophy of the various cellular components of the gill and predominance of the necrosis in this vital organ.

3.4.2.2 Statistical analysis of histopathological changes in the gills:

Six parameter were studied during the histopathological examination of the gills. The varied degree of necrosis of the gill filaments. Necrosis of haemocytes, proliferation of haemocytes, distention of pillar cells, shrinkage of pillar process cells and sloughing off filaments in the various treatments were compared using the Wilcoxon Signed Rank test. Average ranks obtained have been presented in Table 6.

a) Results of the analysis of variance (ANOVA):

Analysis of variance of the ranks obtained through Wilcoxons Signed Rank test indicated that the average effects of all the treatments were significantly different from each other since $F_{11,48}$ obtained for all the treatments were significant at the 1% level. The values are shown in Table 7 and 8.

1) **Necrosis of gill filaments:** Significantly low damage was observed in the controls and in the starved (5 days) treatments. The maximum damage was observed in 8 ppm (8 days) followed by 4 ppm (15 days), 8 ppm (5 days), 1 ppm (5 days) and 4 ppm (10 days).

2) **Necrosis of haemocytes:** Significantly low damage was observed in both the starved treatments as also in the controls. Severe damage was observed in 8 ppm (8 days) followed by 4 ppm (15 days), 4 ppm (10 days), 1 ppm (15 days) and 8 ppm (5 days).

3) **Proliferation of haemocytes:** Among the different treatments employed severe damage was observed in 8 ppm (8 days) followed by 4 ppm (15

Table 6: Average ranks obtained through Wilcoxons Signed Rank Test for the histopathological parameters observed in the gills of *M. dobsoni* (Miers) when subjected to various treatments with PHCs:

	TI	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
A	1	1	2	3	4	2	3	3	2	2	3	1
B	1	1	1	3	4	2	3	3	2	2	3	1
C	1	2	3	3	4	2	3	3	2	3	3	1
D	1	1	1	3	2	3	4	3	2	3	3	1
E	1	3	4	3	4	1	1	1	1	1	1	1
F	1	2	3	4	4	2	3	4	2	2	3	1

Legend:

	Parameter		Treatment		Treatment
A	Necrosis of secondary gill filaments	T1	Control (0 day)	T7	4 ppm (10 days)
B	Necrosis of haemocytes	T2	Starved (5 days)	T8	4 ppm (15 days)
C	Proliferation of haemocytes	T3	Starved (8 days)	T9	1 ppm (5 days)
D	Swelling up of pillar process cells	T4	8 ppm (5 days)	T10	1 ppm (10 days)
E	Shrinkage of pillar process cells	T5	8 ppm (8 days)	T11	1 ppm (15 days)
F	Sloughing off of filaments	T6	4 ppm (5 days)	T12	Control (15 days)

Table 7: Analysis of variance of the effect of PHCs on various histopathological parameters observed in the gills of *M. dobsoni*

:

	P1	P2	P3	P4	P5	P6
T1	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
T2	1.00 (1.00)	1.00 (1.00)	1.90 (1.38)	1.00 (1.00)	2.58 (1.61)	2.06 (1.43)
T3	1.90 (1.38)	1.00 (1.00)	2.84 (1.69)	1.00 (1.00)	3.72 (1.93)	3.06 (1.75)
T4	2.80 (1.67)	2.72 (1.65)	2.96 (1.72)	2.88 (1.71)	2.92 (1.70)	3.00 (1.97)
T5	3.50 (1.87)	3.84 (1.96)	3.90 (1.97)	2.00 (1.41)	3.56 (1.89)	3.90 (1.97)
T6	2.16 (1.45)	1.88 (1.37)	1.88 (1.37)	2.90 (1.70)	1.56 (1.25)	1.84 (1.35)
T7	2.54 (1.58)	2.76 (1.66)	2.80 (1.67)	4.00 (2.00)	2.50 (1.56)	2.50 (1.58)
T8	2.82 (1.66)	3.14 (1.77)	3.18 (1.78)	2.94 (1.71)	3.72 (1.93)	3.64 (1.91)
T9	2.00 (1.41)	2.24 (1.51)	1.76 (1.33)	2.00 (1.41)	2.12 (1.45)	2.00 (1.41)
T10	2.14 (1.45)	1.94 (1.39)	2.72 (1.65)	2.20 (1.48)	2.36 (1.53)	2.00 (1.41)
T11	2.74 (1.65)	2.76 (1.66)	2.98 (1.73)	3.00 (1.73)	2.84 (1.68)	2.92 (1.71)
T12	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
F11, 48	19.18**	328.53**	655.29**	301.61**	75.69**	270.59**
SE	0.07	0.02	0.02	0.02	0.04	0.02
CD	0.189	0.054	0.034	0.058	0.105	0.058

** $p > 0.05$

Note:- Values within paranthesis are the respective values transformed to their square roots

Table 8: Ranking order of the various treatments with PHCs in relation to histopathological changes effected in the hepatopancreas of *M. dobsoni* (Miers):

	1	2	3	4	5	6	7	8	9	10	11	12
p1	T5	T8	T4	T11	T7	T6	T10	T9	T3	T1	T2	T12
p2	T5	T8	T7	T11	T4	T9	T10	T6	T2	T3	T1	T12
p3	T5	T8	T11	T4	T3	T7	T10	T2	T6	T9	T1	T12
p4	T7	T11	T8	T6	T4	T10	T9	T5	T1	T3	T2	T12
p5	T3	T8	T5	T4	T11	T2	T7	T10	T9	T6	T1	T12
p6	T5	T4	T18	T3	T11	T7	T2	T10	T9	T6	T1	T12

Legend:

	Parameter		Treatment		Treatment
p1	Necrosis of secondary gill filaments	T1	Control (0 day)	T7	4 ppm (10 days)
p2	Necrosis of haemocytes	T2	Starved (5 days)	T8	4 ppm (15 days)
p3	Proliferation of haemocytes	T3	Starved (8 days)	T9	1 ppm (5 days)
p4	Swelling up of pillar process cells	T4	8 ppm (5 days)	T10	1 ppm (10 days)
p5	Shrinkage of pillar process cells	T5	8 ppm (8 days)	T11	1 ppm (15 days)
p6	Sloughing off of filaments	T6	4 ppm (5 days)	T12	Control (15 days)

days) as observed in earlier parameters. None of the values obtained were comparable to the controls signifying a high degree of proliferation in all treatments.

4) **Distention of pillar cells:** Low damage was observed in the starved and control conditions. Most sever damage was observed in 4 ppm (10 days), followed by 1 ppm (15 days), 4 ppm (15 days) and 4 ppm (5 days) in a mildly decreasing order.

5) **Shrinkage in pillar cells:** Significantly high damage was observed in the starved (8 days) condition followed by 4 ppm (15 days), 8 ppm (8 days), 8 ppm (5 days) and 1 ppm (15 days). The damage in the other treatments was not significant.

6) **Sloughing off of filaments:** The damage in all treatments except for the controls was severe. Significantly high damage was observed in 8 ppm (8 days) followed by 8 ppm (5 days), 4 ppm (15 days), starved (8 days) and 1 ppm (15 days) in a mildly decreasing order. In all other treatments also the values obtained were significantly different from the controls.

Summarising the results, there is a clear indication that among the different treatments exposure to 8 ppm PHCs for 8 days inflicted the maximum damage in four out of six parameters followed by 4 ppm PHCs (15 days).

Table 9: Correlation matrix for histopathological changes occurring in the gills on exposure to various concentrations of WAF of BHC for varying lengths of time and other treatments:

	A	B	C	D	E	F
A	1.000					
B	0.841**	1.000				
C	0.787**	0.781**	1.000			
D	0.617**	0.703**	0.500**	1.000		
E	0.632**	0.541**	0.859**	0.262*	1.000	
F	0.764**	0.742**	0.906**	0.434**	0.884****	1.000

r**> 0.332 (Significant at 1% level)

r** = 0.255 to 0.332 (Significant at 5% level)

Legend:

A Necrosis of secondary filaments
B Necrosis of haemocytes
C Proliferation of pillar process cells
D Swelling up of pillar process cells
E Shrinkage of pillar process cells
F Sloughing off of filaments

Table 10: Index of efficiency of the different treatments over the twelve parameters of histopathological change in the gills of *M. dobsoni* (Miers) exposed to PHC and other stress factors:

Exposure level and duration	**Score**	**Ranking order**
Control (0 day)	28.540	11
Starved (5 days)	46.998	10
Starved (8 days)	64.035	7
8 ppm (5 days)	89.446	3
8 ppm (8 days)	102.024	1
4 ppm (5 days)	58.075	8
4 ppm (10 days)	84.065	4
4 ppm (15 days)	93.151	2
1 ppm (5days)	55.414	9
1 ppm (10 days)	66.296	6
1 ppm (15 days)	83.839	5
Control (15 days)	28.541	12

c) Results of the Spearmans Rank Correlation test:

Table 10 presents the correlation matrix indicating the changes in the gill caused by exposure to PHCs and the relationship among the variables.

The necrotic secondary gill filaments were found to be highly correlated with necrotic haemocytes (r=0.841), proliferation of haemocytes, sloughing off of filaments, shrinkage of pillar cells (r= 0.632), distention of pillar cells (r=0.617).

Necrotic haemocytes were found to be significantly correlated at 1% level with proliferation of haemocytes (r= 0.781), sloughing off of filaments (0.742), distention of pillar cells (r=0.703) and shrinkage of pillar cells (r=541).

The proliferation of haemocytes is associated at 1% level significance with sloughing off of filaments (r=0.906), shrinkage in pillar cells (0.859) and to a lesser extent with distention of pillar cells (r=0.500). Distention of pillar cells is significantly associated at 1 % level of significance with sloughing off of filaments (r= 0.434). The shrinkage of pillar cells is highly correlated at 1% level of significance to sloughing off of filaments (r=0.889).

b) Results of the determinant function test:

The index score obtained for this experiment are shown in Table 7 and vary from 28.541 to 102.024. In this experiment, maximum damage was caused by exposure to 8 ppm PHCs for 8 days, followed by 4 ppm for 15 days, 8 ppm for 5 days, 4 ppm for 10 days, 1 ppm for 15 days, 1 ppm for 10 days, starvation for 8 days, 4 ppm for 5 days, 1 ppm for 5 days and starvation for 5 days. Control specimen on 0 and 15 days recorded the lowest scores.

3.5 DISCUSSION

Light microscopic assessment of histopathological changes provides visibly tangible evidence of damage by stressors on organisms, substantiating the use of cytological changes as biomarkers. Cajaraville *et. al.* 1993 recommended measurement of cellular responses with organismal responses to xenobiotics for accurate predictions in population and community structure. Varanasi *et al.* (1992) proffered the utility of specific pathological conditions such as neoplasms, non-neoplastic proliferative lesions, degenerative and necrotic lesions and hydropic vacuolation of biliary epithelia as biomarkers for contaminant exposure effects. Moore and Lowe (1985) also opined that cytology offers a useful approach following which the adaptive capability of a stressed animal and the eventual cellular changes could be measured. They identified histopathological conditions such as granulocytomas, haemopoetic neoplasms, haemocytic infiltration of tissues, alterations in reproductive tissues, loss of synchrony in digestive tubules, destabilisation of lysosomes and parasitic infestations in marine organisms as biomarkers. Several physiological mechanisms orchestrate the removal of xenobiotics from the body. The mode of intake, transport within, biotransformation and elimination are key factors influencing toxicokinetics. Histological alterations can be a consequence of these functions or a result of a failure, inadequacy or malfunction of defense/ compensatory mechanisms induced by the presence of the xenobiotic.

Until the early 1990s there was general agreement in the sequence of formation of cells within the hepatopancreatic tubules in the order E → R and E→ F → B cell sequence (Loizzi, 1971; Hopkin and Nott, 1980). Loizzi (1971) ascribed absorptive and storage functions to R-cells along with the metabolisation of lipids and glycogen. F-cells. He theorized that the F-cells synthesied digestive enzymes which accumulate in vacuoles that enlarge by pinocytosis of nutrients and fluids from the tubular lumen, with the vacuoles enlarging and coalescing until only the nucleus and a pinocytically active apical complex remain, completing its transition into a B-cell. Hopkin and Nott (1980) on the contrary believed that the secretion of digestive enzymes was done by F-cells alone and did not ascribe any secretive activity to the B-cells. Al-Mohanna and Nott (1985) described the functions of the B-cell as uptake, intracellular digestion, assimilation and finally elimination of the residual products of digestion, with the process being "fluid phase pinocytosis" for substances entering into the fluid of the vesicle and "absorptive pinocytosis" for those bound to the inner aspect of the vesicle membranes. This observation regarding the excretory function of the B-cell was also apparent in the present study with the tubules in relatively less stressed animals, which were presumably attempting the elimination of PHCs from the body, showing a significantly higher number of B-cells The nature of the contents of the B-cell vacuoles in stressed animals was in contrast with that of the normal B-cells where the amorphous material was

more homogeneous as compared to vacuoles with clumps of darkly staining material. Vogt (1994, 1996) proved that there is no conversion of F-cells to B-cells and that B-cells also arose from E-cells.

No single view has emerged in the mechanism of digestion in crustaceans with several workers favouring the holocrine mode (Hirsch & Jacobs, 1930) where the entire B-cell is dislodged into the lumen intact while others propounded a merocrine mode (Dall, 1967) where the B-cell vacuole along with other products are liberated into the lumen with the B-cell remaining fully functional. The apocrine mode, which is not clearly distinct from the merocrine mode, suggests that only the distal part of the B-cell is secreted (Loizzi, 1971). Gibson and Barker (1979) concluded that merocrine or apocrine secretion is normal but that intensive stimulation by starvation (fasting) may cause extensive holocrine discharge. Al-Mohanna and Nott (1986) provided evidence through electron microscopy for the holocrine mode, rejecting any secretory role for the B-cells. However, the true mechanism remains in doubt. In the present study, attempts have been made to delineate changes caused by PHC toxicity from that of normal digestion. As the digestive cycle of the shrimps is completed within 8 hours of feeding, sampling was done only after this stipulated period to minimize the chances of encountering digesting cells. While the extrusion of B-cells from the proximal end of the tubules and the presence of darkly staining inclusions within the hepatocytes is part of the natural progression of the digestive cycle, the mass delamination of hepatocytes, presence of clumps

of dislodged cells within the tubule lumens and the proliferation of darkly staining bodies can only be attributed to endotoxic response of the hepatocytes.

Histopathological alterations in shrimps due to toxicants have not received as much attention as in fish and in molluscs. Hypertrophy, atrophy of cells, changes in the lipid contents, proliferation of smooth ER, increased lysosomal autophagy, accumulation of lipofuscin in shrimp hepatopancreas (Manisseri, 1993) and the same has also been observed along with aging and neoplastic transformations in fish (Kohler, 1989,1990; Varanasi, 1989). The histopahological effects of crude oil on fishes at the light microscopic level observed by Prasad (1988) were that the changes associated commonly with the gills were lesions, oedema and mucous cell hypereplasia. Positive correlation between levels of sediment PAHs and prevalence of liver lesions in *Parophrys vetulus* from contaminated sites Puget Sound (Myers *et al.* 1990) and flounder *Pleuroicthys fleus* from similar regions in the Elbe estuary (Kohler, 1990) were reported. Bayne *et al.* (1980) listed several histological changes such as hyaline degeneration of the collagenous connective tissue of the gills, occurrence of parasites, production of mucus by gills, gonadal and haemopoetic neoplasms, granulocytomas, haemocyte infiltration of the tissues and loss of synchrony in digestive cells of mussels as useful for monitoring pollution effects.

As the B-cells are the excretory cells their proliferation in the dosed shrimps as compared to their numbers in the control shrimps where there is

a normal rate of progression within the tubules is expected, that is young B-cells are found to be abundant in the middle region whereas no or few mature B-cells are found in the middle or distal regions of the tubules. The current results evidence that there is a pronounced increase in the number of mature B-cells with exposure to PHCs and these are present closer to the distal region in dosed animals than in control animals.

The substantial proliferation of haemocytes in the starved specimen could not be explained. The increase of numbers in haemocytes noticed in the 4 ppm 15 days dose may be indicative of the activation of immune responses in the shrimp keeping in mind the occurrence of disease in animals subjected to the same dose beyond 18 days. The same response was however, not seen in the 1 ppm dose (15 days). The effect of stress on proliferation of shrimp haemocytes in the hepatopancreas needs further investigation. Auffret (1988) found a haemocytic diapedesis in the digestive tubules of *Mytilus edulis* to be a synergestic response to exposure to copper and diesel oil.

"Evidence of the value of lysosomal stability as a measure of cellular condition and catabolic potential is provided by significant positive linear correlation between this index and the physiological scope for growth" (Bayne *et al.*, 1979). Lysosomal latency was classified as a nonspecific general response to stress (Moore *et al.*, 1984). Moore and Lowe (1985) linked such degradative damage to functional physiological modules within the cell with eventual wide- ranging effects on the integration of cellular,

tissue and ultimately, whole-organism physiological processes. The damage inflicted by destabilisation of lysosomal membranes or "leaky lysosomes" is through the activation and release of lysosomal degradative enzymes induced by stressors. The activity of autolysosmes in severely stressed cells represent a pathological condition different from the survival mechanism effected by heterolysosomes or even autolysosmes in mildly stressed cells or cells attempting recovery from stressors (Moore *et al.*, 1982; Moore & Viarengo, 1987; Moore, 1991).

Halliwell & Gutteridge, (1986) found damaged cells and tissues to undergo lipid peroxidation, presumably secondary to membrane disruption by enzymes released from lysosomes and failure of antioxidant mechanisms. Viarengo (1984) demonstrated that lipofuscin granules trap toxic metals in a relatively stable form and opined that they may subsequently be eliminated by exocytosis of the residual bodies. Influx of water and ions were cited as the cause for swelling and damage of cell membranes with leaked intracellular ions, enzymes and other proteins in snail *Planorbis corneus* exposed to PCP (Klobucar *et al.*, 1997). Low pH inside the cell was also seen to stimulate lysosomal enzymes and cellular lesions in turn increasing lipid peroxidation within the cells. Destabilisation by lysosomal membranes may be linked to lysosomal enlargement and lipofuscin accumulation, both of which are indicative of autophagy. Autophagy is directly linked to digestive cell atrophy. (Moore, 1988; Lowe

& Pipe, 1994). Seven years after the *Amoco Cadiz* spill, oysters sampled off the site of the wreck in Brittany showed that accumulated hydrocarbons were found to interact with cellular hydrocarbons leading to different histopathological lesions with the highest necrosis index of cells being recorded in the digestive tract followed by interstitial tissues and gills (Berthou *et al.*, 1987). Klobucar *et al.* (1997) also found that a deficiency in ATP that stimulates glycolysis promotes the accumulation of lactic acid and a shift towards acidosis and also inhibits the cationic pump within cells allowing an influx of water and ions. Gross inundation of cells may lead to bursting and loosening from the cell membrane or myothelial fibres, ultimately leading to cytorrhexis and delamination.

The indices selected for the identification of the severity of cell damage and tissue damage following rank correlation method have limitations. It is assumed that the various parameters selected give an accurate picture of the health of the tissue and the organ. The limitations in identifying the various parameters that should form an integral part of rank analysis could sometimes mislead interpretations of the extent and gravity of tissue damages which in turn could give wrong signals on the condition of the tissues One such parameter in the present study is the proliferation of connective tissue in the 4 ppm PHC doses which does not occur in the other treatments. Therefore it becomes imperative to depend on first hand visual appraisal along with evaluation methods.

Proliferation of haemocytes observed both in the gills of dosed and

starved shrimps may be due to independent reasons. This may explain the seemingly erroneous correlation observed between proliferation of haemocytes and distention of pillar cells as well as shrinking of pillar cells. However, similarities observed in shrinkage of pillar cells and atrophy of hepatocytes in both starved and heavily dosed shrimps suggests that "starvation" and lack of nutrient availability from reserves (due to both reduced food intake and inefficient assimilation of food ingested) is experienced by shrimps exposed to 4 ppm PHCs (10 days) and above. The severity increasing with dosage and period of exposure on this reaction, points at the complexities of nutrient availability, transport and utility. This observation corresponds with behavioural records where the shrimps reduced the intake of food substantially and defecated often in the higher doses. As these parameters observed are caused by PHC exposure, the above factors are the results of a synergy in tissue reactions accompanying PHC toxicity and progressive "starvation".

The results suggest the presence of a mechanism in shrimps, which attempts at detoxification by utilizing the B-cells to expel the PHCs accumulated in the hepatopancreas and removing the cells from the gills by sloughing off the affected tissues in narcotized shrimps. This repair mechanism appears to loose efficiency as and when the tissues are more damaged in animals responding to higher dosages for a longer duration. Further, the proliferation of R-cells suggests that in shrimps the detoxification mechanism of PHCs involves breaking down of the PHCs and storage of

some of the metabolites to a certain extent. Vogt and Quintino (1994) found that the shrimp *Penaeus aztecus* detoxified heavy metals by expelling lead through haemocytes via the gills whereas copper was sequestered in the R-cells of the hepatopancreas. Exposure for prolonged periods or high doses results in large-scale toxicity to cells. An inability to cope with the burden of the toxicant coupled with direct damage to cell structure results in functional and structural breakdown of the tubules. Such damage to a primary organ of the digestive system would reflect in reduced efficiency of absorption, voiding of high caloried faeces and over a period of time this might lead to loss in body condition, diseases and ultimately, death of the shrimp. In addition to inefficient digestion, damage to gill filaments result in a slowing down of oxygen intake and metabolism, affecting the ability of the shrimp to combat stress. The histopathological findings, especially changes in the haemocytes, corroborate the trends identified in the rates of oxygen consumption and bioaccumulation of PHCs.

It is significant to note that in the present experiment, mortality on the 9^{th} day of exposure to the 8 ppm PHCs appeared to due to the inability of the shrimp to activate any compensative mechanism to toxicant uptake. This was evidently due to the overall deterioration of body condition, with the shrimp ultimately succumbing to narcosis on the addition of fresh toxicant. The behavioural changes of the shrimps and the changes to the structure and function of the nervous system in PHC toxicity in shrimps requires to be evaluated.

REFERENCES

*AL-MOHANNA, S. Y., 1983. The Hepatopancreas of *Penaeus semisulcatus* De Haan (Crustacea: decapoda) During the Digestive and Moult Cycles. Ph. D Thesis, University of Wales.

AL-MOHANNA, S. Y., NOTT, J. A. and LANE, D. J. W., 1984. M-'midget' cells in the hepatopancreas of shrimp *Penaeus semisulcatus* De Haan, 1844 (Decapoda, Natantia) *Crustaceana*, 48, 260-268.

AL-MOHANNA, S. Y., NOTT, J. A. and LANE, D. J. W., 1985. Mitotic E- and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* (Crustacea: Decapoda), *J. mar. biol. Ass. U.K.*, 65: 901-910.

AL-MOHANNA, S. Y. and NOTT, J. A., 1986. B-cells and digestion in the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda). *J. mar. biol. Ass.U.K.*, 66: 403-414..

AL-MOHANNA, S.Y. and NOTT, J. A., 1987. R-cells and their digestive cycle in *Penaeus semisulcatus*. *Mar. Biol.* 95 : 129-137.

AUFFRET, M., 1988. Histopathological changes related to chemical contamination in *Mytilus edulis* from field and experimental conditions. *Mar. Ecol. Prog. Ser.* 46: 101-107.

BAYNE, B. L., BROWN, D. A., HARRISON, F. and YEVICH, P. D. 1980. Mussel health. In: The International Mussel Watch. NAS (Ed.), Washington, D. C., pp.163-235.

BAYNE, B. L., MOORE, M. N., WIDDOWS, J., LIVINGSTON, D. R. and SALKEID, P., 1979 Measurement of the responses of individuals to environmental stress and pollution. Studies with bivalve molluscs. *Phil. Trans. R. Soc. Lond.* B. 286, 563-581.

*BELL, T. A., 1948. Handbook of normal penaeid shrimp histology. In: Bell and Lightner 1988 (see below)

BELL, T. A. and LIGHTNER, D. V., 1988. A handbook of normal penaeid shrimp histology. The World Aquaculture Society. Allen Press Inc., Kansas.114 pp.

BERTHOU, F., BALOUET, G., BODENNEC, G. and MARCHAND, M., 1987. The occurrence of hydrocarbons and histopathological abnormalities in oysters for seven years following the wreck of Amoco Cadiz in Brittany (France) *Mar. Environ. Res*, 23: 103-133.

BLISS, D. E. and MANTEL, L. H., 1983. The biology of crustacea, Vol. 5. Internal anatomy and physiological regulation. Academic Press, New York.

CAJARAVILLE, M. P. , MARIGOMEZ, J. A. , DIEZ, G. and ANGULO, E. 1992 Comparative effects of the WAF of three oils on mussels. 2. Quantitative alterations in the structure of the digestive tubules. *Comp Biochem Physiol , 102 C: 113-123.*

CAJARAVILLE, M. P. , MARIGOMEZ, J. A , and ANGULO, E., 1993 Correlation between cellular and organismic responses to oil- induced environmental stress in mussels. *Sci Total Environ Suppl.* Elsevier Science Publishers BV , Amsterdam. p. 1353 - 1371

CHANDY, J. P. and KOLWALKAR, D. G. , 1984. Histological changes in the gill and hepatopancreas of the marine crabs *Charybdis lucifera* (Fabricius) & *Scylla serrata* (Forskal) exposed to crude oil emulsion. *Indian J. Mar. Sci.*, 13: 10-13.

CLIFFORD, H. C. and BRICK, R. W., 1983. Nutritional physiology of the freshwater shrimp *Macrobrachium rosenbergii* (De man) –1: Substrate metabolism in fasting juvenile shrimp. *Comp. Biochem. Physiol.* 74A (3): 561-568.

DALL,W., 1967. The functional anatomy of a digestive tract of a shrimp, *Metapenaeus bennettae* Racek & Dall (Crustacea: Decapoda: Penaidea). *Aust. J. Zool.* 15: 688-714.

DALL,W., HILL,B. J., ROTHLISHERG,P. C. and STAPLES,D. J., 1990. The biology of penaeids. Advances in Marine Biology, Vol. 27 Academic Press London; 488 pp.

DALL, W. and MORIARTY D. J. W., 1983. Functional aspects of nutrition and digestion. In: The biology of crustacea, Vol. 5. Internal anatomy and physiological regulation. Bliss D. E and Mantel L. H. (eds.) Academic Press, New York, p. 215-251.

DALL, W. and SMITH, D. M., 1986. Oxygen consumption and ammonia-N excretion in fed and starved tiger prawns, *Penaeus esculentus* Haswell. *Aquaculture*, 55:23-33.

EVANS, D. H., 1987. The fish gill: Site of action and model for toxic effect of environmental pollutants. *Environ. Health Perspect.*71, 54-58.

GIBSON, R. and BARKER, P. L., 1979. The Decapod hepatopancreas. *Oceanography and Marine Biology, an Annual Review*, 17: 285-346.

HALLIWELL, B. and GUTTERIDGE, J. M. C., 1986. Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. *Arch. Biochem. Biophys.* 246: 501-514.

*HIRSCH, G. C. and JACOBS, W., 1928. Der Arbeitrhythmus der Mitteldarmdruse Von *Astacus leptodactylus. Z. Vergl. Physiol*, 8: 102-144.

*HIRSCH, G. C. and JACOBS, W., 1930. Der Arbeitrhythmus der Mitteldarmdruse

* Not referred to in original

Von *Astacus leptodactylus. Z. Vergl. Physiol*, 12: 525-528.

HOPKIN, S. P. and NOTT, J. A., 1980. Studies on the digestive cycle of the shore crab *Carcinus maenas* (L.) with special reference to the B cells in the hepatopancreas. *J. mar. biol. Ass. UK* 60 : 891-907.

HUMASON, G. L., 1972. Animal Tissue Techniques. 3rd ed. W.H. Freeman and Company. San Fransisco.

*JACOBS, W., 1928. Untersuchungen uber de cytologie der sekretbildung in der Mitteldamdruse von *Astacus leptodactylus. Zeitschrift fur Zellforshung und mikroskopische Anatomie,* 8: 1-16.

JOHNSON, L. L., STEHR, C., OLSEN, O. P., MYERS, M. , PIERCE, S. , MC CAIN, B. , and VARANASI, U. 1992. National Status and Trends Program, National Benthic Surveillance Project : Northeast coast, Fish Histopathology and relations between lesions and chemical contaminants (1987-89). NOAA. Tech. Memo. NMFS F/NWFCS-4. 96pp.

JOHNSON, P. T., 1980. Histology of the Blue Crab, *Callinectes sapidus*. Orager Publishers Division of Greenwood Press, Inc. Westport, Conn.

KLOBUCAR, G.I.V., LAJTNER, J., ERBENER., 1997. Lipid peroxidation and histopathological changes in the digestive gland of a freshwater snail *Planorbarius corneus* L. (Gastropoda, Pulmonata) Exposed to chronic and sub-chronic concentrations of PCP. *Bull. Environ. Contam. Toxicol.* (1997) 58:128-134.

KOHLER, A. , 1989. Cellular effects of environmental contamination in fish from the River Elbe and the North sea. *Mar. Environ. Res.* , 28 : 417-424.

KOHLER, A., 1990. Identification of contaminant induced cellular and sub cellular lesions in the liver of flounder (*Platichthys flesus*) caught at differently polluted estuaries. *Aquat. Toxicol.* , 16 : 271-274.

KUKKONEN, J. V. K. and LANDRUM, P. F., 1998. Effect of particle-xenobiotic contact time on bioavailability of sediment-associated benzo (a) pyrene to benthic amphipod, *Diporeia* spp. *Aquat. Toxicol.* 42: 229-242.

LEE, R. F., SAUERHEBER, R. and BENSON, A. A., 1972a. Petroleum hydrocarbons: Uptake and discharge by the marine mussel *Mytilus edulis*. *Science*, 177: 344-346.

LEE, R. F., SAUERHEBER, R. and DOBBS, G. H., 1972b. Uptake, metabolism and discharge of polyaromatic hydrocarbons by marine fish. *Mar. Biol.*. 17: 201-208.

LANDRUM, P. F., 1988. Toxicokinetics of organic xenobiotics in the amphipod, *Pontoporeia hoyi*: role of physiological and environmental variables. *Aquat. Toxicol.* 12: 245-247.

LEE, R. F., RYAN, C. and NEUHAUSER, M. L., 1976. Fate of petroleum hydrocarbons taken up from food and water by the blue crab *Callinectes sapidus*. *Mar. Biol.* 37: 363-370.

LILIUS, H., SANDBACKA, M. and ISOMAA, B., 1995. The use of freshly isolated gill epithelial cells in toxicity testing. *Toxicol. In Vitro*. 9, 299-296.

LOIZZI, R. F., 1971. Interpretation of crayfish hepatopancreas function based on the structural analysis of epithelial cell lines and muscle network. *Z. Zellforsch. Mikrosk. Anat.* 113: 420-440.

LOWE, D.M. and PIPE, R.K., 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cell: an in vitro study. *Aquat. Toxicol.* , 30: 357-365.

LUCU, C., 1990. Ionic regulatory mechanism in crustacean gill epithelia. *Comp. Biochem. Physiol.* 97: 297=306.

LUNA, L. G. (ed.) , 1968. Manual of Histologic staining Methods of the Armed Forces Institute of Pathology, 3rd ed. McGraw Hill, New York, NY.

MANNISSERI, M. K., 1993. Investigations on heavy metal stress and energetics in *Metapenaeus dobsoni* (Miers). Ph.D. Thesis, Cochin University of Science and Technology, Cochin, India.

MANISSERI, M. K. and MENON, N. R., 1995. Copper induced damage to the hepatopancreas of the penaeid shrimp *Metapenaeus dobsoni* - an ultra structural study. *Dis. Aquat. Org., 22: 51-57.*

MOORE, M. N. , 1988. Cytochemical responses of the lysosomal system and NADPH - ferroprotein reductase in molluscan digestive gland cells to environmental exposure to xenobiotics. *Mar. Ecol. Prog. Ser.* , 46 ; 37-43.

MOORE, M. N., 1991. Lysosomal changes in the response of molluscan hepatopancreatic cells to extra cellular signals. *Histochem. J.* , 23 : 495-500.

MOORE, M. N. and LOWE, D. M., 1985. Cytological and cytochemical measurements. In: The effects of stress and pollution on marine animals. Bayne B. L., Brown, D. A., Burns, K., Dixon, D. R., Ivanovici, A., Livingstone, D. R., Lowe, D. M., Moore, M. N., Stebbing, A. R. D. and Widdows, J. (eds.), Praeger Special Studies, Praeger, New York, pp.46-74.

MOORE, M. N., PIPE, R.K. AND FARRAR, S.V., 1982. Lysosomal and microsomal responses to environmental factors in Littorina littorea from sullum Voe. *Mar. Poll. Bull.* , 13: 340.

MOORE, M. N., WIDDOWS, J. , CLEARY, J. J. , PIPE, R. K. , DONKIN, P. , FARRAR, S. V. , EVANS, S. V. , and THOMSON, P. E. , 1984. Responses of the mussel *Mytilus edulis* to copper and phenanthrene: interactive effects. *Mar. Environ. Res.*, 14: 167-183.

MOORE, M. N. and VIARENGO, A., 1987. Lysosomal membrane fragility and catabolism of cytosolic proteins: evidence for a direct relationship. *Experientia*, 43 : 320-323.

MYERS, M. S., LANDAHL, J. T., KRAHN, M. M., JOHNSON, L. L., and MC CAIN, B. B., 1990. Studies on liver carcinogenesis in English sole from Puget Sound, Washington, USA: Evidence for a xenobiotic chemical etiology. Pathology and epizootiology. *Sci. Tot. Environ. , 94: 33-50*

PRASAD, M. S., 1988. Sensitivity of branchial mucous to crude oil toxicity in a fresh water fish, *Colisa fasciatus. Bull.Environ. Contam. Toxicol.* , 47: 754-758.

SANDBACKA, M., PART, P. and ISOMA A., 1999. Gill epithelial cells as a tool for toxicity screening- comparison between primary cultures, cells in suspension and epithelia on filters. *Aquat. Toxicol.* 46(1) 23-32.

SPEARMAN, C., 1904. Rank Correlations. In: Statistical Methods. Snedecor (ed.)G. W. (1946). Iowa State College Press, Ames, Iowa, USA

VARANASI, U., STEIN J. E., and NISHIMOTO, M., 1989. Biotransformation and disposition of PAH in fish. In: Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. Varanasi, U.(Ed.) 93-149 pp. CRC Press, Boca Raton , FL.

VARANASI, U., STEIN,J. E., JOHNSON,L. L., COLLIER, T. K., CASILLAS, E. and MYERS, M. S., 1992. Evaluation of bioindicators of contaminant exposure and in coastal ecosystems. In Ecological indicators , Vol.I. Proceedings of the International Symposium on Ecological Indicators,. D. H. Mc Kenzie, D. E. Hyatt, and V. J. Mc Donald (Eds.):461-498 pp.

VIARENGO, A., PERTICA, M., MANTINELLI, G. and ORUNESU, M., 1984. Possible role of lysosomes in the detoxification of copper in the digestive gland cells of metal exposed mussels. *Mar.Env.Res.*, 14: 469- 70.

VOGT, G., 1994. Life-cycle and functional cytology of the hepatopancreatic cells of *Astacus astacus* (Crustacea, Decapoda). *Zoomorphology*, 114:83-101.

VOGT, G., 1996. Morphology and physiology of digestive epithelia in Decapod crustaceans. *Pflugers Arch*, 1996, 431: 239-240.

VOGT, G. and QUINITIO, E. T., 1994. Accumulation and excretion of metal granules in the prawn, *Penaeus monodon*, exposed to water-borne copper, lead, iron and calcium. *Aquatic Toxicology*, 28:223-241.

WIDDOWS, J. , BAKKE, T. , BAYNE, B.L. , DONKIN, P. , LIVINGSTONE, D. R. , LOWE, D. M. , MOORE, M. N. , EVANS, S. W. and MOORE, S. L. , 1982. Response of *Mytilus edulis* to the water accomodated fraction of North Sea oil. *Mar. Biol. , 67 : 15 - 31.*

WIDDOWS, J., DONKIN, P. and EVANS, S.V., 1985. Recovery of *Mytilus edulis* L. from chronic oil exposure. *Mar. Environ. Res.* 17: 250 - 253.

YOUNG, J. H. 1959. Morphology of the white shrimp, *Penaeus setiferus* (Linnaeus, 1758). *Fish. Bull.* 59: 1-168.

Chapter 4:

ULTRASTRUCTURE

4.1 INTRODUCTION

Structural and functional integrity of cell organelles and cells is fundamental to the smooth orchestration of physiological functions in an organism. Measurable physiological and cytochemical stress indices are the cumulative manifestation of sustained disruption and disintegration of the structure and functions of cell organelles. Therefore subcellular assay techniques like the ultrastructure studies serve as an early warning system for the presence of harmful xenobiotics within a system, the pathological manifestations of which take place at the histological, biochemical or behavioural level. Cellular ultrastructure studies provide a means to identify possible routes of entry of xenobiotics into cells, target organelles of specific toxicants and subcellular changes indicative of detoxification or adaptive mechanisms. The sensitivity of ultrastructure studies is superior to and more accurate than light microscopic studies. Electron microscopy can reveal what light microscopy cannot since cell can be in a necrotic stage for several hours without showing gross structural changes detected under light microscopy (Trump & Arstila, 1975) or even before the effects of the toxicant become discernable at the behavioural level (Vogt *et al.*, 1987).

4. 2 REVIEW OF LITERATURE

Ultrastructure studies of biological tissues have been in use for the past four decades. However not much attention has been focused on histopathology of marine organisms, with the bulk of research being directed at mammalian tissues and cancer research.

Several workers have documented the ultrastructural studies on crustaceans, especially the structure of the hepatopancreas of the decapod crustaceans (Miyawaki and Tanoue, 1962; Bunt, 1968; Stainer *et. al.*, 1968; Gibson and Barker, 1979; Hopkins and Nott, 1980, Bliss and Mantel, 1983). The TEM and SEM studies of hepatocytes reveals that small lipid droplets and glycogen granules are numerous within R-cells with their numbers increasing with maturation of the cell. A number of electron dense zymogen granules are frequently encountered, particularly during the digestive phase in the F-cells. A supranuclear vacuole with a concentration of ferretin granules is usually present. The cell membrane is continuous, often with basal invaginations and with several pinocytic vesicles running deep into the F-cells during digestion. Digestive enzymes synthesised by these cells accumulate into vacuoles that enlarge by pinocytosis of nutrients and fluids from the lumen. These vacuoles enlarge and coalesce until only a single large vacuole, a nucleus and a pinocytically active apical complex remain when the F-cell completes its transition into the B-cell. Cell organelles within

B-cells showed degradative changes such as degeneration of cytoplasm and ER during the last phase of digestion when B-cells are ejected into the lumen Loizzi (1971).

Bunt (1968), who was one of the first to conduct detailed TEM studies of the crustacean hepatopancreas, confirmed the absorptive and lipid storage functions of the R- cells and attributed an exocrine role to F-cells. The hepatopancreas of the shrimp *Penaeus semisulcatus* was investigated in detail for determining the roles of the various cell types during the digestive cycle. It was confirmed that E-cells are not concerned directly with digestion but with mitosis and maturation into F and R-cells. F- cells are secretory (Al-Mohanna *et al.*, 1985a), R-cells have absorptive and storage functions (Al-Mohanna & Nott, 1987) and B-cells are excretory in function (Al-Mohanna & Nott, 1986). A new type of cell with large electron dense bodies occupying most of the area was also identified in starved individuals (Al-Mohanna, 1985b) and termed as M-cell.

Fine structure of the crustacean gills has received less attention than the hepatopancreas, though some detailed studies pertaining to morphology are available (Fisher, 1972; Green & Neff, 1972; Bubel & Jones, 1974; Finol & Croghan, 1983). Dickson *et. al.* (1991) stated that unlike the dorsal carapace, whose underlying epithelium is dedicated to secretion of cuticle deposition, the epithelium underlying the gill cuticle is predominantly concerned with respiration and ion transport and is consequently much reduced in thickness to facilitate these functions effectively. Andrews and Dillman (1993) have reported that the gill epithelium underlying the cuticle

and overlying the haemocoel is a continuous layer that remains relatively unchanged during the moult cycle. The respiratory epithelial cells are squamous with limited organelles and no elaborate Golgi or ER systems. The ion transporting epithelia have a characteristically convoluted apical membrane and are greater in height than respiratory epithelia. The large numbers of mitochondria present in it are suggestive of their function in active transport. Here also elaborate Golgi and ER systems are absent. In the examination of pre and post moult related changes in the gills, it was found that there is no postexuvial deposition of cuticle and the preexuvial cuticle deposition is greatly delayed, perhaps restricted to the last 10% of the moult cycle in both respiratory and ion transporting gill filament types.

Three types of haemocytes have been identified in decapod crustaceans: hyaline haemocytes which are the smallest and have small (~50 nm) granules and a nucleocytoplasmic ratio >0.35, the small granulocyte which have granules of varying sizes (generally 0.4 μm and above) and a centrally located nucleus, and the large granular haemocyte which have only large sized granules of 0.8 μm and above and an eccentric nucleus. The nucleocytoplasmic ratio of both granulocytes is <0.35. The hyaline haemocytes compose 5-10% of the circulating haemocytes in a normal decapod and are involved in the initiation of haemolymph coagulation. The granuloctyes are part of the defence mechanism of cells against alien material, which are neutralised or removed by phagocytosis or encapsulation. In the above evaluation, the small granular haemocytes are the most abundant types and comprise 75% of all haemocytes in several decapods,

often reducing the quantum of large granular haemocytes. On the contrary, hyaline haemocytes were found to be above 54% of the total haemocytes present in healthy *Sicyonia* shrimps (Martin & Graves, 1985; Hose *et. al.*, 1990; Martin & Hose, 1992).

One of the earlier studies in ultrastructural changes induced by toxicants in fish was by Baker (1969) who found myelin- like bodies and membrane bound vesicles containing elements of the endoplasmic reticulum (ER) in the epithelial cells of the gill lamellae of the winter flounder (*Pseudopleuronectes americanus*) subjected to copper poisoning. Leland (1983) observed only subtle changes in the ultrastructure of hepatocytes of juvenile rainbow trout and amature brown trout exposed to copper or zinc. Kohler (1990) found enlarged Golgi bodies and proliferation of rough endoplasmic reticulum in the livers of flounder *Platichthys flesus* caught from the Elbe estuary and other areas polluted by mercury and chlorinated hydrocarbons and interpreted them as adaptive sublethal responses indicating successful detoxification. Prasad (1991) conducted SEM studies on the effects of crude oil on the gills and air breathing organs of climbing perch, *Anabas tesudineus.*

During and subsequent to the Mussel Watch programme, investigations on the fine structure changes in molluscs gained momentum. The first discernable histopathological ultrastructural changes induced by xenobiotics were observed in subcellular organelles like mitochondria, lysosomes, endoplasmic reticulum and biological membranes (Moore, 1985). SEM and TEM studies done on the gill epithelium of mollusc *Patella vulgata*

after exposure to North Sea Crude and dispersants revealed a number of pathobiological changes ranging from damaged microvilli and cilia to severe disruption of normal cellular organisation, proliferation of lysosomes, vacuolation of mitochondria and extrusion of cytoplasm and damaged organelles. Difficulty in distinguishing primary effects caused by pollutants from the secondary changes occurring due to cell death was reported. However, the disappearance of apical granules, appearance of lipid- like droplets and an increase in number, size and apparent activity of lysosomes have been identified as primary changes. (Nuwayhid *et al.*, 1980). Structural deformities in ER, mitochondria, basal lamina and nucleus in varying degrees were seen in the shrimp *Metapenaeus dobsoni* exposed to copper (Manisseri & Menon, 1995).

The lysosomal–vacuolar system has been accepted as a biomarker for xenobiotic induced stress as it has been identified as the most sensitive among cell organelles to stress. Moore (1982) found that the stability of lysosomes in the digestive cells of *Mytilus edulis* is affected by changes in both the chemical and physical conditions of the environment. Lysosomes of digestive cells of the hepatopancreas of the mussels have the ability to accumulate organic contaminants like PHC, resulting in enhanced toxicity and cell injury through lysosomal damage (Moore, 1990; Pipe & Moore, 1985). Nott *et. al.* 1985 demonstrated through a procedure using cryopreservation and electron microscopy that phenanthrene caused proliferation of smooth endoplasmic reticulum (SER) and pathological

alterations in lysosomal membranes and that anthracene has no effect on the lysosomal membranes. Multiple lysosomal membrane layers may also be indicative of the addition of membrane-bound material to the lysosome, the surplus membrane being returned to the ER and outer cell membrane, with the blebbing phenomenon being a part of this process.

Several workers have reported the proliferation of SER in stressed cells. There is evidence that in mussels that the induction of phenobarbital and phenanthrene into the cell results in the stimulation of NADPH-neotetrazolium (cytochrome P-450) reductase (Moore, 1980). This enzyme is associated with the ER and structural evidence of proliferation of SER may be linked to the increase in enzyme activity (Nott *et al.,* 1985).

Exposure of *Littorina littorea* to 30 μg/l of naphthalene in the laboratory resulted in ultrastructural changes within secretory cells that manifested as increased formation of accumulated lipid droplets, increased occurrence of membrane bound dark bodies that became further enlarged after 96 hours of exposure to naphthalene, distortion of the Golgi complex and altered mitochondrial structure (Cajaraville *et al.*,1990).

Proliferation of basophilic cells have been reported with the possibility of their being associated with the simultaneous loss of digestive cells after injury and with disintegration or regenerative processes of digestive tubules in PHC treated mussels. Thompson *et al.* (1974) could not clearly establish whether the increase in basophil cells was absolute or if their proportion in relation to numbers of digestive cells had increased. Cajaraville *et al.* (1993)

found that the volume density of basophilic cells of the digestive cells in mussels exposed to crude oil WAF correlated with catalase levels in the tissue.

Fine structure studies on the changes accompanying starvation in isopod *Eurydice pulchra* show a general reduction and damage to cellular inclusions, a thickening of basal lamina and proliferation of vacuoles in the hepatopancreatic cells (Jones *et al.*, 1969). In juvenile *Penaeus monodon* starved for 7 days, a decrease in the size of the R -cell, depletion of the stored lipid inclusions and an increase in the thickness of the basal lamina was noticed (Storch *et al.*, 1984). Accumulation of residual glycogen and fat was seen in the initial phase of starvation in *Palaemon serratus.* (Papathanassiou & King, 1984). Manisseri (1993) has opined that an increase in the number of vacuoles or vesicles and general reduction in the cellular inclusions in *Metapenaeus dobsoni* starved for eight days may indicate utilisation of reserve food, burning out of mitochondria or spent lysosomes.

Aggregations of haemocytes to produce clots is described as a response to the presence of foreign material in the gills of crustaceans. While some of these haemocytes phagocytose the material, others are involved in the encapsulation of the larger haemocyte-foreign material aggregation. Such aggregations would subsequently occlude the gill vasculature (Smith & Ratcliffe, 1980). In addition to phenoloxidases, which may initiate immune responses in crustaceans, haemocytes contain a cell adhesion protein that may serve to bind haemocytes and foreign material in tight aggregations

called nodules (Johansson & Sodehall, 1992). Martin *et al.*, (2000) described this nodule formation as a fast and effective mechanism in crustaceans to eliminate foreign particles from circulation, with the sequestered material in the nodules being eliminated from the gills following the subsequent moult. Deposit cells or hyaline haemocytes probably form clots, consisting of coagulin polymers that cross link to form a gel matrix, which are initiated by cytoplasmic factors released by cell lysis. They subsequently merge to form a single continuous clot within a tissue (Omori *et al.*, 1989).

4. 3 MATERIALS AND METHODS

Juvenile *Metapenaeus dobsoni* of the size range 30-35 mm were collected and acclimated to laboratory conditions as described in Chapter 1. Healthy intermoult juveniles were selected from these and were exposed to the WAF of BHC diluted to 1 ppm, 4 ppm and 8 ppm concentrations for a period of 15 days in a semi- flow through system used for all previous experiments. Three replicate experiments for each dose were performed. The shrimps were fed on boiled clam meat *ad libitum.* Faecal matter was siphoned out daily. A set of shrimps was maintained under control conditions and another set was subjected to starvation for comparative studies. The shrimps subjected to starvation were maintained individually to avoid cannibalism.

One shrimp each was sacrificed from the replicates of each dose on the 5^{th}, 10^{th} and 15^{th} day in the case of those exposed to 1 ppm and 4 ppm PHCs and those maintained under control conditions. Sampling was done on

the 5th and 8th day for starved shrimp and those exposed to 8 ppm of PHCs. 2.5% gluteraldehyde stored at 4°C was injected into the cephalothorax while the animal was still alive. The hepatopancreas and gills was excised and fixed immediately in 2.5% gluteraldehyde buffered in 0.1M cacodylate solution at pH 7.2 for 30 minutes. The tissues were then washed three times in cacodylate buffer, trimmed to size, washed again in fresh buffer and post fixed in the secondary fixative (1% osmium tetroxide in 0.1M cacodylate buffer) for two hours at 4°C. After draining out the secondary fixative, the tissues were washed in several changes of fresh buffer solution and then dehydrated in an ascending series of acetone (30 minutes each in 30%, 50%, 70%, 90% and absolute acetone) at 4°C, giving two washes at each step. The tissues were infiltrated in varying proportions of acetone and Spurr media without DMAE (75:25, 50:50, 25:75). Special care was taken to infiltrate the gill tissue as it had a tendency to float. The tissues were finally embedded in Spurr Embedding Medium in plastic moulds and allowed to polymerise at 70°C temperature for 24 hours in a hot air oven.

The blocks were trimmed and semithin sections were cut from them, stained using methylene blue and viewed under a light microscope. Areas identified were marked and ultrathin sections of these were cut using an "Ultracut E " ultramicrotome, mounted on the matted side of copper grids, which were dried on a filter paper. The sections were then stained using uranyl acetate (Watson, 1968) and lead citrate (Reynolds, 1963). The sections were then examined under a Philips EM 300 Transmission Electron

Microscope at 60 and 80 KV and relevant areas photographed.

As E-cells are found only at the proximal tip of the tubules and were often absent in the sections observed, they were not included in the present study. Since the disintegration and extrusion of B-cells during the normal course of digestion is a common occurrence and distinguishing between such processes from the effect of PHCs proved to be difficult, they were also excluded from the study. Attention was mainly focused on F- and R-cells.

4. 4 RESULTS

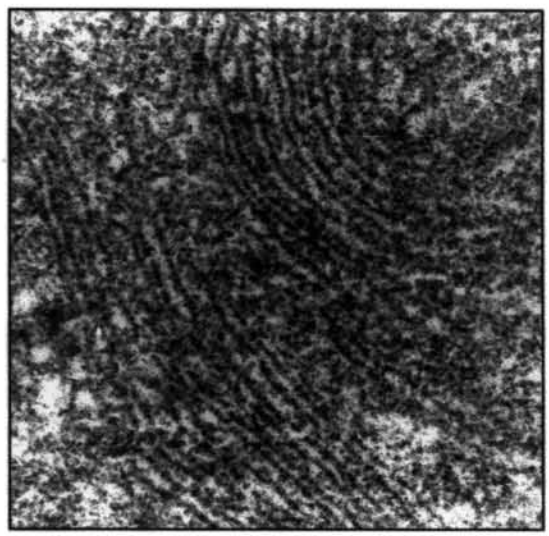
4.4.1 Subcellular changes in the hepatopancreas:

The morphology of normal F-, R-, and B-cells has been described in the previous chapter dealing with histopathology and the literature cited above. In the normal F- and R-cells of the hepatopancreas of shrimps, the cell organelles were well discernable with large networks of SER and RER (Fig 1 a) with uniformly placed intact ribosomes, Golgi apparatuses budding off primary lysosomes, lysosomes with electron dense material, intact mitochondria and basal lamina separating the cells from the haemolymph spaces. The nucleii were found to be intact with the nucleolemma adhering close to the nucleoplasm and in contact with adjacent SER. Chromatin was distributed in a characteristic pattern with one aggregation in the centre and the rest abutting the nuclear membrane. The nucleolus was not discernable. A supranuclear vacuole was often seen in F-cells. Mitochondria, though of

various sizes, had intact cristae. In B-cells the electron dense material present in the large vacuole were evenly distributed, giving the contents of the vacuole a homogeneous appearance.

The most discernable change in cells damaged as a consequence of exposure to PHCs was damage to nuclei, disintegration of endoplasmic reticulum (Fig. 1 b), increase in electron dense material and the presence of cellular debris. In shrimps dosed with 4- ppm PHCs for 10 and 15 days and 8 ppm PHCs for 5 and 8 days, a majority of F-and R- cell had disintegrating cytoplasm and were denuded of subcellular components, leaving behind large lipid droplets and pyknotic nuclei (Fig 1 c). However, the damage to hepatocytes in most of the damaged tubules did not appear wholly uniform with several cells still retaining the semblance of still functional entities (Fig.1c). A proliferation of microsomes was seen in all cells subjected to 1-ppm PHCs. Beyond this dosage it becomes difficult to discern microsomes from the debris of disintegrating SER and RER. Heavy vacuolation of the cytoplasm was seen in lower doses. Starvation induced changes within the cell that were distinctly discernable from the effects of the PHCs. Increased vacuolation and cellular breakdown were noticed (Fig. 8 b & c) but the storing of electron dense material was far reduced. Overall reduction in the size due to atrophy of cells was evident (Fig.8 a & c). A peculiar proliferation of wandering haemocytes in the intertubular spaces and in extruding B-cell vacuoles was also noticed.

The resting phase nucleus of the hepatopancreatic cells under normal conditions is an elliptical organelle, with a tensile nuclear membrane. The



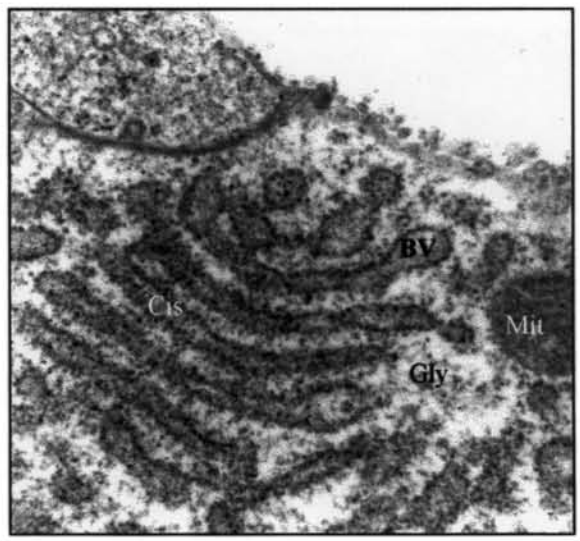
1 a

1 b

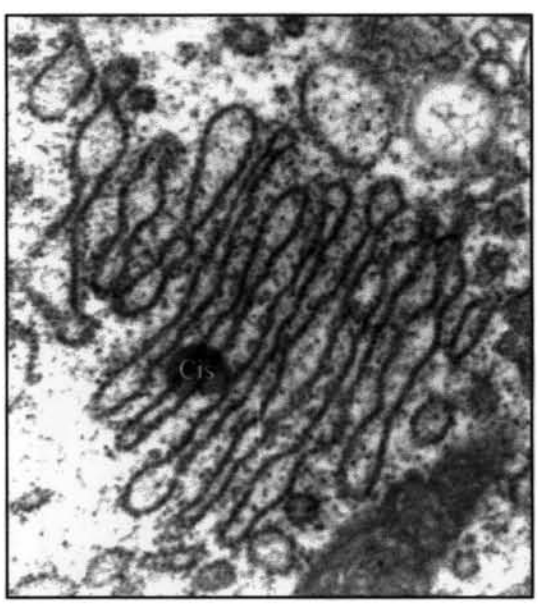
1 c

Fig.1. ***Metapenaeus dobsoni*** Miers: Ultrastructure of hepatocytes:

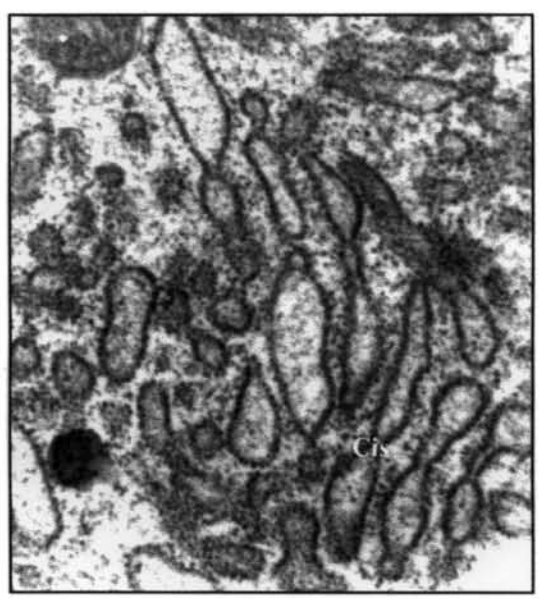
a. R-cell: Rough endoplasmic reticulum (control conditions, X 37800)
b. R-cell: Damaged rough endoplasmic reticulum (4 ppm PHCs, 15 days. X 37800)
c. Mid-region of tubule: Severely damaged R-cell with large lipid droplets (L) and a pyknotic nucleus (N) adjacent to a relatively less damaged F-cell (4 ppm PHCs, 10 days. X 6900)



2 a



2 b



2 c

Fig 2: *Metapenaeus dobsoni* Miers: Ultrastructure of hepatocytes:

a. F-cell: Golgi apparatus with cisternae (Cis) producing primary vesicles through blebbing (BV) and increased presence of glycogen granules (Gly) in the cytoplasm. A mitochondrion (Mit) is also seen. (1 ppm PHCs, 5 days, X 69000).
b. F-cell: Golgi apparatus with swollen cisternae (1 ppm PHCs, 15 days, X 69000).
c. F-cell: Golgi apparatus with increasingly swollen cisternae (1 ppm PHCs, 15 days, X 69000).

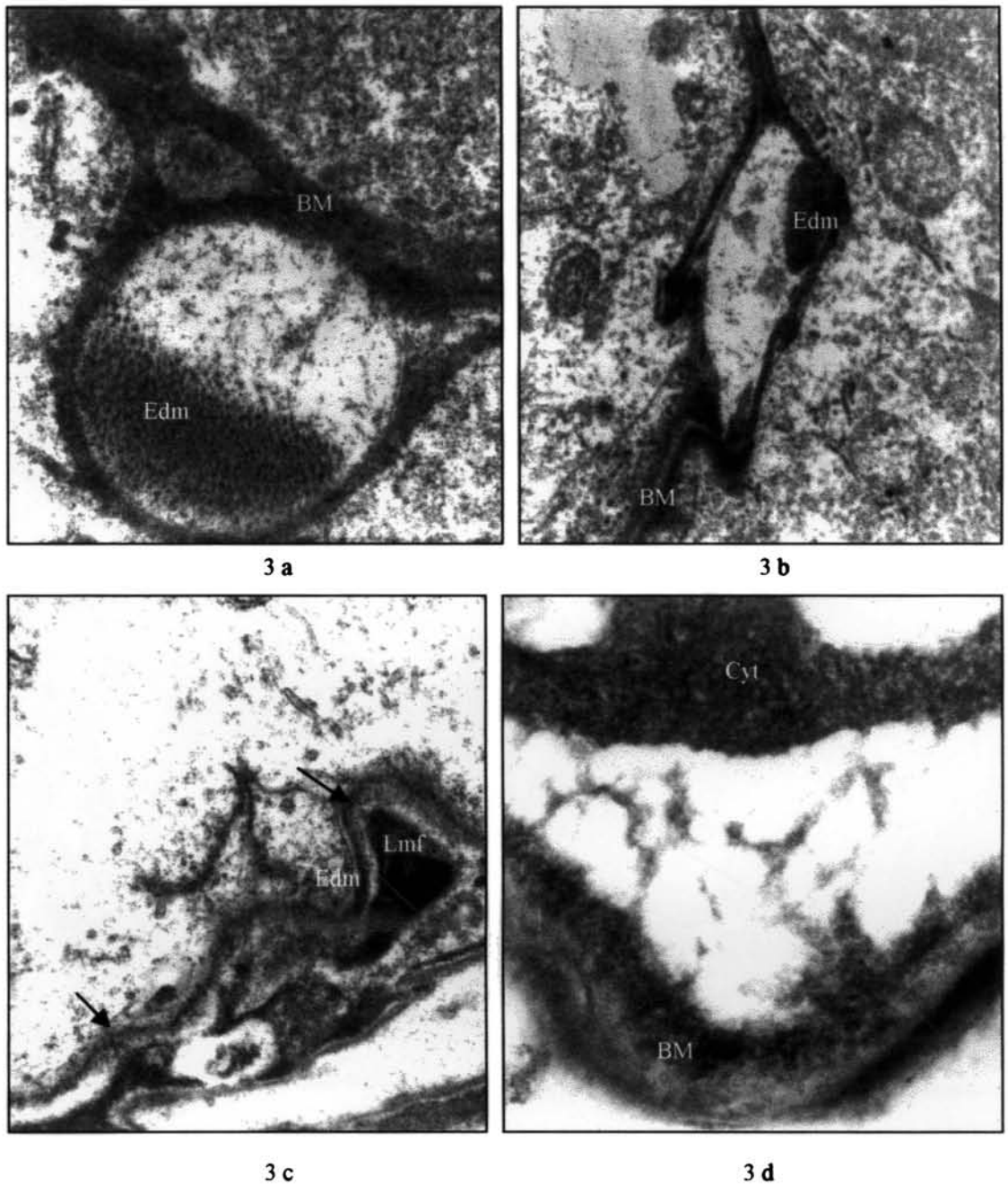
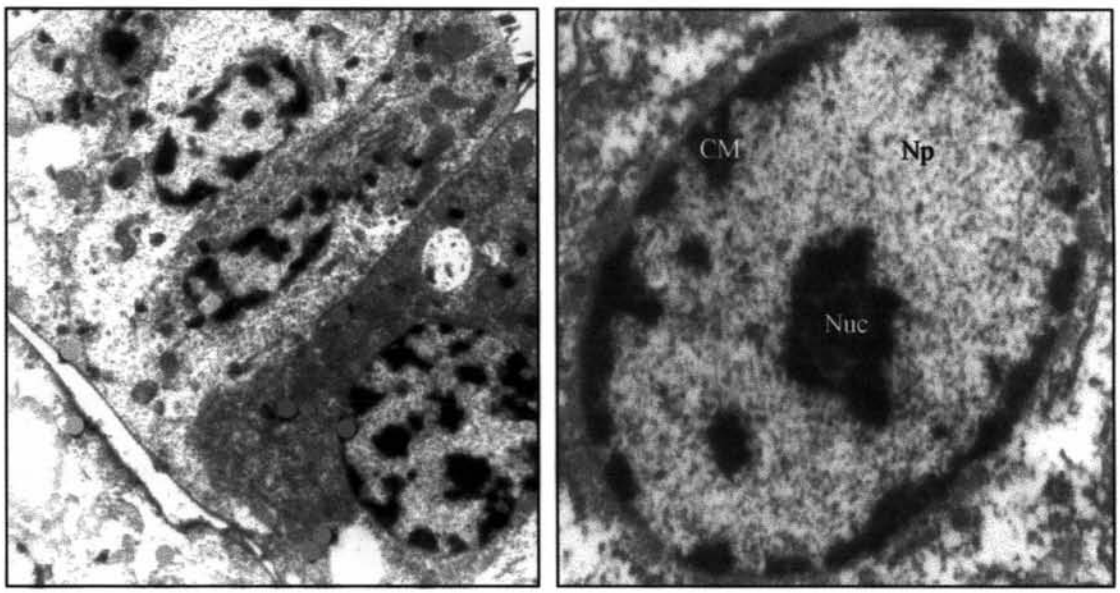
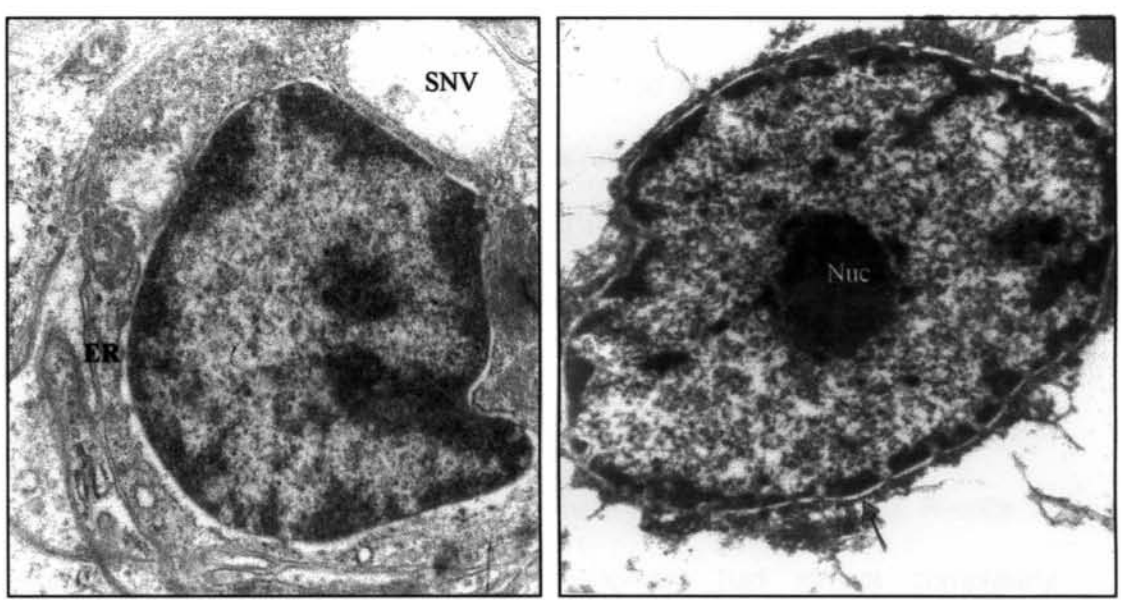


Fig.3. *Metapenaeus dobsoni* Miers: Ultrastructure of hepatocytes:

a. F-cell: Basal membrane (BM): Extrusion of a vesicle filled with electron dense material (Edm) (4 ppm,10 days. X 93000).
b. F-cell: Shearing of abutting basal membranes (BM) between hepatocytes with accumulation of electron dense inclusions (Edm) (4 ppm : 15 days. X 27600).
c. F-cell: Basal membrane: increased folding and blebbing with accumulation of electron dense material (Edm) along the folds, in the vicinity of a longitudinal muscle fibre (Lmf) (4 ppm, 15 days. X 37800).
d. R-cell: Separation of basal membrane (BM) from the cytoplasm (Cyt) (20 ppm, 8 days. X 1,20,000).



4 a 4 b

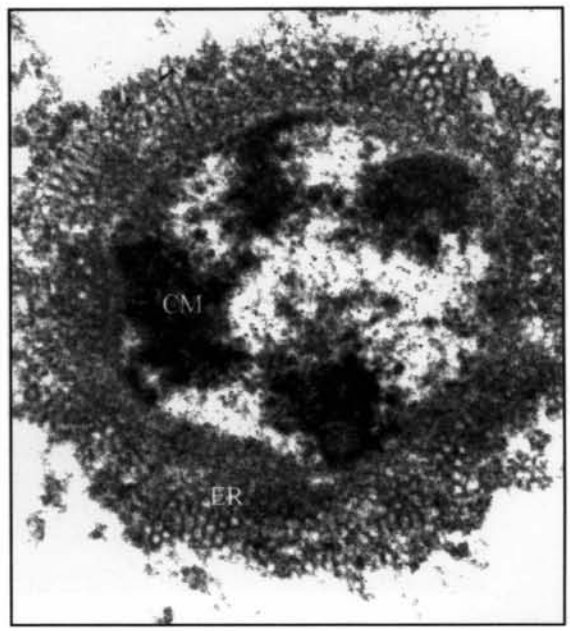


4 c 4 d

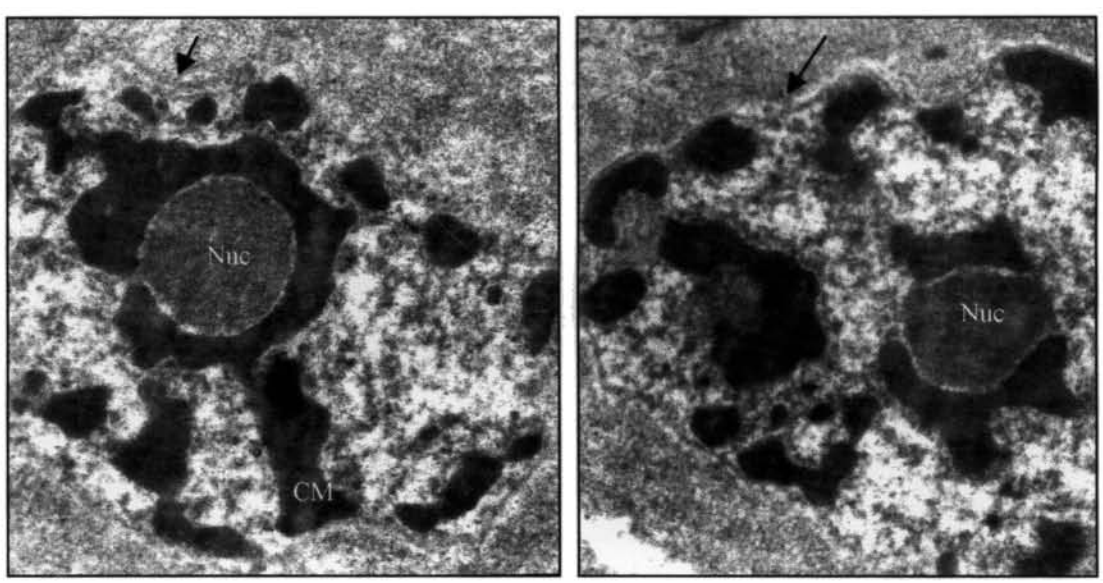
Fig. 4: *Metapenaeus dobsoni* Miers. Ultrastructure of hepatocytes:

a. Mid - region of tubule: A grossly enlarged nucleus (N) in an R cell adjacent to an F cell. (1ppm, 15 days. X11700)
b. F-cell: Nucleus showing a discernable nucleolus (Nuc), chromatin material (CM) and initiation of disintegration of nucleoplasm (Np) and surrounding endoplasmic reticulum (ER). (1 ppm. 5 days. X 20400)
c. F-cell: Nucleus (N) showing mild blebbing and distortion of nuclear membrane, surrounded by relatively intact endoplasmic reticulum (ER). The supranuclear vacuole (SNV) is also seen. (1 ppm, 10 days. X 27600)
d. F-cell: Nucleus showing a distinct nucleolus, disintegrating nucleoplasm and blebbing of nuclear membrane (↑). (4 ppm, 10days.X 20400)

karyoplasm communicates with the cytoplasm at intervals through pores. A network of SER usually surrounds the nucleus. A nucleolus surrounded by chromatin material is generally located in the centre and more chromatin material appears distributed along the periphery. The granular karyoplasm appears more or less evenly distributed. The most dramatic dose and time related changes were seen in the nuclei of the hepatopancreatic cells. An increase in size or overall swelling of the nucleus was noticed in the 1-ppm dose in samples from the 5th (Fig. 4 a) and 10th day of exposure. The opposite was noticed from the 15th day with shrinking and distortion of the nuclear membrane becoming evident (Fig. 4 c) and the degree of deterioration directly correlated with further increase in dose and time. The nucleoplasm remained in communication with the cytoplasm at intervals even through the detached nuclear membrane (Fig. 4 d). Chromatin stained more darkly with increase in stress. Chromatin material seemed to escape the nuclear envelope in some R- and F-cells of the hepatopancreas of shrimps exposed to 8-ppm PHCs for 5 days. A peculiar proliferation and crowding of SER around the nucleus was noticed in severely stressed nuclei of R-cells in which other cells organelles and cytoplasm had almost completely disintegrated in shrimps exposed to 4 ppm PHCs for 15 days (Fig.5 a). The nuclear envelope appeared wavy, perhaps losing its tensile strength in the F- and R- cells and even haemocytes present in the sinuses between tubules in the 8- ppm dose (Fig. 5 b), with multiple nucleoli appearing by the 8th day (Fig. 5 c). In starved cells, a slight shrinking and mild puckering of the nuclear membrane was noticed on the 8th day of starvation. In shrimps



5 a



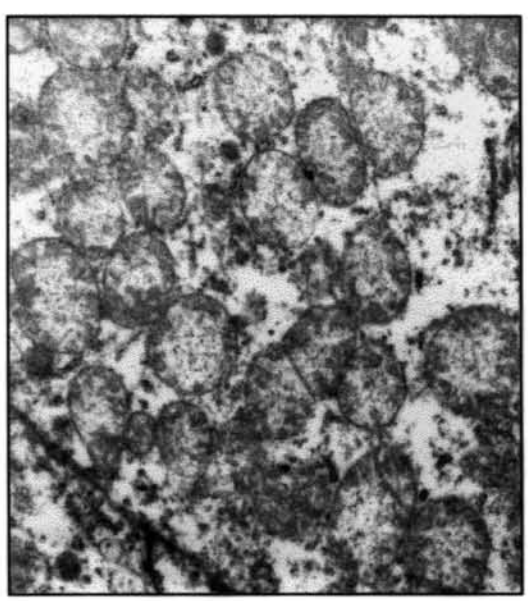
5 b

5 c

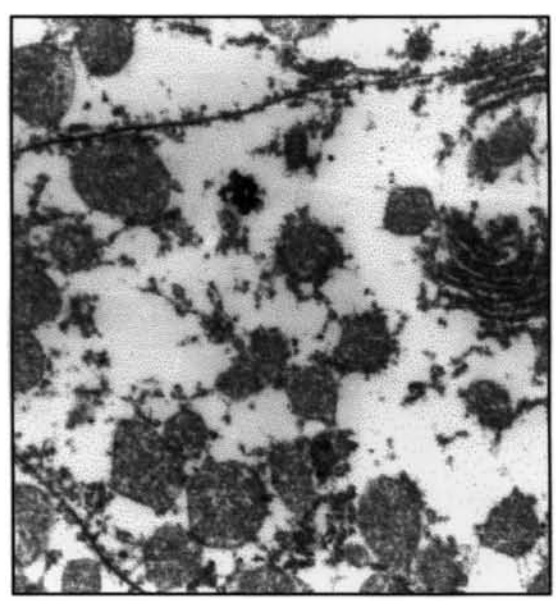
Fig.5. *Metapenaeus dobsoni* Miers. Ultrastructure of hepatocytes:

a. R-cell : Clumping of chromatin material (CM) and a peculiar proliferation of ER around the nucleus (4 ppm,15 days. X 50400)
b. Hyaline haemocyte: Nucleus showing distortion of nuclear membrane (↑), disorganisation of nucleoplasm and chromatin and a prominent nucleolus (8 ppm, 5 days X 44000)
c. Hyaline haemocyte: Nucleus showing severe distortion nuclear membrane (↑), disorganisation of nucleoplasm and chromatin (8 ppm, 8 days X 44000)

6 a



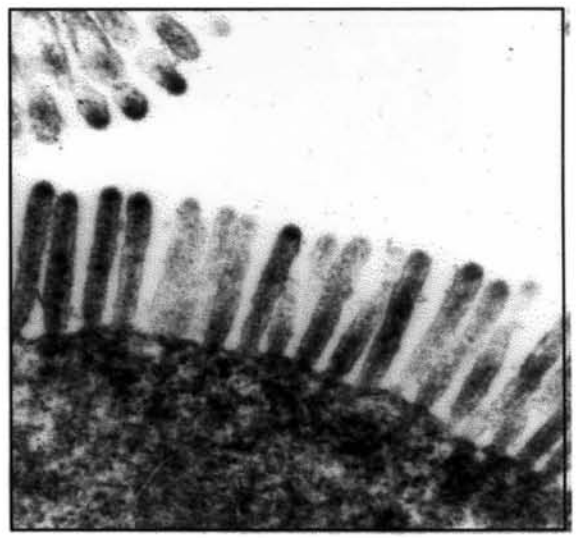
6 b



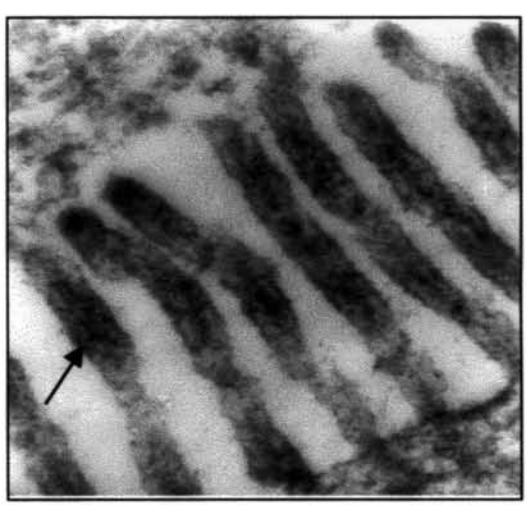
6 c

Fig.6. *Metapenaeus dobsoni* Miers: Ultrastructure of hepatocytes:

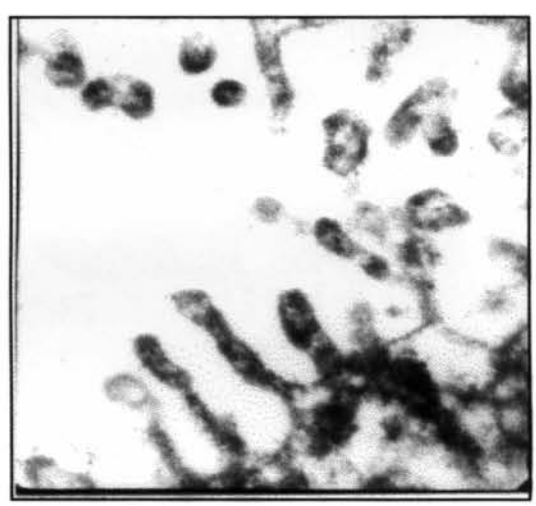
a. R-cell: Distortion in shape of the mitochondria (8 ppm, 5 days. X 78000)
b. R-cell: Proliferation of mitochondria (1 ppm : 10 days X 27600)
c. R-cell: Proliferation and mild distortion in shape of mitochondria (4 ppm, 10 days. X 20400)



7 a



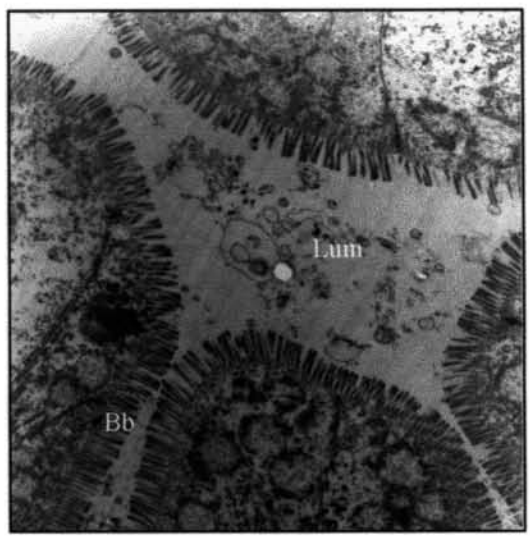
7 b



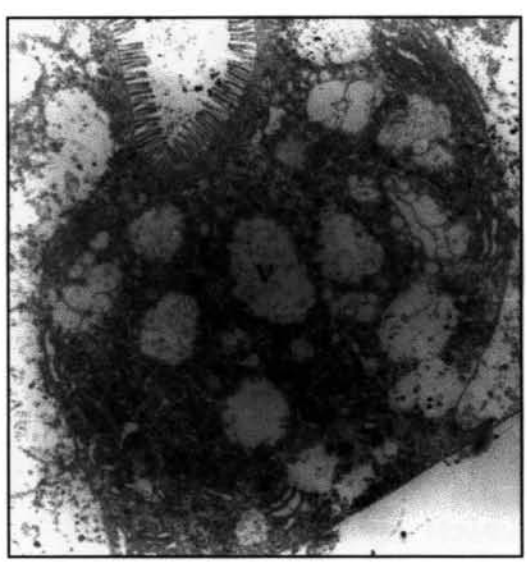
7 c

Fig.7. ***Metapenaeus dobsoni*** Miers: Ultrastructure of hepatocytes:

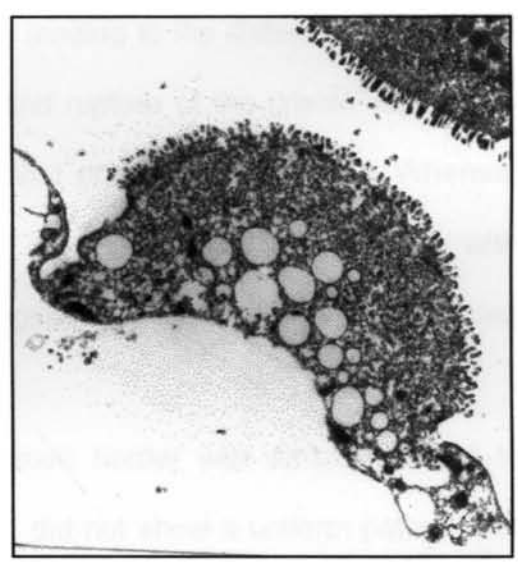
a. R-cell: Microvilli of brush border (Control conditions, X 69000)
b. R-cell: Microvilli showing increased formation of desmosome -like structures (↑) (1 ppm PHCs: 5 days. X 69000).
c. R-cells: Damaged microvilli (8 ppm PHCs: 5 days, X 69000).



8 a



8 b



8 c

Fig. 8. *Metapenaeus dobsoni* Miers. Ultrastructure of hepatocytes:

a. Shrunken tubules within the hepatopancreas of a shrimp (Starved: 5 days X 15900).
b. R-cell: Proliferation of vacuoles (Starved: 5 days X 15900).
c. Tubule lumen: Apical complex of a cell sloughed off into the lumen with numerous empty vacuoles (Starved: 8 days X 15900).

subjected to a lethal dose of 20 ppm for 15 minutes, the nucleus appeared heavily shrunk with disintegrating chromatin material and the presence of large vacuoles within the nucleus.

Numerous large electron dense dark bodies were accumulated in R-cells of *M. dobsoni* subjected to 8 ppm PHCs on the 5^{th} and 8^{th} days. They were distinctly different from those seen in the shrimps exposed to 1 ppm PHCs, where the small dark bodies seen could also be zymogen granules associated with regular digestion. A proliferation of lysosomes was noticed only in the shrimp exposed to1 ppm PHCs and those dosed for 5 days in 4 ppm PHCs (Fig. 6 a).

Proliferation of mitochondria was noticed in all hepatocytes exposed to PHCs Fig. 6 b). Swelling and shrinking, leading to the distortion of shape of mitochondria (Fig. 6 a & c) and loss and rupture of the cristae within was noticed in shrimps exposed to more than 1 ppm PHCs for 5 days. Whereas the changes related to energy release are also similar, the frequency with which it appeared in dosed shrimps suggests that these changes are induced by PHCs.

Distortion of microvilli of the brush border was directly related to increase in PHC dosage. However this did not show a uniform pattern with some intact cells retaining their microvilli in an undamaged condition even when those of several adjacent ones showed extensive distortion, shrinkage or detachment (Fig. 7 c). In the initial stages no distortion was seen but an increased number of desmosomes (Fig. 7 b) were present.

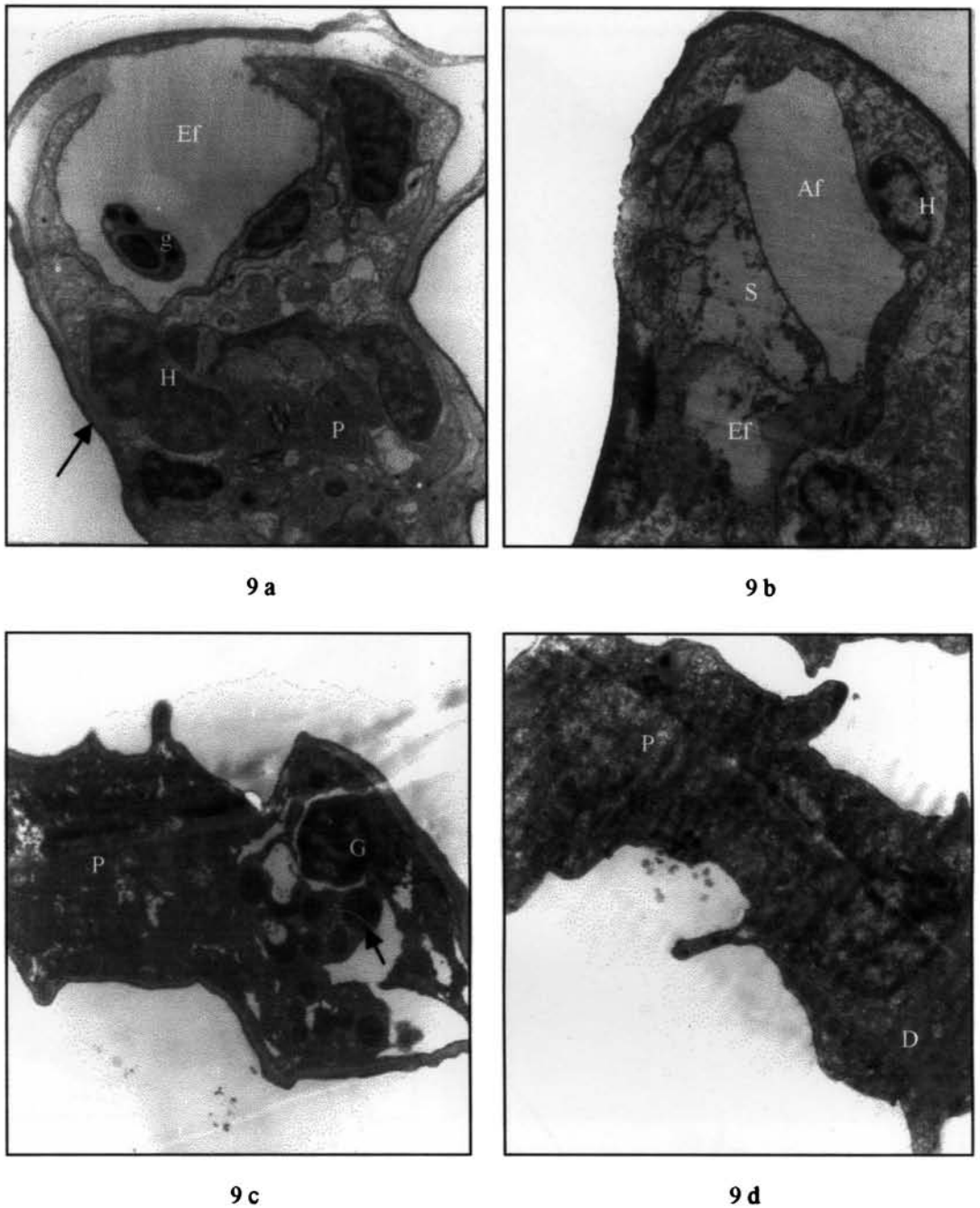
Ruffling and blebbing of the basal lamina along with the accumulation

of electron dense material was seen in all doses and periods of exposure with the severity being dose and time related (Fig. 3 c). Shearing and separation of the limiting membranes between adjacent cells was also seen (Fig. 3 b.). In one instance the basal lamina formed a vacuole-like bulge filled with electron dense material in the F-cell of a shrimp dosed with 4 ppm PHCs for 10 days (Fig. 3 a.). Total separation of the basal membrane from the cytoplasm was noticed in hepatocytes subjected to the lethal toxicity of 20 ppm PHCs for 15 minutes (Fig.3 d).

4.3.2 Changes in the cell organelles of gills:

In shrimps maintained under control conditions, the primary, secondary and secondary branching filaments appeared undamaged. Cell organelles of the epithelial pillar cells in secondary branching and secondary filaments, such as mitochondria, lysosomes and cytoplasmic vacuoles, were readily discernible and the circulating haemocytes were intact (Fig. 9 a).

Proliferation of haemocytes appeared to be the most discernible change brought about by exposure to PHCs. Shrinkage of pillar cells and the enlargement of the lacunae in the distal tips of secondary and secondary branching gill filaments also were observed as prominent structural response. (Fig. 10 b). Several gill filaments with severely shrunken pillar cells were noticed in shrimps exposed to lower doses of PHCs after 15 days (Fig. 10 c). However, necrotised and atrophied filaments were absent in the shrimps dosed with 1 ppm PHCs upto 10^{th} day of exposure. The incidence of such filaments was directly proportional to increase in dose and duration, though



9 a

9 b

9 c

9 d

Fig.9. *Metapenaeus dobsoni* Miers: Ultrastructure of the gils.

a. Secondary filament: Distal end showing efferent blood vessel (Ef), small granular haemocytes (g), hyaline haemocytes (H), an intact cuticle (↑) and other organelles of a pillar cell (P) (control conditions. X 6900)
b. Secondary filament: Distal end showing efferent (Ef) and afferent (Af) blood vessels, and disruption of cytoplasm of cell organelles of pillar cell and the septum (S) between the blood vessels (1 ppm: 10 days. X 6900)
c. Secondary filament: Distal end showing atrophy of pillar cell (P), necrosis of large granular haemocytes (G) and accumulation of electron dense bodies (↑). (4 ppm: 15 days. X 11700)
d. Secondary filament: Necrosis of pillar cell (P) and distal tip (D). (8 ppm: 8 days. X 11700)

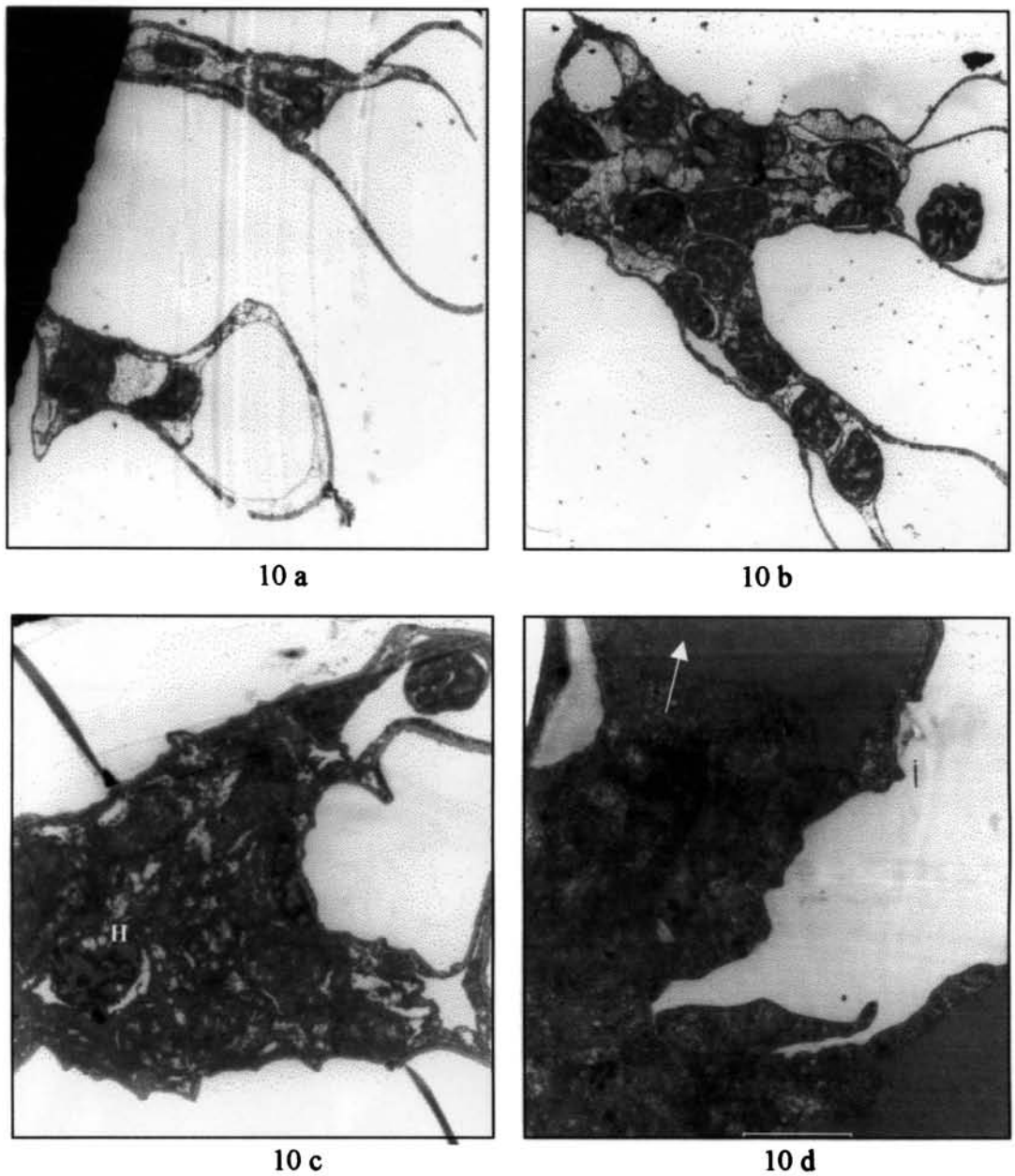
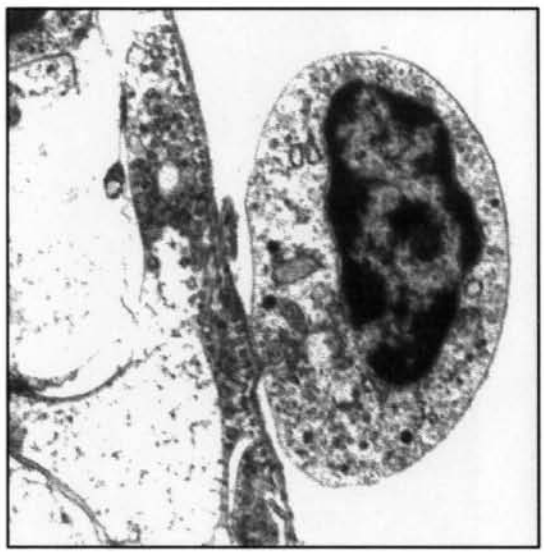
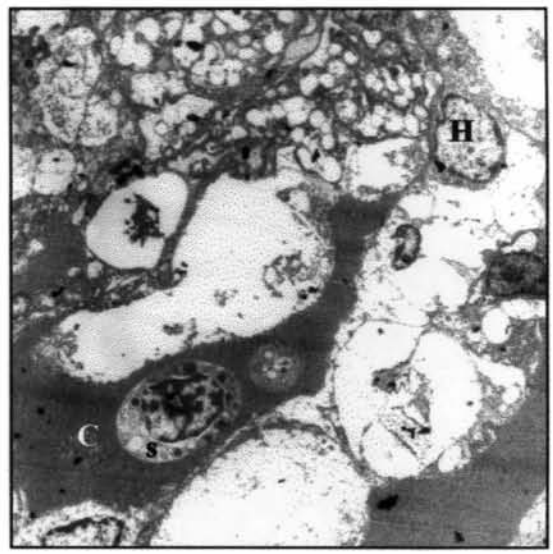


Fig. 10 *Metapenaeus dobsoni* Miers: Ultrastructure of the gills:

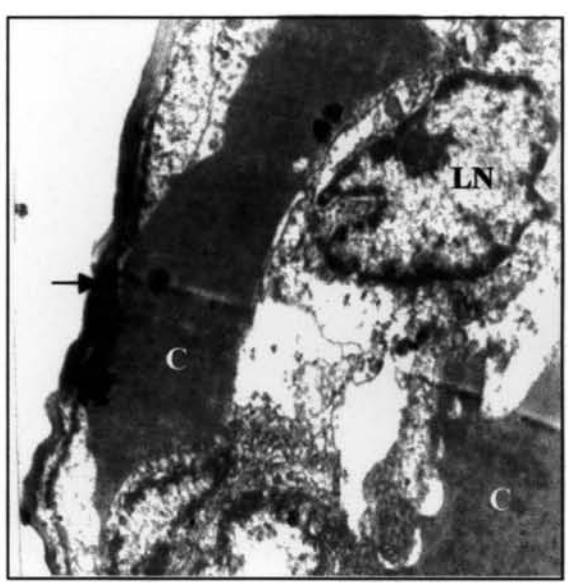
a. Secondary branching filaments: Shrunken pillar cells with a reduced number of organelles (starved 8 ppm, 8 days, X 6900)
b. Secondary branching filaments: Presence of numerous hyaline haemocytes (1ppm, 10 days, X 6900)
c. Secondary branching filaments: Partially necrotised pillar cells and haemocytes (H) (4 ppm, 15 days, X 6900)
d. Secondary branching filaments: Necrotised filaments with distal tips filled with coagulated haemolymph -like substance (↑ (8 ppm, 8 days, X 11700)



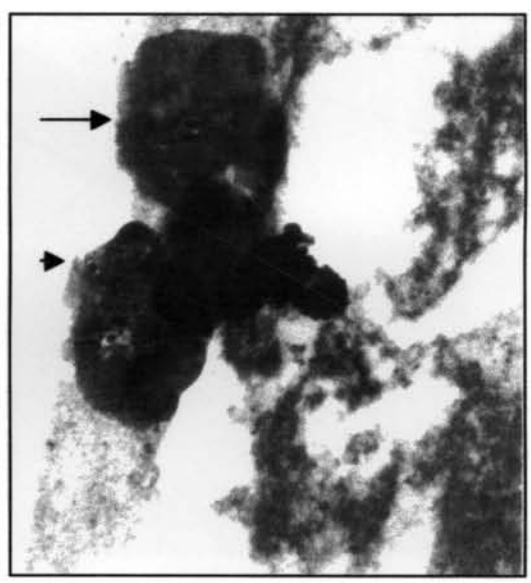
11 a



11 b



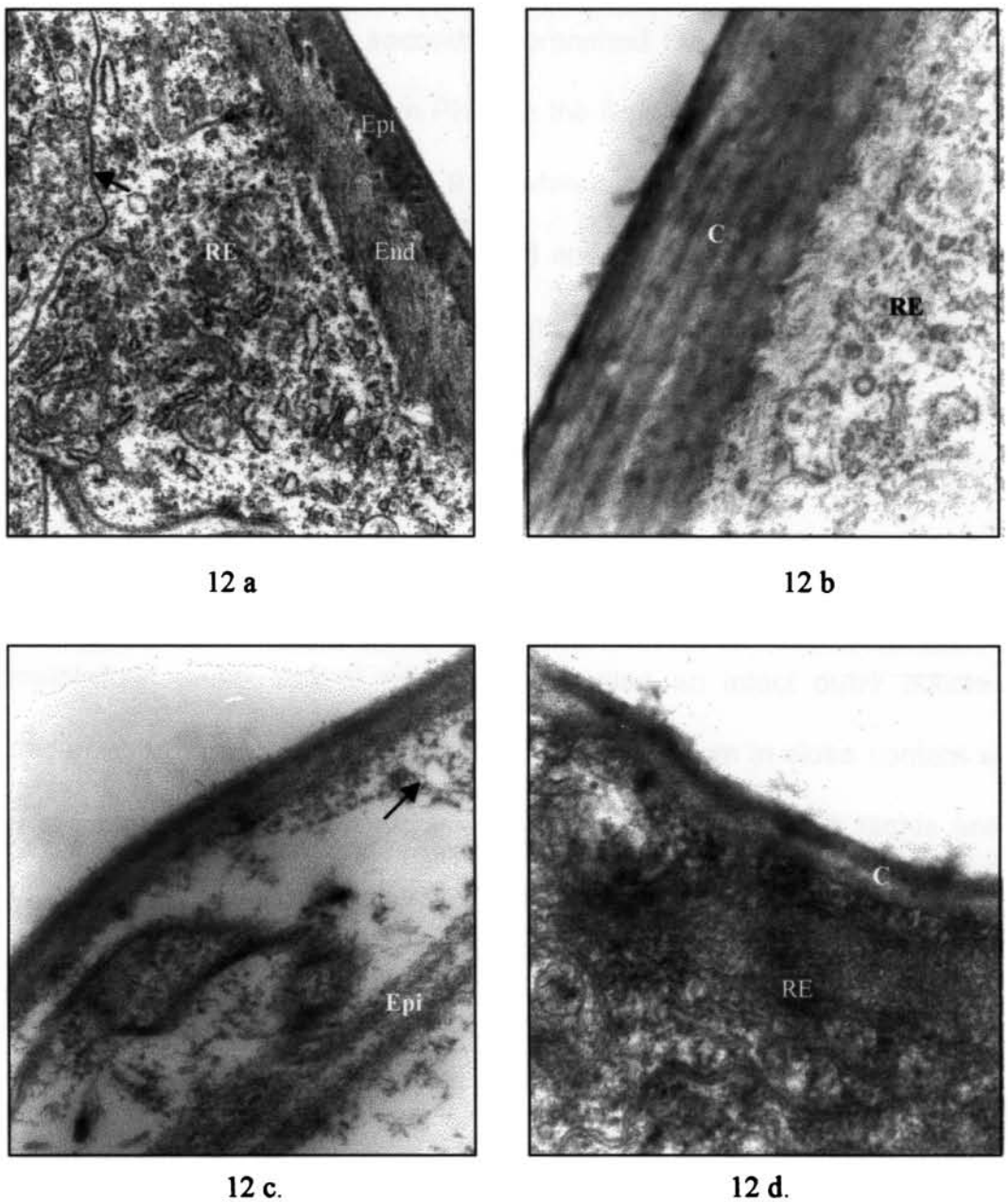
11 c



11 d

Fig. 11 *Metapenaeus dobsoni* Miers: Ultra structure of gills:

a. Small granular haemocytes: Intact nucleus and cytoplasm with small granules (control conditions, X 44000)
b. Coagulated haemolymph (C), a small granular haemocytes (s) and collapsed hyaline haemocytes (H) (1 ppm, 15 days, X 27800)
c. Lysing small granular haemoctes with pyknotic nuclei (LN), coagulated haemolymph (C) and the presence of a melanised plaque in the epithelial layer (↑) (4 ppm, 10 days, X 44000)
d. Melanised plaque (↑) formed in the epithelial layer (4 ppm, 15 days, X 93000)



12 a

12 b

12 c.

12 d.

Fig. 12. *Metapenaeus dobsoni* Miers: Ultra structure of the gills:

a. Secondary filament: Epicuticle (Epi), endocuticle (End) and underlying respiratory epithelium (RE) (control conditions, X 93000)
b. Secondary filament: Cuticle (C) and respiratory epithelium (RE) showing a mild degree of degeneration of organelles (1 ppm, 5 days, X 93000)
c. Secondary filament: Disruption of epicuticle (Epi) and damaged respiratory epithelium (RE) (8 ppm, 5days, X 93000)
d. Secondary filament: Necrotic cuticle (C) and respiratory epithelium (RE) (8 ppm, 8 days, X 93000)

total necrosis in all filaments were not seen in any shrimp. Necrosis of secondary filaments and secondary branched filaments was visible by the 15th day of exposure to 4 ppm PHC. In the 8 ppm dose, several gill filaments appeared wholly necrotic (Fig. 9 d) while some appeared partially necrotic with atrophied pillar cell, and enlarged and distorted haemocytes. (Fig. 9 c). The lacunae of the distal tips of some necrotic secondary branching filaments appeared to be filled with electron dense material similar to coagulin gel in shrimp exposed to 4 ppm for 15 days and 8 ppm PHCs for both 5 and 8 days (Fig. 10.d).

Cross section of a secondary filament from the gill of a shrimp maintained under control conditions revealed an intact outer cuticle and undamaged respiratory or ion transporting epithelium in close contact with it (Fig 12 a). Vacuolation was noticed in the cytoplasm of the rachis and the proximal tips of secondary filaments of gills in shrimps dosed for 5 days with 1 ppm PHCs. Epithelium underlying the cuticle showed signs of disintegration. This layer was found to degenerate further with increasing PHC dosage and time of exposure. By the 5th day of exposure to 8 ppm PHCs, the epithelial and the cuticular layers was greatly disrupted. Dissolution of the old cuticle was apparent in several regions of the filaments with the epithelium underlying the epicuticle unable to continue with the polymerisation and secretion of the new cuticle due to severe disorganisation of the cytoplasm (Fig.12 c). Large lacunae filled with patches of dissolving cuticle and cytoplasm appeared in both these layers above the haemocoel. By the 8th day of exposure to 8 ppm PHCs, several filaments showed acute

necrosis with the layers of the filaments including the cuticle being atrophied and indistinguishable. A few distorted haemocytes were discernible within these necrotised areas and in the tips of the filaments (Fig. 12 d.). Increased presence of granulocytes was evident in the haemocoel, especially that underlying ion transporting epithelium, and in distal tips of filaments in shrimps dosed with 4 ppm and 8 ppm PHCs.

Large-scale nodule formation was another notable change in gill filaments exposed to 1 ppm PHCs for 15 days and all higher doses (fig 11 b & c). Clot formation around lysed and damaged haemocytes was also common (Fig. 11 a & b).

4. 5 DISCUSSION

Juvenile shrimps were exposed to three concentrations of PHCs for varying periods. The results show that the concentrations employed did not exceed the genetic adaptive capabilities of the shrimps. However, examination of important target organs evinced various stages of cellular changes and organelle distortions. It is assumed that these were the manifestations of subacute toxic effects of PHCs under which juvenile shrimps can exist for variable durations. In this context the remark made by Bayne (1985) that the adaptive capability of mussels can be judged by cell death, which according to him should happen as a function of dose and time. Chronic exposure of mussels to copper polluted waters of Plymouth estuary was found to lead to severe damages of cells in the case of *Mytilus edulis*.

Lysosomes and endoplasmic reticulum are the most prominent subcellular organelles which can be used for early detection of toxic effects by way of change in structure (Slater, 1976). Discussing on the utility of using biomarkers for pollution monitoring studies, Lowe and Pipe (1994) felt that the digestive cells of the hepatopancreas are useful in the case of mussels. Kohler (1989) experimenting with flounder *Platychthys fleus* L. showed that pathological changes of liver could be judged by the disorganisation of RER, lysosome –lipofuscin and defects in the cytoskeleton. However, she felt that these were cellular responses showing initiation of detoxification processes, since the animals were sampled along a pollution gradient in the Elbe estuary which contained pollutants at different levels. These findings establish the utility of looking into ultrastructure responses of target organs of intoxicated organisms as biomarkers. Varanasi (1989) has remarked about stressor targets often as a specific cell type of the target organ. The utility of this according to her is that the primary intracellular disturbances triggered by the presence of xenobiotics or their derivatives could spread rapidly into a complex network of associated secondary and higher order disturbances. Autolysis and atrophy of important tissues do occur in bivalves facing starvation, which Bayne (1985) believes, is due to reduced capacity for metabolism and exhaustion of stored energy. It is also interesting to note that Thompson (1974) found major changes associated with starvation in protein secretory cells. Assuming that there is a time course in the pattern of

congregation of endoplasmic reticulum in the periphery of the nuclei, toward the nuclei and abutting the nuclear membrane. The evidence from the micrographs indicate that the disturbances expressed by way of a reorientation of chromatin granules to form clumps could be indictors of the nucleus facing a total disruption after reacting hard to safe guard its integrity by direct response or through the endoplasmic reticulum. This aspect needs further investigation which could prove whether the noticed changes can be categorised as biomarkers of xenobiotic reactions in general or as PHC intoxication in peculiar to shrimps.

Structural damages in nuclei owing to PHC contamination have been noticed by various authours (Jones and Huffman, 1957, Bayne *et.al.*, 1985). Varanasi (1986) even claimed that PAH could ultimately damage the DNA. The role of endoplasmic reticulum as an organelle involved in detoxification with reference to PHCs is lipophilic. One of the physiological mechanisms involved would be binding with membrane lipids. Goldacre (1958) found that hydrocarbons along with several aromatics caused swelling and disruption of plasma membranes of protozoans. Roubal (1974) and Roubal and Collier (1975), using spin-labelling techniques found that paraffins penetrate into and were associated with the hydrophobic interiors of membranes whereas aromatic hydrocarbons were bound to various nonploar and electron-interactive sites on the membrane surface., which caused perturbations on the membrane surfaces. Osmotic and ionic exchanges between the cell and the extracellular milieu is mediated by the cell membrane and is essential for

maintenance of cellular integrity and for such physiological functions as neurotransmission, muscle contraction and osmoregulation. Aromatic hydrocarbon induced changes in the surface organisation of membranes may interfere with these essential processes. In addition many enzymes associated with membranes are weakly bound to membrane lipids. Polar and other interactions between the membrane and enzymes may alter enzyme activity. In organisms exposed to cadmium, mercury and copper, the rate of protein synthesis reduces by slowing down the rate of RNA synthesis and by influencing the attachment of polyribosomes to the RER. (Viarengo *et al.*, 1987)

Cellular injury can induce dramatic alteration in ER with swelling and rupture of other membranous structures like the mitochondria, which additionally show lack of cristae was well as general cytoplasmic disorganisation (Bayne *et. al*, 1985). Activated derivatives of xenobiotics are retained within the lipoprotein membranes of the endoplasmic reticulum where they may enter a self sustaining redox cycle and give rise to potentially damaging oxyradicals which react with many biological molecules, leading to protein degradation, lipid peroxidation, DNA damage and cell death (Winston & Di Guilio, 1991). The resulting bulging and disintegration of the endoplasmic reticulum may have deleterious effects on the vital function of this organelle. Hinton and Lauren (1990) found that on exposure to xenobiotics, endoplasmic reticulum in cells can undergo proliferation, markedly altering the internal organisation. Proliferation of SER is an

indication of induction and increased activity of the MFO system. Detoxifying enzymes of PAH mixed function oxidases are localised in the microsomes in the SER of the hepatopancreas of bivalves (Varanasi 1989).

The findings of Viarengo *et. al.* (1987) that the rate of protein synthesis could be related to the rate of RNA synthesis in the case of the organisms exposed to cadmium, mercury and copper and to the reactions to RER integrity probably indicate that these organelles are directly involved in dealing with all xenobiotics.. Bayne (1985) opined that injury of cell induces structural alterations in the mitochondria. Early morphological changes preceding cell death include cytoplasmic oedema, dilation of endoplasmic reticulum and disaggregation of polysomes accompanied by accumulation of triglycerides as fat droplets. Late changes are progressive swelling of mitochondria, cristae disruption, cytoplasmic swelling, dissolution of organelles and nucleus and rupture of plasma membrane (Bridges *et. al,* 1983).

The seriousness of various types of liver lesions differs markedly. Necrosis may or may not be serious, depending on, for example the extent of the effect. New cells through mitosis of adjacent hepatocytes may replace local necrotic cells. An accumulation of triglycerides per se is not necessarily indicative of damage. Under certain conditions the hepatocytes with accumulated fat function normally. (Ingelfinger, 1971).

Increase of lipid droplets in the digestive cells is seen in PHC exposed molluscs. (Pipe & Moore, 1986; Cajaraville *et. al.* 1990). Kohler (1990) found that enlarged lipid droplets resembling large vacuoles filled with finely

granular material accompany accumulation of lipophilic substances in flounder *P. flesus* caught from a highly contaminated site and suggested that these vacuoles might play a role in the transport of lipoproteins.

Lysosomes can accumulate and sequester heavy metals and organic pollutants (Moore, 1985, Moore *et al.*, 1982, 1986). Damage to the lysosomal system is often apparent at the ultrastructural level where it involves the integrity of lysosomal membranes, resulting in "leaky" lysosomes, which can thus release their hydrolytic enzymes into the cytosol. Moreover, changes such as the swelling of the cells can occur while at the same time the cytoplasm becomes granular and eventually disintegrates, thus giving rise to a dense, opaque acidophilic mass (Bayne *et. al*, 1985). Examining the lysosomal membranes of *Mytilus edulis* subjected to PHC exposure Nott and Moore (1987) noticed discontinuities appearing as ruptures in the lysosomal membranes. A comparison of the involvement of lysosomes in the digestive apparatus of molluscs and crustaceans has an inherent lacuna. This is brought about by the variation in the mode of digestion resorted to by these two groups of invertebrates. Carles *et al.* (1986) explained the mechanism of lysosomal membrane injury involving lipid peroxidation and combination of lipofuscin as an after effect of PHC intoxication. Throughout the examination of ultrastructure of the hepatopancreas of shrimps show that the role of lysosomes in handling the toxicity of PHC is limited, evidenced by the generally reduced number of lysosomes encountered as compared to the pronounced increases seen in that of mussels.

The cuticle of the gill filaments in shrimp is much thinner than the protective cuticle of the carapace, abdomen and telson, largely to facilitate the effective diffusion and ion transport (Burggren *et. al.*, 1974). Consequently, its effectiveness as a barrier to toxicant entry is also far lesser than that of the exoskeleton. While damage to the cuticle is evident only in the higher dose of 8 ppm PHCs and towards prolonged exposure in 4 ppm, the damage to the underlying epithelium is more discernible. It can be concluded that the inability of the epithelium underlying the epicuticle to synthesis and polymerise chitin due to disruption of cytoplasm and organelles is the major factor that disallows the regeneration of damaged cuticle. This evidently would inhibit the inherent capacity of the cuticular layer to reduce the entry of toxicant which when comes in contact with the cells of the filaments destroys their integrity. The most dramatic changes noted in the formation of melanised nodules along the gill cuticle and widespread formation of clots by lysed hyaline haemocytes are indicative of attempts made by the shrimps to eliminate the toxicant and to control toxicant induced damage within the organs. Bauer (1998) described the formation of nodules, which follows degeneration of the gills and influx of haemocytes caused by toxicant exposure, as a well developed mechanism to keep the external and internal (Martin *et.* al., 2000) surfaces of the gill clean.

However, beyond a "point of no return" these mechanisms fail to protect the tissues from necrosis. The fine structure studies substantiate the observations made in the experiments on physiological responses and light

microscopic histopathology examinations discussed earlier. Martin *et. al.* (2000) opined that heavy bacterial infections which resulted in a high load of foreign material into the gills of shrimps, lobsters and crayfish could results in breakage of the exoskeleton, and formation of nodules far larger than the diameter of the vasculature, impinging on the normal functioning of the gill and hampering respiration and ion regulation by restricting blood flow. The hepatopancreas and in particular the gills have been proven to be the major organs involved in detoxification and elimination of PHCs from the shrimps. In comparison to other types of toxic responses such as those induced by heavy metals (Victor, 1993; Manisseri & Menon, 1995; Vogt & Quintino, 1994) it can be said that there is less sequestration of the toxicant in the hepatopancreas with the toxicant being metabolised to a large extent and stored in lipid droplets only in the higher doses. Also a great amount of the toxicant is eliminated via sloughing off of necrotised epithelium in the higher doses and nodule formation in all doses.

REFERENCES

AL- MOHANNA, S. Y. and NOTT, J. A., 1986. B-cells and digestion in the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda). *J. mar. biol. Ass. U. K.* 66: 403-414.

AL- MOHANNA, S.Y. and NOTT, J. A., 1987 R-cells and their digestive cycle in *Penaeus semisulcatus*. *Mar. Biol.* 95: 129-137.

AL-MOHANNA, S. Y., NOTT, J. A. and LANE, D. J. W., 1985 a. Mitotic E- and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus*

(Crustacea: Decapoda), *J. mar. biol. Ass. U. K.*, 65: 901-910.

AL-MOHANNA, S. Y., NOTT, J. A. and LANE, D. J. W., 1985 b. M-'Midget' cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* de Haan, 1844 (Decapoda, Natantia), Crustaceana 48 (3): 260- 268.

ANDREWS, S. C. and DILLAMAN, R. M., 1993. Ultrastructure of the gill epithelia in the crayfish *Procambarus clarkii* at different stages of the molt cycle. *J.Crust. Biol.* 13 (1): 77-86.

BAEUR, R. T., 1998. Gill- cleaning mechanisms of the crayfish *Procambrus clarkii* (Astacidea: Cambaridae): Experimental testing of setobranch function. *Invertebrate Biol*: 117: 129-143.

BAKER, J. T. P., 1969. Histological and electron microscopical observations on copper poisoning in the winter flounder (*Pseudopleuronectes americanus*). *J. Fish. Res. Bd. Can.* 26: 2785-2793.

BAYNE, B. L., BROWN, D. A., BURNS, K. A. , DIXON, D. R. , IVANOVICI, A. , LIVINGSTONE, D. R. , LOWE, D. M. , MOORE, M. N. , STEBBING, A. R. D. and WIDDOWS, J., 1985. The effects of stress and pollution on marine animals. Praeger Publishers, New York.

BAYNE, B. L., 1985 Responses to the environmental stress: Tolerance, Resistance and Adaptation. In: Marine Biology of Polar regions and Effects of Stress on Marine Organisms. Gray, J. S. and Christiansen, M.E. (eds.), John Wiley and Sons Ltd. pp. 3331-3349.

BLISS, D. E. and MANTEL, L. H., 1983. The biology of crustacea, Vol.5. Internal anatomy and physiological regulations. Academic Press, New York

BUBEL, A. and JONES, M. B., 1974. Fine structure of the gills of *Jaera nordmanni*. *J. mar. Biol.* 54: 737- 743.

BUNT, A. H.,1968. An ultrastructural study of the hepatopancreas of *Procambarus clarkii* (Girard) (Decapoda, Astacidea). *Crustaceana* 15: 282-288.

BURGGREN, W. W., MAC MOHAN,B. R., and COSTERTON, D. B.,1974. Branchial water and blood flow patterns and structures of the gill of the crayfish *Procambrus clarkii, Can. J. Zool.*52: 1511-1518.

CAJARAVILLE, M. P., DIEZ, G. , MARIGOMEZ, J. A. , and ANGULO, E., 1990. Responses of the basophilic cells of the digestive gland of mussels to petroleum hydrocarbon exposure. *Dis Aquat Org* , 9: 221-28.

CAJARAVILLE, M. P., MARIGOMEZ, J. A. , and ANGULO, E., 1993 Correlation between cellular and organismic responses to oil- induced environmental stress in mussels. *Sci. Total Environ. Suppl.* 1353 - 1371. Elsevier Science Publishers BV , Amsterdam.

CARLES, D., HENRY, M., SIRON, R. and GUISTI, G., 1986. Induction of the ultrastructural alterations in the digestive gland of *Mytilus galloprovincialis* by crude oil. In : Strategies and advanced techniques for marine pollution studies : Mediterranean Sea. Giam, C. S. and Dou, H. J. M. (eds.), NATO ASI Series, Vol. G 9, Springer - Verlag, Berlin/Heidelberg.

DALL, W. and MORIARTY, D. J. W., 1983. Functional aspects of nutrition and digestion. In: The biology of crustacea, Vol.5. Internal anatomy and physiological regulations. Bliss D.E. and Mantel, L. H. (eds.) Academic Press, New York. p. 215-251.

DICKSON, J. S., DILLAMAN, R. M., ROER,R. D. and ROYE, D. B.,1991.Distribution and characterisiation of ion transporting and respiratory filaments in the gill of Procambarus clarkii. *Biological Bulltn.* 180: 154-166.

FINOL, H. J. and CROGHAN, P. C..1983. Ultrastructure of the branchial epithelium of an amphibious brackish-water crab. *Tissue & Cell.*15: 63-75.

FISHER, J. M., 1972. Fine-structure observations on the gill filaments of the freshwater crayfish, *Astacus pallipes*. *Tissue & Cell*, 4: 287-299.

GIBSON, R. and BARKER, P. L., 1979 . The decapod hepatopancreas. *Oceanogr. mar. Biol. Ann. Rev* 17:285-346.

GOLDACRE, R. J.,1968. The effects of detergents and oils on the cell membrane. In: The Biological Effects of Oil Pollution on Littoral Communities. Carthy J. D. and Arthurs D. R. (eds.) London: Field Studies Control.

GREEN, J. P. and NEFF, M. R., 1972. A survey of the fine structure of the integument of the fiddler crab. *Tissue & Cell* 4: 137-171.

HINTON, D. E. and LAUREN, D. J.,1990. Liver structural alterations accompanying chronic toxicity in fishes: potential biomarkers of exposure. In: Biomarkers of environmental contamination. Mc Carthy, J. F. and Shugart, L. R. (eds.). Lewis Publishers, Boca Raton, pp 17-57.

HOPKINS, S. P., and NOTT, J. A., 1980. Studies on the digestive cycle of the shore crab *Carcinus maenas* (L.) with special reference to the B cells in the hepatopancreas. *J. Mar. Biol. Ass U.K.* 60 : 891-907.

HOSE, J. E., MARTIN G. G. and GERARD, A. S., 1990. A decapod classification scheme integrating morphology, cytochemistry and function. *Biol. Bull.* 178: 33-45.

INGELFINGER, F. J.,1971. Forward . In: Regeneration of liver and kidney. Butcher N. R. L. and Malt. R. A. (eds.) Little, Brown, Boston.

JOHANSSON, M. W., and SODERHALL, K., 1992. Cellular defense and cell adhesion in crustaceans. *Anim. Biol.* 1: 97-107.

JONES, D. A., BABBAGE, P. C. and KING P. E.,1969. Studies on digestion and the fine structure of digestive caeca in *Eurydice pulchra* (Crustacea: Isopoda).

Mar. Biol. 2: 311-320.

JONES, R. W. and HUFFMAN, M. N., 1957. Fish embryos as bioassay material in testing chemicals for effects on cell division and differentiation. *Trans. Amer. Micros. Assoc.* 76: 177-183.

KOHLER, A., 1990. Identification of contaminant induced cellular and subcellular lesions in the liver of flounder (*Platichthys flesus*) caught at differently polluted estuaries. *Aquat. Toxicol.* , 16: 271-274.

LELAND, H. V., 1983. Ultrastructural changes in the hepatocytes of juvenile rainbow trout and mature brown trout exposed to copper or zinc. *Environ. Toxicol. Chem.*, 2: 253.

LOIZZI, R. F., 1971. Interpretation of crayfish hepatopancreas function based on the structural analysis of epithelial cell lines and muscle network. *Z. Zellforsch. Mikrosk. Anat.* 113: 420-440

LOWE, D. M. and PIPE, R. K., 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cell: an in vitro study. *Aquat. Toxicol.* , 30: 357-365.

MANISSERI, M. K., 1993. Investigations on heavy metal stress and energetics in *Metapenaeus dobsoni* (Miers). Ph.D. Thesis, Cochin University of Science and Technology, Cochin, India

MANISSERI, M. K. and MENON, N. R., 1995. Copper induced damage to the hepatopancreas of the penaeid shrimp *Metapenaeus dobsoni* - an ultra structural study. *Dis. Aquat. Org.*, 22 : 51-57.

MARTIN, G. G. and GRAVES, B. L., 1985. Fine structure and classification of shrimp haemocytes. *J. Morphol.* 185: 339-348.

MARTIN, G. G. and HOSE, J. E., 1992. Vascular elements and blood (Haemolymph). In: Microscopic anatomy of invertebrates. Vol. 10: Decapod, Crustacea, pp. 117-146.

MARTIN, G. G., QUINTERO, M., QUIGLEY, M. and KHDSROVIAN H., 2000. Elimination of sequestered material from the gills of decapod crustaceans. *J. Crust. Biol.*, 20 (2): 209-217.

MIYAWAKI, M. and TANOUE S.,1962. Electron microscopy of the hepatopancreas in the crayfish, *Procambrus clarkii. Kumamoto J. Sci.*, (B) 6: 1-11.

MOORE, M. N.,1980. Cytochemical determination of cellular responses to environmental stressors in marine organisms. In : Biological effects of marine pollution and the problems of monitoring. Mc Intyre, A.D. and Pearce, J.B. (eds.) Rapp. P.V. Reun. Cons. Int. Explor. Mer., 179 : 7.

MOORE, M. N., 1982. Lysosomes and environmental stress. *Mar. Pollutn. Bull.* , 13:42.

MOORE, M. N., 1985. Cellular responses to pollutants. *Mar. Pollutn. Bull.* , 16 : 134.

MOORE, M. N., 1988. Cytochemical responses of the lysosomal system and NADPH - ferriprotein reductase in molluscan digestive gland cells to environmental exposure to xenobiotics. *Mar. Ecol. Prog. Ser.*, 46; 37-43.

MOORE, M. N. , PIPE, R. K. and FARRAR, S. V., 1982. Lysosomal and microsomal responses to environmental factors in *Littorina littorea* from Sullum Voe. *Mar. Pollutn. Bull.* , 13: 340.

MOORE, M. N., PIPE, R.K. , FARRAR, S. V. , THOMSON, S. and DONKIN, P., 1986. Lysosomal and microsomal responses in *Littorina littorea* : Further effects of environmental effects in the vicinity of the Sullom Voe oil terminal and the effects of experimental exposure to phenanthrene. In : Oceanic processes in marine pollution - Biological processes and waste in the ocean, Vol I. Capuzzo, J.M. & Kester, D. R. (eds.), pp 89-96. Melbourne, Florida.

Krieger Publishing.

MOORE, M. N.,1990. Lysosomal cytochemistry in marine environmental monitoring. *Histochem. J.*, 22 : 189-191.

NOTT, J. A. and MOORE, M. N.,1987. Effects of polycyclic aromatic hydrocarbons on molluscan lysosomes and endoplasmic reticulum. *Histochem. J.* 19: 357-368.

NOTT, J. A., MOORE, M. N., MAVIN, L. J. and RYAN, K. P.,1985. The fine structure of lysosomal membranes and endoplasmic reticulum in the digestive cells of *Mytilus edulis* exposed to anthracene and phenanthrene. *Mar. Environ. Res.* 17: 226-229.

NUWAYHID, M. A., DAVIES, P. S. and ELDER, 1980. Changes in he ultrastructure of the gill epithelium of *Patella vulgata* after exposure to North Sea crude oil and dispersants. *J. mar. biol. Ass. U. K.* Vol. 60, 439-448.

OMORI, S. A., MARTIN, G. G. and HOSE, J. E., 1989. Morphology of haemocytes lysis and clotting in the ridge back prawn *Sicyonia ingitis. Cell Tissue Res.* 255: 117-123.

PAPTHANASSIOU, E. and KING, P. E., 1984. Effects of starvation on the fine structure of the common prawn *Palaemon serratus* (Pennant). *Comp. Biochem. Physiol.* 7(A) 2: 243-249.

PIPE, R. K. and MOORE, M. N., 1985. Ultrastructural changes in the lysosmal vacuolar system in digestive cells of *Mytilus edulis* as a response to increased salinity. *Mar. Biol.* 87: 157-163.

PRASAD, M. S., 1991. SEM study on the effects of crude oil on the gills and air breathing organs of climbing perch, *Anabas tesudineus. Bull. Environ.*

Contam. Toxicol., 47 : 882-889.

REYNOLDS, E. S.,1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell. Biol.* 17: 19-58.

ROUBAL, W. T. and COLLIER,T. K.,1975. Spin- labeling techniques for studying mode of action of petroleum hydrocarbons on marine organisms. *Fish. Bull.* 73: 299-305.

ROUBAL, W. T.,1974. Spin labeling of living tissue- a method for investigating pollutant caused interaction. In: Pollution and physiology of marine organisms. F.J. Vernberg and W. B. Vernberg (eds.) pp: 367-380. Academic Press, New York.

SLATER, T. F.,Free radical mechanisms in tissue injury. Pion Ltd. London. 1976.

SMITH, V. J. and RATCLIFFE, N. A., 1980. Cellular defense reactions of the shore crab, *Carcinus maenas*; *in vivo* haemocytic and histopathological to injected bacteria. *J. Invertebr. Pathol.*, 35: 65-74.

STAINER, J. E., WODHOUSE, M. A. and GRIFFIN, R. L.,1968. The fine structure of the hepatopancreas of Carcinus *maenas* (L.) (Decapoda: Brachyura). *Crustaceana* 14: 56-66.

STORCH, V., JUARIO, J. V. and PASCAL, F., 1984. Early effects of nutritional stress on the liver of milk fish *Chanos chanos* and on the hepatopancreas of the tiger prawn *Peneaus monodon*. *Aquaculture*, 36: 229-2

THOMPSON, R. J., RATCLIFFE, N. A. and BAYNE, B. L.,1974. Effects of starvation on structure and function in the digestive gland of the mussel (*Mytilus edulis* L.) *J. mar. biol. Ass. U. K.* 54: 699-712.

TRUMP, B. F. and ARSTILA, A. V.,1975. Cell membranes and disease processes. In: Pathobiology of cell membranes Vol. I Trump, B. F. and Arstila, A. V. (eds.) Academic Press, New York.

VARANASI, U., 1989. (eds.) Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. pp. 328. CRC Press, Boca Raton, Florida.

VIARENGO, A., MOORE, M. N., MANCINELLI, G, MAZZUCOTELLI, A., PIPE, R. K. and FARRAR, S. V., 1987. Metallothioneins and lysosomes in metal toxicity and homeostasis in marine mussels: the effects of cadmium in the presence and absence of phenanthrene. *Mar. Biol.* 94: 251-257.

VICTOR, B., 1993. The responses of haemocytes and gill tissues to sublethal cadmium chloride poisoning in the crab *Paratelphusa hydrodromous* (Herbst). *Arch. Environ. Contam. Toxicol.*, 24: 432-439.

VOGT, G., 1987. Monitoring of environmental pollutants such as pesticides in prawn aquaculture by histological diagnosis. *Aquaculture*, 67 (1-2): 157-164.

VOGT, G. and QUINTINO, E. T., 1994. Accumulation and excretion of metal granules in the prawn, Penaeus monodon, exposed to waterborne copper, lead, iron and calcium. *Aquat. Toxicol.*, 28: 223-241.

WATSON, W. L., 1968. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem Cytol.* 4: 475-478.

WINSTON, G. W. and DIGUILIO, R. T., 1991. Pro oxidant and anti oxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* , 19: 137 - 161.

Chapter 5:

ENVIRONMENTAL LOAD OF PETROLEUM HYDROCARBONS IN THE COCHIN BACKWATERS

5. 1 INTRODUCTION

Continuous monitoring of ecosystems for the presence and degree of contamination of pollutants allows the formulation of projects and policies to safeguard resources and amenities within them. An understanding of the dynamics of petroleum hydrocarbon (PHC) metabolism in the environment is fundamental to the assessment of its effects on the biota. Monitoring the total concentrations, routes of influx and efflux and spatial and chronological variations in PHC quantities provide baseline information for assessing and predicting the health of the ecosystem. Evaluation of the presence of PHCs in the natural habitat of a species is also necessary for correlating laboratory findings with possible effects under natural conditions. A monitoring study was conducted for a short period of time over one set of postmonsoon, winter and premonsoon months to determine the PHC load and distribution within the habitat of *M. dobsoni*, the Cochin backwaters.

5. 2 REVIEW OF LITERATURE

Much attention has been focussed on the nature and fate of PHC pollution in the marine environment since the advent of pollution research. Clark and Brown (1977) reviewed the work of various researchers contributing to the understanding of inputs, transport mechanisms and levels of hydrocarbons in the marine environment and provided an exhaustive review of research done until the late seventies in the fields of physical and chemical properties of petroleum and petroleum byproducts, methods of analysis and the presence of hydrocarbons in animals.

The amount of oil reaching the marine environment through transportation activities has shown a decrease over the past decade as compared to the 1970s and '80s. A study conducted by the U. S. National Academy of Science showed that oil input to the tune of 1.47 metric tonnes per annum arising from transportation activities had reduced to 0.57 metric tonnes by 1989 (IMO, 1990). Though attracting the most media attention, the contribution of episodic oil spills to the total marine contamination by oil is far less as compared to that arising from shipping, urban run-offs, industrial discharges and offshore production (GESAMP, 1993). The seas along unpopulated regions where there are no major shipping channels are remain relatively uncontaminated with low levels of PHCs, most of which is of biological origin (Pendoley, 1992; Cripps, 1992). This is in direct contrast with densely populated shores with industrial development and sea transport routes (Law, 1981; Zhijie & Zhang, 1988; Balci, 1993; Al-Saad & Al-Timari, 1993).

The Arabian Gulf is arguably the most contaminated region in the world in term of oil pollution with its massive and regular oil production operations and has been subjected to large-scale sabotage of the same during the Gulf War in 1991 (Gundalch *et. al.*, 1993). Emara (1990) recorded an average of 30,000 tonnes of oil per annum entering the western Gulf coastal waters. Another estimate (Heinrichson, 1990) gave the figure of 5 million tonnes of PHCs entering the Arabian Gulf waters per annum. However the total PHC concentrations values for the water column was 4.4-63 μg/l, which is comparable with figures worldwide. During the Gulf war in 1991 an estimated

6-8 million barrels of oil were released into the Northern Arabian Gulf from damaged oil fields in Kuwait contaminating large areas along the coasts and offshore. Pre-war offshore sediments showed concentrations ranging from 0.1-1.5 μg/g (Fowler, 1988) and was termed as very low in the sediments off Kuwait, Bahrain and Oman, chiefly arising from biological sources and land runoffs (Mille *et al.*, 1992). After the war, 14.0-56.2 μg/g TPHC were recorded from the surface sediments of the Arabian Gulf (Abdali *et. al.*, 1993; Al-Lihaibi and Al-Omran, 1995). In 1997 PHC concentration in sediments reported were 5.4-92.0 μg/g PHC concentration from sediment with an average of 32.6 μg/g (Al-Lihaibi and Ghazi, 1997). The effect of the Gulf war spills were however not felt in the northern Arabian Sea along the Indian coast, probably owing to the prevalent physical oceanographic conditions within the Gulf favouring the containment of spilled oil (Sen Gupta *et. al.*, 1993).

Along the Indian coasts, values of PHC concentrations reported are 22.8 - 42.8 μg/l in Goan coastal waters (Fondekar *et.al.*, 1980), 3.9-18.1 μg/l for Okha - Ratnagiri (Kadam & Bhangale, 1993), 2.9-93.5 μg/l along the Bombay coast (Ingole *et al.*, 1995), 1.6 - 11.1 μg/l for the Northern Arabian Sea (Sen Gupta *et al*, 1990) and 15.9 - 108 μg/l in the harbour waters of Vishakapatnam (Mohan and Prakash, 1998). Heinrichson (1990) reported 40,000 tonnes of oil entering the Bay of Bengal per annum with several harbours showing above 100 μg/l concentrations of TPHC in surface waters. Kadam (1987) reported 6.5 - 23.3 μg/g PHCs in the surface sediments of Kandla creek, Gujarat. A study conducted by Nair *et. al.* (1995) cites a relatively high value for sediments of Vembanad estuary (249-570 μg/g), the

method of analysis adopted being gravimetric. Most other studies employ spectrophotometric methods and specifically measure petrogenic hydrocarbon content. Values from 2.9-10.3 μg/g wet weight were reported from sediments along Bombay coast (Ingole *et al.*, 1995). An account of the major oil pollution incidents in the Indian Ocean has been compiled by Ramamurthy (1991).

Various methods for the collection, sampling, preservation, extraction, separation of, and identification and quantification of components of hydrocarbons have been evolved over the years. Methods employed for all of these are defined by the objective of the investigation and the nature of the sample (Clark & Brown, 1977). Methods for estimation of hydrocarbons in seawater and sediment include organic solvent extraction, alkali digestion combined with solvent extraction (Warner, 1975; Farrington & Tripp, 1975), head space gas stripping, direct heating of sample into the inlet system of a mass spectrophotometer or gas equilibration techniques. Two methods are commonly used for extraction of hydrocarbons from biological material namely, extraction with organic solvents in a refluxing apparatus and alkali digestion combined with organic solvent extraction (Donkin & Evans, 1984). Steam distillation has also been used (Ackman & Noble, 1973). Component hydrocarbons within an extract are separated using column chromatography with silica gel, alumina or both, high pressure liquid chromatography, gel permeation, molecular sieve method charge transfer complexion with nitro-compounds or a combination of these methods (Farrington *et. al.*, 1976). A range of techniques and instruments are employed for analysing the

components of hydrocarbon mixtures. The oldest among these are the gravimetric method and thin later chromatography (Brown *et. al.*, 1978). Earlier workers also relied on absorption spectrophotometry for estimation of PHC concentration (Levy, 1971). Gas chromatography is a popular method employed for source identification of the PHC contaminants (Ehrhardt & Blumer, 1972; Wang & Fingas, 1997). Infrared spectrophotometry (Jeltes & den Tonkelsar, 1972) and mass spectrometry are also employed especially for "fingerprinting" individual hydrocarbons (Giger & Blumer, 1974, Hansen, *et al.*, 2000). Fluorescent spectrophotometry is commonly employed as a rapid screening technique for detecting petroleum hydrocarbons (Wakeham, 1977; Law, 1981). Greater sensitivity and selectivity in measurements is achieved by an integration of methods (Readman, 1986), for example, a combination of HPLC with fluorescence detection and capillary gas chromatography with mass spectrophotometry (GCMS) (Mason, 1987; Law and Biscaya 1994), which give relatively good interlaboratory comparison results. It was found that discrepancies are mainly due to differences in calibration procedures and errors made during calculations (Law *et al.*, 1997). Some of the latest techniques employed in petroleum characterisation and identification involve hyphenated analytical and chemometric methods using the GCMS (Christensen *et al.*, 2000) and the carbon isotopic sampling method using gas chromatography- isotope ratio mass spectrophotometry (Philip *et. al.*, 2000)

The Vembanad estuary is the largest among backwater lakes of Kerala (235 sq.km) and runs parallel to the coast from Munambam in the north to Alapuzha in the south, lying within the co-ordinates latitude 9 ° 28′ and 10°

10′ N, longitude 76° 13′ to 76 ° 31′ E. The Cochin backwater is the northward extension of the Vembanad Lake. The lake is a typical tropical positive estuary (Ramamritham and Jayaraman, 1963) which has been explained using synoptically synergic modulation processes by Balchand *et. al.* (1991). Two major rivers, the Periyar and the Muvattupuzha, discharge into it. Four more rivers discharge into the estuary on the opening of the Thaneermookam bund in the monsoons- the Achankovil, the Meenachil, the Manimala and the Pamba (Nair *et. al.,* 1995). It is connected to the Arabian Sea through a permanent "gut =tidal inlet" at the Cochin Harbour entrance (9° 58′ N and 76° 15′ E). Two other inlets are present, one in the north at Azhikode and another seasonal one in the south at Thurava. Of its three dredged channels, one is around 10 km long, with 500 m width and an east- west orientation. The two inner channels, the 5 km long and 250-500 m wide Ernakulam channel and 3 km long 170-250 m wide Mattancherry channel run on either sides of the Wellingdon island, which is in itself is formed from the dredged material over the century. The channels are constantly dredged to maintain a depth of 10-13 m to facilitate the entry of vessels. Investigations on the freshwater and sweater mixing and stratification in the estuary have revealed the existence of a salt wedge from August to October. A partially mixed condition is prevalent from November to January whereas in June a partially mixed condition is seen. The geographical features and anthropogenic infrastructure have significant influence on the circulation within the estuary (Joseph & Kurup, 1987, 1989; Ajith, 1996; Menon *et. al.*, 2000). The Kochi

harbour is situated close to the barmouth and offers berthing facilities to cargo containers, oil containers, passenger liners, naval warships, mechanized trawlers, research vessels and small fishing crafts. An oil terminal for transportation of crude and refined products and with pipelines running to Kochi Refineries Limited, exists close to the barmouth. More than seventeen major industrial units and two shipyards release effluents into the estuary with the industrial area lying mostly in the barmouth area and along the northern stretches of the estuary. (Unnithan *et al.,* 1977, Sarala Devi *et al.,* 1989). The backwaters face serious threats from intertidal land reclamation, effluent discharge and runoffs, harbour maintenance and expansion activities and urbanization (Gopalan, *et al.*, 1983). The barmouth and numerous smaller inlets along the coast serve as points of entry and exit for the larval and juvenile stages of penaeid shrimps that arrive at the estuary for feeding and metamorphosing into subadults. The Vypeen-Cherai shrimp fields are amongst the most productive shrimp fields in the world. An active fishery for *Metapenaeus dobsoni* exists through the entire estuary and the barmouth region (Menon & Menon, 2000).

5. 3 MATERIALS AND METHODS

The PHC content of the surface water, subsurface water and surface layer of sediment was monitored for a total of 12 stations for a period of 9 months from September 1995 to May 1996.

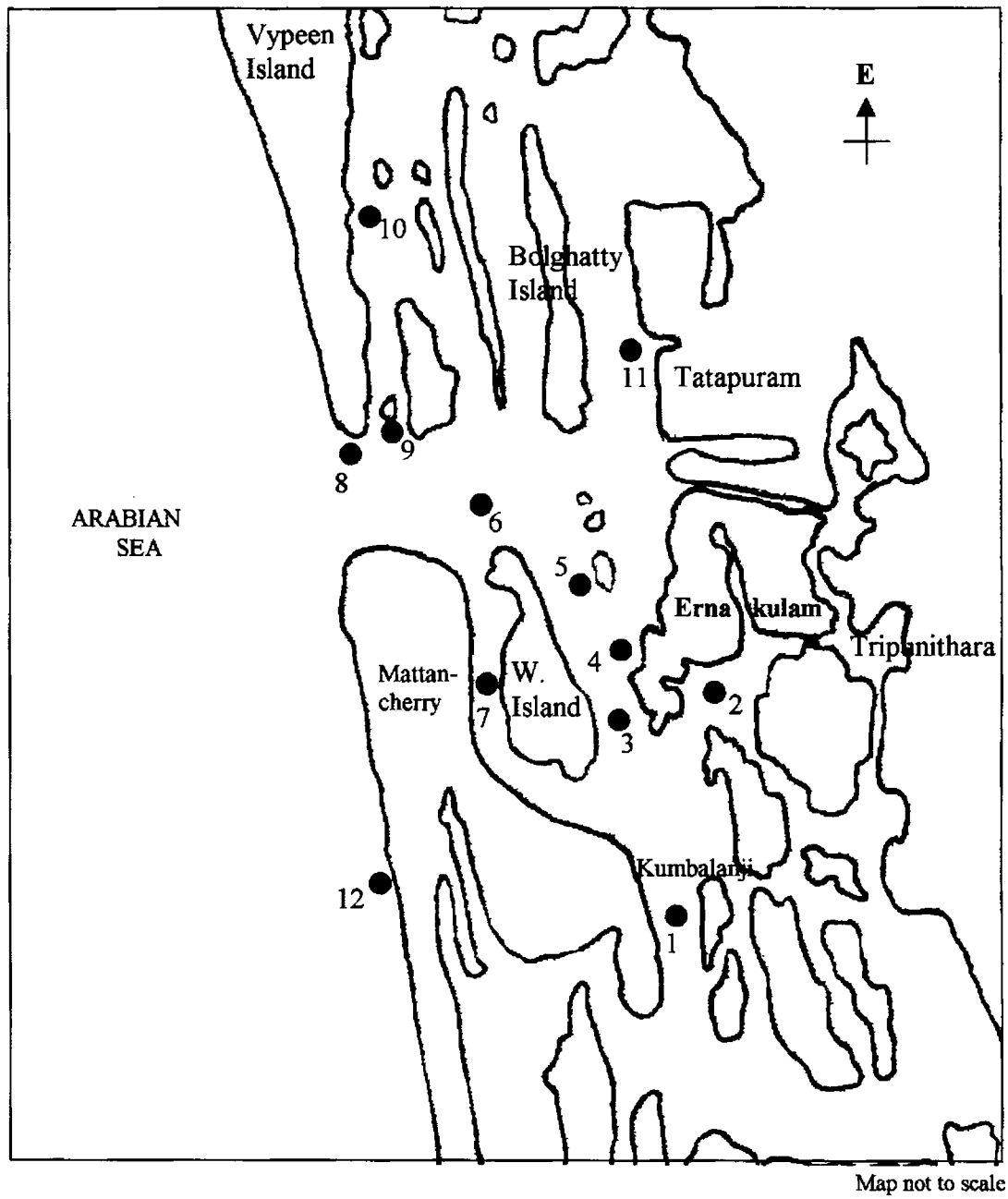


Fig. 1: Location of stations within the Cochin backwaters

5.3.1. Selection of stations:

Two stations were monitored during the first month. Subsequently, 11 stations were chosen within the estuary in a manner so as to cover representative localities like areas receiving discharges from the northern and southern regions, maritime installations situated within the estuary, etc. Station 1 (Kumbalanji, depth 5 m) and Station 2 (Chambakkara channel, depth 2.5 m) were located in the Muvattupuzha river. Station 3 (Vendurthy bridge, depth 8 m), Station 4 (Marine Science Jetty, depth 2 m) and Station 5 (Oil terminal, depth 5 m), were situated in the vicinity of oil transportation and shipping infrastructure. Station 6 (Backwater Buoy No.4, depth 3 m) was close to the bifurcation of the Ernakulam and Mattancherry channels and Station 7 (Mattancherry channel, depth 5 m) was located downstream the main fishing harbour in the Mattancherry channel. Station 8 (Barmouth) was located off the mudflats at the barmouth (depth 4 m), Station 9 (Gundu island, depth 2 m) was located in the easternmost distributary of the Periyar in the vicinity of the Vypeen fishing harbour, Station 10 (Mulavukadu, depth 3 m) further upstream towards the prawn fields region and Station 11 (Tatapuram, depth 2 m) between the Bolghatty island and the Tomco plant in the westernmost distributary of the Periyar (depth 2 m). The Reference Station was located in the nearshore region off Kanamally, south of Fort Kochi, at 1.5 m depth. Locations of the various stations are given in Fig.1.

5.3.2. Collection of water and sediment samples:

Fortnightly samples of surface water, sub surface water and sediments were collected. Sampling of surface water was done by allowing the surface layer of water to drain directly into a sampling bottle cleaned with n-hexane. Subsurface samples were collected from a depth of about 1 m depth above the bottom using a subsurface sampler. A van Veen grab was used to collect sediment samples. Macrobenthos was removed from the grab sample. Subsamples of approximately 100 g in triplicate were made from the surface layer, wrapped in aluminum foil and stored at –20°C in a deep freezer.

The water samples were analysed within 4 hours of sampling and the sediment the within 24 hours.

5.3.3. Analysis of samples:

5.3.3.1. Preparation of Chrysene standard

A stock solution of chrysene was prepared by dissolving 1.0 mg chrysene in 100 ml n-hexane (HPCL grade) in a 100 ml standard flask. This was allowed to stand overnight before dilution to ensure complete dissolution (IOC, 1984). A blank solution of pure hexane and a range of standard dilutions of 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0, 2.0 and 3 μg/l from the stock solution were prepared and their fluorescence determined spectrophotometrically. A standard graph was drawn by plotting the concentration of chrysene (μg/l) with the x-axis against fluorescence on the y-axis. This graph was used for computing the PHC concentration of samples analysed with the values being expressed in μg/l or μg/g in chrysene units.

5.3.3.2. Analysis of seawater

The procedure recommended by IOC (1984) was used for extraction of PHCs from water samples. 500 ml of the sample was taken in a 1 l separatory funnel and the PHC content extracted with 25 ml. Of the organic solvent n-hexane (HPCL grade) by shaking vigorously for 10 minutes. The procedure was repeated to effect maximum extraction. The pooled concentrate was chemically dried using anhydrous sodium sulphate and made upto 50 ml. by addition of n-hexane. The concentration of PHC was determined against a standard of chrysene, using a fluorescent spectrophotometer (Hitachi model F-3010) at wave lengths 310 nm (Ex) and 360 nm (Em).

5.3.3.3 Analysis of sediment:

50 g of wet sediment sample were weighed out and digested for 2 hours with 50 ml of 0.5N methanolic KOH (HPCL grade). The resultant organic phase was extracted twice with 25 ml n-hexane (HPCL grade). The combined extracts were dehydrated using anhydrous sodium sulphate and subjected to a clean up procedure using activated alumina columns (2g, 10 cm). The PHC extract was made up to a final volume of 25 ml each with n-hexane and the fluorescence measured as mentioned above. The concentration of PHC is expressed in μg/g chrysene equivalents.

An oil spill occurred in the Cochin harbour on 15.03.1996 at the North tanker Jetty (near Stations 4, 5 and 6) due to the accidental bursting of a supply hose during bunkering operations of an oil tanker, spilling an unestimated quantity of Furnace Fuel oil into the harbour (Anon, 1996; Paul *et. al.*, 1998).

5.3.4 Statistical analysis of results:

The values obtained for PHCs in the samples were subjected to statistical analysis by performing analysis of variance- ANOVA (Two factor ANOVA without replication) test. ANOVA tests were also performed after filtering the values that were perceived to be caused by the oil spill. Values for September and October were not included for computing the F and P-values as all stations were not monitored during this month. Correlation was also computed for obtaining the effect of rainfall on the PHCs values in various stations and depths. All calculations were performed using an MS Excel, Microsoft Windows (95) software package.

5. 4 RESULTS

The variance between stations and variation of PHC concentration within stations over time were found to be significant when tested at 1% level. Analysis of the values obtained for water and sediment from the various stations show an extraordinary increase during the second fortnight of March 1996, which is clearly the effect of the oil spill mentioned. Intrastation variance is also high in a majority of the sampled areas excepting Stations 1, 2, 10, 11 and the Reference Station during the second half of March and in the first fortnight of April. The effect of the spill was detectable in surface waters only immediately after the spill in stations with higher depths and was not discernable in any of the stations in April. In subsurface waters higher than normal values were seen in Stations 3, 4, 5, 6, 7, 8, and 9 upto April with the

Table 1: Concentration of dissolved and dispersed petroleum hydrocarbons in the surface water at various stations in the Cochin backwaters (in ug/l chrysene units)

Month	PHC concentration (ug/l)											
	Stn 1	Stn 2	Stn 3	Stn 4	Stn 5	Stn 6	Stn 7	Stn 8	Stn 9	Stn 10	Stn 11	Stn 12
Sep	*N.A	N.A	N.A	20.16	N.A	N.A	N.A	N.A	N.A	N.A	N.A	2.8
Oct	N.A	N.A	N.A	35.84	N.A	N.A	N.A	24.08	N.A	N.A	N.A	3.36
Nov	N.A	N.A	20.72	43.68	N.A	N.A	N.A	19.88	N.A	N.A	N.A	2.8
Dec	12.32	10.64	16.24	54.6	14	29.96	62.44	26.88	14.84	15.4	14.56	3.36
Jan	14.56	11.48	27.44	61.88	17.36	29.4	63.28	34.16	20.72	15.12	16.8	4.48
Feb	12.32	11.2	26.32	35.28	19.32	28.28	44.24	36.68	21.28	16.52	10.64	5.04
Mar I	12.3	11.34	28.42	52.34	22.34	34.32	57.57	43.18	22.63	17.12	12.56	4.75
Mar II	12.42	11.55	42.37	298.46	98.28	140.56	87.92	287.28	197.96	17.36	12.88	4.48
Apr I	11.67	11.54	37.89	60.34	28.53	37.89	59.55	44.34	36.82	17.02	11.72	3.42
Apr II	9.8	9.24	30.8	45.64	18.76	32.48	61.04	45.92	39.2	17.36	10.64	3.36
May	11.76	12.04	24.36	36.96	14.84	27.44	76.44	40.88	22.4	19.88	14	3.36

*Not available

ANOVA

Source of Vari	SS	df	MS	F	P-value	F crit
Rows	498.11	6	83.018	2.693	0.0212	2.2395
Columns	22057	11	2005.1	65.044	5E-31	1.937
Error	2034.6	66	30.827			
Total	24589	83				

Table 2: Concentration of dissolved and dispersed petroleum hydrocarbons in the subsurface waters at various stations in Cochin backwaters (in ug/l chrysene units)

Month	PHC concentration (ug/l)											
	Stn 1	Stn 2	Stn 3	Stn 4	Stn 5	Stn 6	Stn 7	Stn 8	Stn 9	Stn 10	Stn 11	Stn 12
Sep	*N.A	N.A	N.A	21.28	N.A	N.A	N.A	N.A	N.A	N.A	N.A	2.24
Oct	N.A	N.A	N.A	49.28	N.A	N.A	N.A	24.36	N.A	N.A	N.A	1.4
Nov	N.A	N.A	24.64	33.88	N.A	N.A	N.A	16.8	N.A	N.A	N.A	1.4
Dec	11.76	11.48	23.24	46.76	13.16	28.84	39.2	21.56	14.84	14.84	6.72	1.4
Jan	13.44	13.44	29.68	38.64	14	22.4	42.28	21.56	20.44	14.28	6.44	1.68
Feb	14	13.16	30.52	31.36	14.84	28.84	34.44	31.92	20.72	15.96	6.72	1.4
Mar I	14.73	15.37	34.28	39.27	16.22	30.73	35.74	36.23	21.36	16.23	5.95	1.4
Mar II	12.15	9.27	73.8	573.93	93.54	141.19	79.62	467.77	305.12	15.69	5.81	1.4
Apr I	14.83	13.26	52.67	142.83	53.12	47.83	58.22	102.84	82.28	16.12	4.92	1.36
Apr II	14	12.32	39.2	72.464	18.76	32.76	44.52	50.68	40.6	16.24	5.6	1.12
May	14	12.88	31.36	52.92	16.52	27.44	40.04	29.4	21.84	17.64	11.76	1.4

*Not available

ANOVA

Source of Vari	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	121.74	4	30.436	2.4342	0.0613	2.5837
Columns	8246.8	11	749.71	59.959	9E-23	2.014
Error	550.16	44	12.504			
Total	8918.7	59				

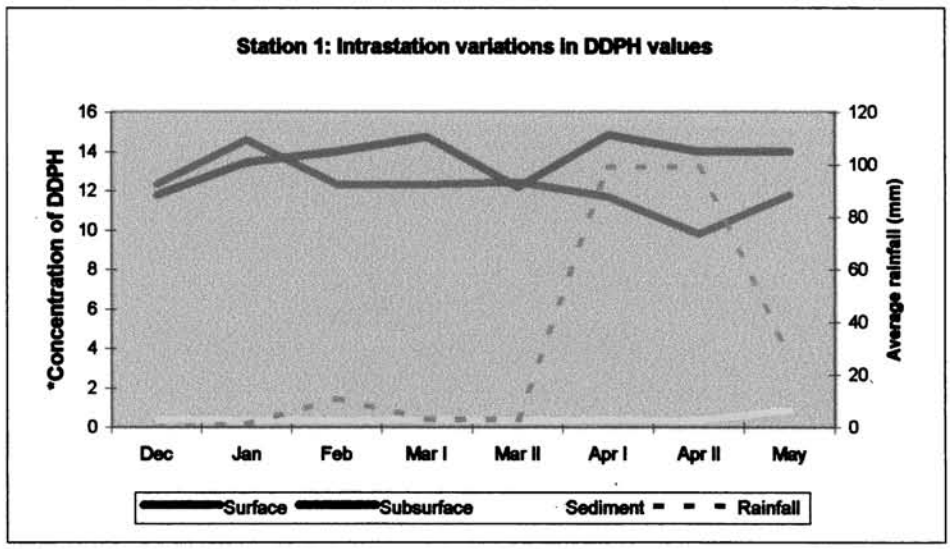
Table 3: Concentration of dissolved and dispersed petroleum hydrocarbons in the sediment at various stations in the Cochin backwaters (in ug/g chrysene units)

Month	PHC concentration (ug/g)											
	Stn 1	Stn 2	Stn 3	Stn 4	Stn 5	Stn 6	Stn 7	Stn 8	Stn 9	Stn 10	Stn 11	Stn 12
Sept	N.D	N.D	N.D	19.6	N.D	N.D	N.D	N.D	N.D	N.D	N.D	0.672
Oct	N.D	N.D	N.D	18.7	N.D	N.D	N.D	15.792	N.D	N.D	N.D	0.672
Nov	N.D	N.D	N.D	24.08	N.D	N.D	N.D	13.104	N.D	N.D	N.D	0.672
Dec	3.25	8.624	18.592	23.3	11.984	19.936	21.84	16.912	13.328	13.216	7.95	0.672
Jan	2.58	7.504	24.64	19.152	12.544	17.584	21.28	20.384	16.016	14.224	5.74	0.672
Feb	2.58	6.16	20.72	23.3	14.112	18.704	22.96	21.168	14	14.672	8.96	0.672
Mar	2.58	7.616	24.64	24	15.12	19.824	21.168	28.112	14.672	15.008	9.29	0.672
Mar II	2.8	6.384	48.272	652.96	73.136	93.968	67.424	237.44	90.16	15.232	8.74	0.672
Apr	2.8	7.168	20.832	285.82	25.76	25.2	29.344	43.12	40.768	18.48	9.07	0.672
Apr II	2.8	8.288	20.048	88.2	10.64	20.496	29.232	20.272	12.992	13.328	9.52	0.896
May	6.61	8.6688	18.144	57.34	9.52	17.248	28.896	20.272	11.76	13.664	9.86	0.784

*Not available

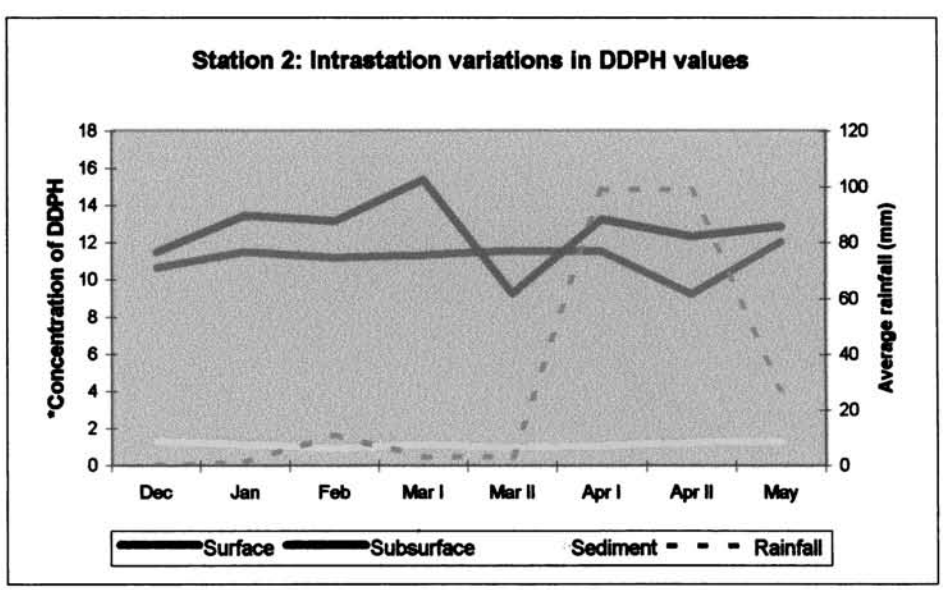
ANOVA

Source of Vari	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	24.153	3	8.0512	2.5155	0.0753	2.8916
Columns	2628.7	11	238.98	74.665	5E-20	2.0933
Error	105.62	33	3.2006			
Total	2758.5	47				



Correlation of values:

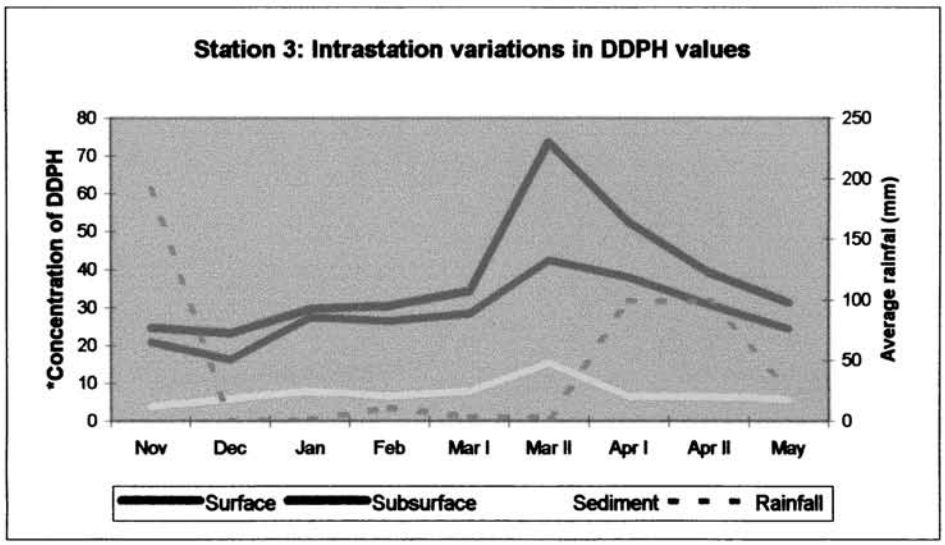
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	-0.24864	1		
Sediment	-0.15937	0.031776	1	
Rainfall	-0.7197	0.50762	-0.02259	1



Correlation of values:

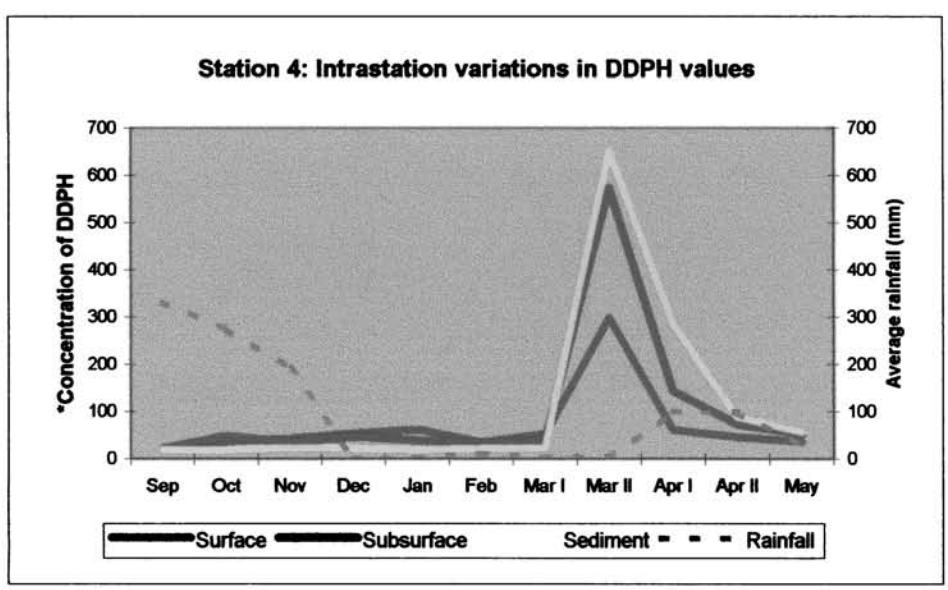
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.106119	1		
Sediment	-0.28716	0.161603	1	
Rainfall	-0.45665	0.073248	0.160479	1

*Units in ug/g for sediment and ug/l for surface and subsurface water



Correlation of values:

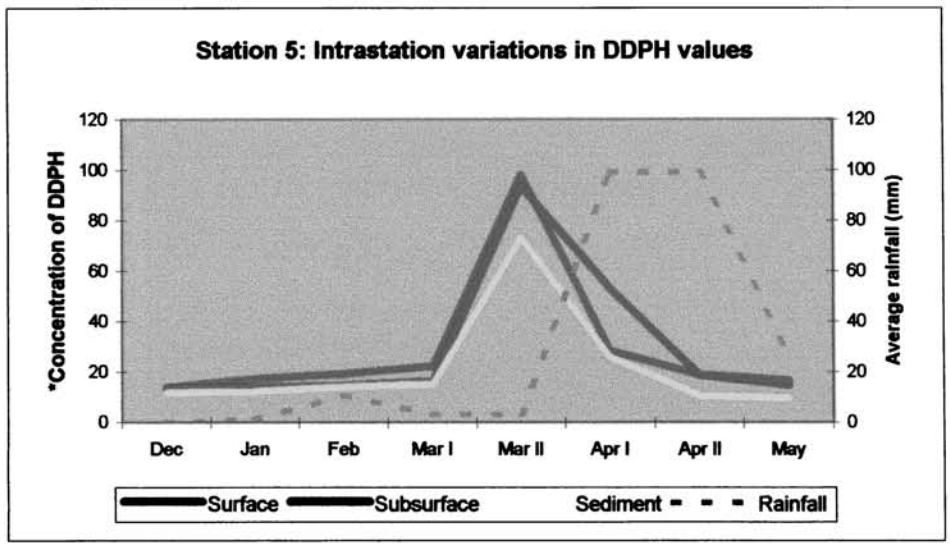
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.938164	1		
Sediment	0.741867	0.848501	1	
Rainfall	-0.07643	-0.11889	-0.49301	1



Correlation of values:

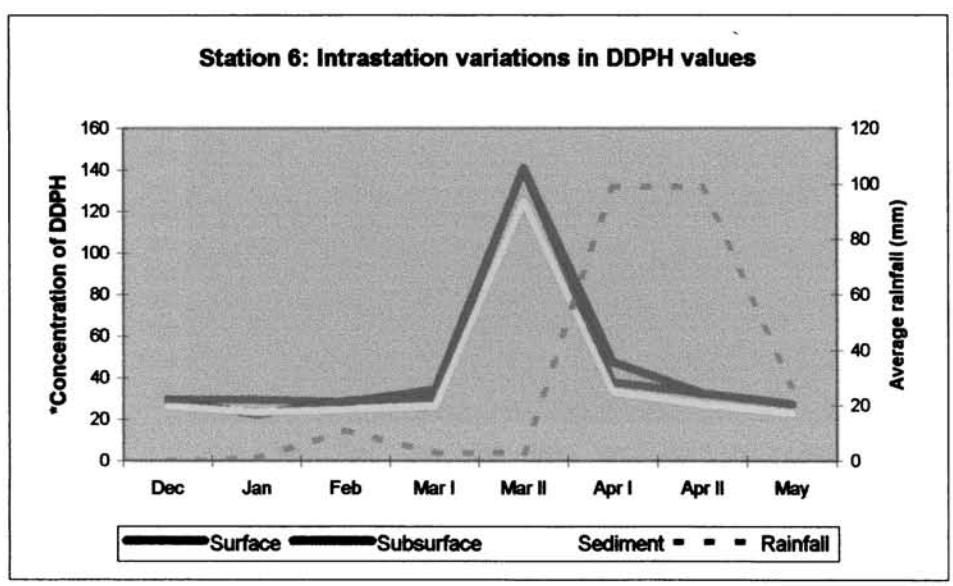
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.982854	1		
Sediment	0.930008	0.975967	1	
Rainfall	-0.3506	-0.2713	-0.25458	1

*Units in ug/g for sediment and ug/l for surface and subsurface water



Correlation of values:

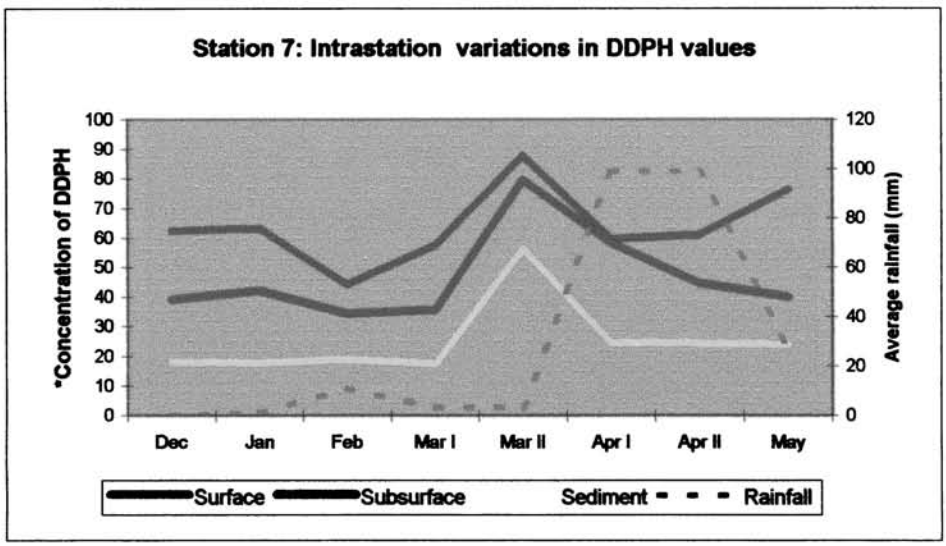
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.106119	1		
Sediment	-0.28716	0.161603	1	
Rainfall	-0.45665	0.073248	0.160479	1



Correlation of values:

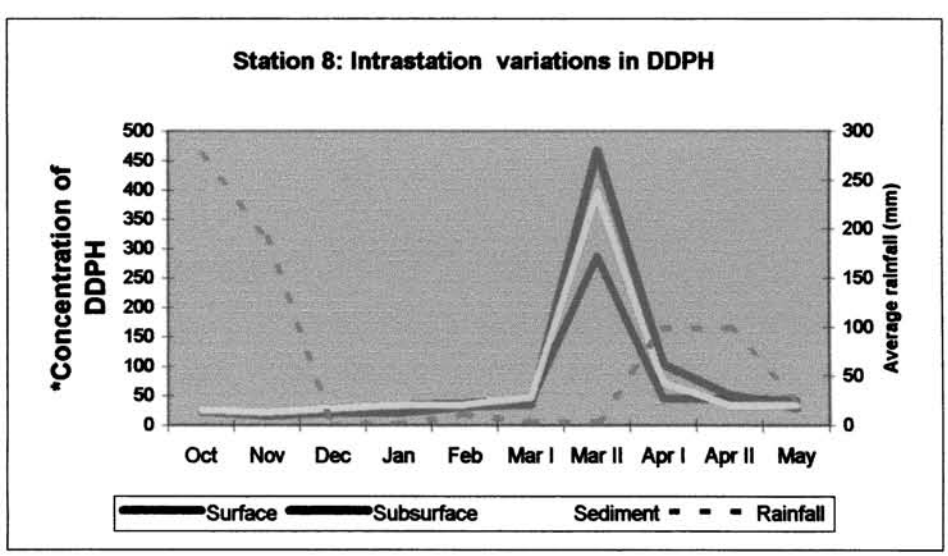
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.992633	1		
Sediment	0.999191	0.994893	1	
Rainfall	-0.20226	-0.11367	-0.19103	1

*Units in ug/g for sediment and ug/l for surface and subsurface water



Correlation of values:

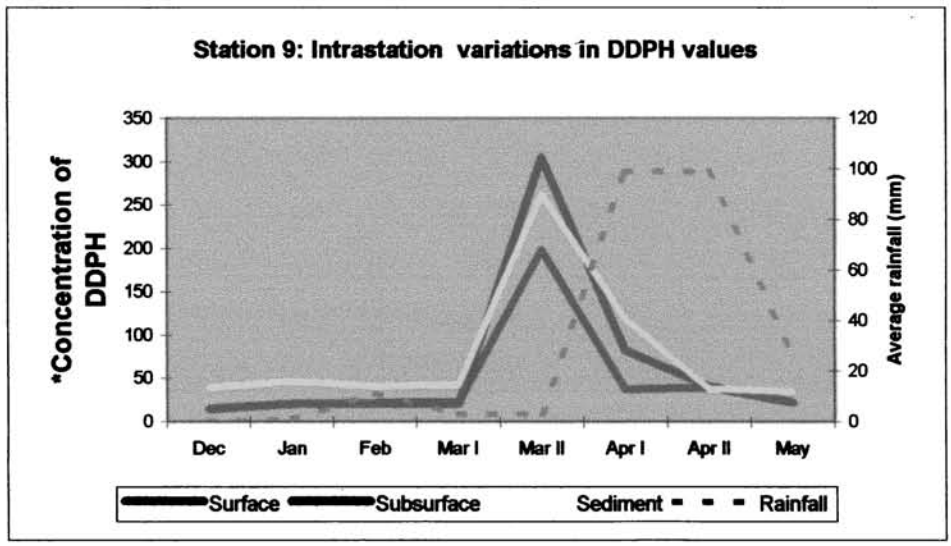
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.712888	1		
Sediment	0.780674	0.923501	1	
Rainfall	-0.14641	0.14314	-0.0505	1



Correlation of values:

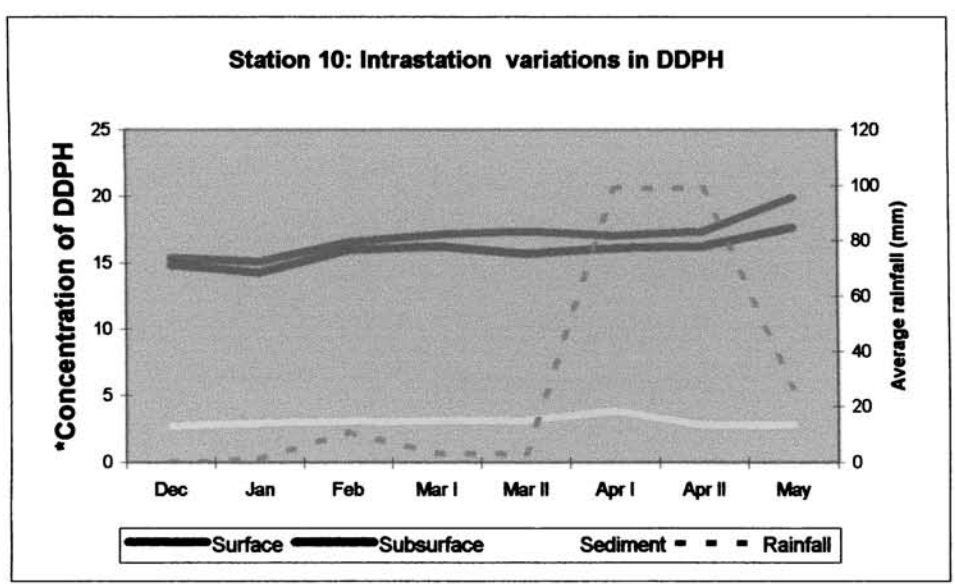
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.990176	1		
Sediment	0.995552	0.996439	1	
Rainfall	-0.30239	-0.24472	-0.27494	1

*Units in ug/g for sediment and ug/l for surface and subsurface water



Correlation of values:

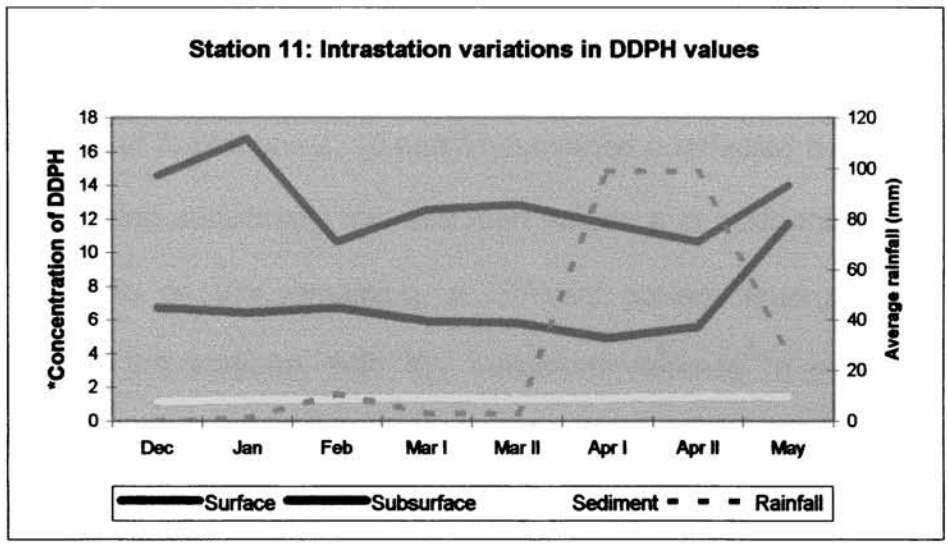
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.990379	1		
Sediment	0.953739	0.985222	1	
Rainfall	-0.12711	-0.06769	-0.0443	1



Correlation of values:

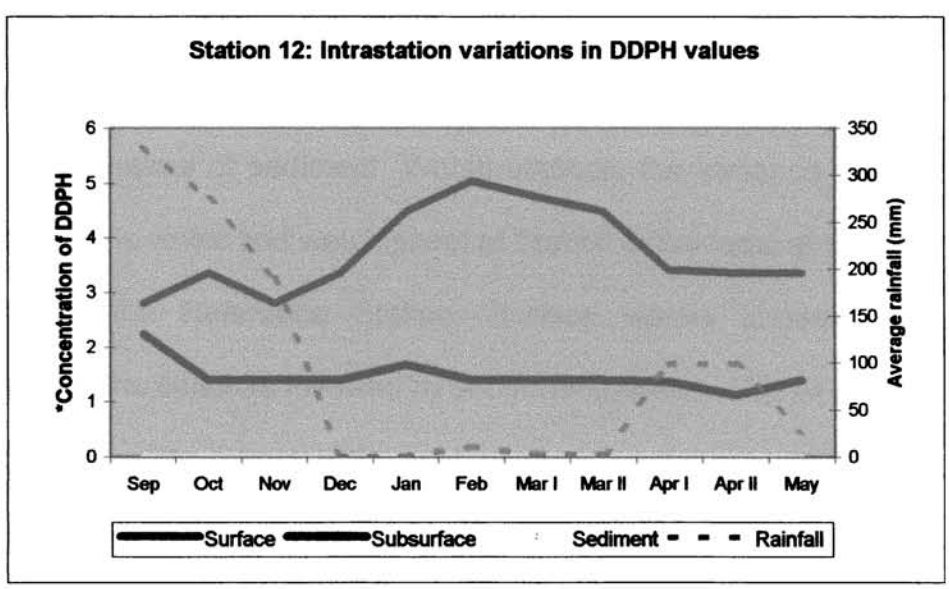
	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.954712	1		
Sediment	0.006678	0.075528	1	
Rainfall	0.263551	0.354139	0.40089	1

*Units in ug/g for sediment and ug/l for surface and subsurface water



Correlation of values:

	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	0.30124	1		
Sediment	-0.36565	0.430686	1	
Rainfall	-0.55251	-0.25456	0.441886	1



Correlation of values:

	Surface	Subsurface	Sediment	Rainfall
Surface	1			
Subsurface	-0.20863	1		
Sediment	-0.22669	-0.42392	1	
Rainfall	-0.69717	0.502332	-0.07561	1

*Units in ug/g for sediment and ug/l for surface and subsurface water

effects being negligible in May. In the case of sediment high values were recorded from the time of the spill upto May, though decreasing substantially, in Stations 4 and 7. Stations 2, 10 and 11 remained unaffected by the spill.

On filtering abnormally low and high values and performing a second set of ANOVAs on the remaining, a different pattern emerged for PHC distribution in the stations, with the maximum variance in surface water concentrations of PHCs between stations occurring in May and the minimum in September. In surface waters, the highest fluctuations were seen at Station 7 and the least at Station 2 after the Reference station. In the case of subsurface waters the maximum and minimum variance were seen again in May and March respectively, with Station 4 showing the maximum variance and Station 2 the minimum. As the sediment values did not return to normal in the month of May, it was not taken into consideration. Among the values compared, maximum variance was seen in March and the minimum in October in the case of sediment. Within stations, the variance was far less than that seen in water and was highest at Station 8 and least at Station 11, in exclusion of the Reference Station. Surface waters showed maximum variance over the seasons followed by subsurface waters. Within stations also the pattern was the same with least variance noticed in PHC values in sediments. The results of the monitoring of PHCs in the surface waters, subsurface waters and sediment of Cochin estuary from the postmonsoon season of September 1995 to the premonsoon season up to May 1996 are presented in Fig. 2 - 4 respectively, along with the corresponding results of

the ANOVA performed after filtering abnormally low and high values are given below each table. The values highlighted in red are those ascribed to the effects of the oil spill.

Stations 4, 7 and 8 appear to be the most polluted showing consistently higher values for surface, subsurface and sediment PHC concentration. In contrast the Stations 1, 2 and 11, besides the reference station, appear reasonably unpolluted.

A strong negative correlation was seen between rainfall and the PHC concentrations in a majority of the stations, indicating diluting effects by precipitation. No correlation is seen between subsurface waters of the Reference station and Station 11 with rainfall. Negative correlation of sediment values with rainfall exists though to a much lesser magnitude than in surface waters. Correlation between rainfall received and the concentration of PHCs in the surface water, subsurface water and sediment layers for each station is depicted graphically. Correlation was seen between surface water, subsurface water and sediment concentrations of PHCs in all stations except in Stations 1 and 2 where no correlation was seen between the PHC concentration in the water and sediment in Station 1 and 2. No correlation was also seen between values for PHCs in the subsurface layer and sediment in the Reference Station.

5. 5 DISCUSSION

The introduction of hydrocarbons into the marine environment takes place through natural and anthropogenic processes, the chief sources being

petrogenic, pyrogenic and biogenic. The anthropogenic sources of environmental contamination by aromatics stem from the isolation, processing, combustion and disposal of fossil fuels. In addition, the production of value added petroleum products such as polymers, plastics, pesticides, solvents, explosives and even pharmaceuticals can lead to the release of hydrocarbons in the environment (NAS, 1975; Johnston, 1984; Zylstra,1994). Two broad categories of oil pollution can be outlined namely, (1) episodic /accidental incidents involving acute short term and subsequently long-term sub lethal toxicity in the affected environment. and (2) chronic low level sub lethal toxicity producing long term effects in the affected ecosystem which may not be as easily discerned as in the first case.

An oil slick is formed during the initial phase following accidental oil spills. Complex and interrelated physical, chemical and biological processes determine the fate of the slick. The degradation routes and rates of these are influenced largely by the ambient abiotic environment and to a smaller extent the biotic environment and by the properties of the oil. Physical and chemical changes occur in the spilt oil as a result of interactions with environmental parameters and system fluxes (Fay, 1971).

The major processes involved in the degradation of spilt oil are surface drifting and spreading by winds and currents, evaporation, dissolution, dispersion, dilution, emulsification, photochemical oxidation, auto oxidation, biodegradation, adsorption in particles, ingestion by zooplankton and higher forms (crustaceans, fishes etc.), sinking, initial sedimentation, subsequent resuspension of sediments into water- sediment interface and interstitial voids

within sediments (Geyer, 1980). Spatial differences exist in the degradation processes with the main among these dominating degradation in the surface layers being evaporation, dissolution, emulsification, photochemical oxidation and microbial degradation. Evaporation alone is responsible for the removal of volatile components (20-50% for crudes and up to 75% for fuel oils, with C15 to C 25 range of hydrocarbons evaporating to a limited extent. Studies following *Amoco Cadiz* spill showed that approximately 30% oil evaporated, 14% dissolved in water, 8% incorporated in sediment, 28% washed up on shore (Marchand, 1981). The rate of evaporation is affected by temperature, wind speed, solar radiation, thickness of slick and composition of oil. The dissolving and dispersal of hydrocarbons beneath the surface depends upon the solubility of the hydrocarbons, which decreases exponentially as a function of their molecular volume. Consequently low weight aromatics such as benzenes, xylenes and naphthalenes have a relatively higher solubility (McAuliffe, 1966). The vapour pressures and solubility of individual hydrocarbons govern evaporation and dissolution. Naturally occurring surfactants such as humic acid and fatty acids tend to accumulate in the surface layers and they, along with solar radiation, aid in increasing dissolution by forming polar, surface active compounds. UV and near UV rays provide energy to transform hydrocarbons in the presence of oxygen to oxygenated compounds with aromatics being more easily oxidized than aliphatic.

Certain surface-active compounds found in crude oils such as porphyrins and carboxylic acids cause the formation of a water-in-oil emulsion

popularly known as "chocolate mousse" (Canevari, 1969). The same are not found in fuel oils and refined petroleum products and hence no mousse formation takes place. PAHs are hydrophobic with a high affinity for adsorption onto particulates. Hence the low molecular weight and more soluble PAHs, mainly phenathrenes, are found in the water column in relative abundance (Baumard *et al.*, 1998). In both episodal and chronic oil pollution suspended solid particulates play an important role in sedimentation of hydrocarbons. Scanning electron microscope studies reveal that the fibrillar structures on the surface of particulates provides the surface for adsorption of hydrocarbons (Lee, 1980). This phenomenon is of greater importance along coastal regions and in intertidal zones and estuaries than in subtidal or off shore regions where the load of suspended particles is far lesser. In the subtidal regions the primary factor is oxidation of hydrocarbons which leads to increase in density due to addition of polar groups, which in turn increases settling velocity (Lee & Page, 1997).

Mackay *et al.* (1983), using partitioning models of the distribution of compounds in particulate and dissolved phases of seawater, predicted that the lower molecular weight compounds with higher water solubilities would readily be desorbed from the sediment into the dissolved phase. Dominant sources of particles in the water column are mainly phytoplankton and microzooplankton production, and near shore sediments. Sediment resuspension plays a major role in supplying particles to the water column in shallow areas. The persistence of PAHs in sediments depend mainly on the sorption, volatilasation, leaching and biological or chemical degradation

processes which may be influenced by a variety of environmental factors. Only a fraction of the total measured sediment PAH is available for equilibrium partitioning. With the aqueous phase this will depend mainly on the sediment-water partitioning qualities of PAH. The organic carbon content in the water and the sediment plays a major role in the distribution of PAH. Cochin estuary being highly productive, has a very high quantity of suspended solids contributed by phytoplankton debris, lipids, proteins etc. These contribute to the rich organic load of sediment (Nair *et. al.*, 1993).

Most PAHs being relatively water insoluble are ultimately deposited in sediment. In areas of major oil spills or in localised or enclosed areas, sediments are shown to have high concentrations of PAH (Maccubn *et. al.*, 1985). The geography of the area in which the spill occurs or the discharge takes place is of the greatest significance to the fate of the oil as in the open seas with good water circulation the result is very quick dispersal and degradation (Rashid, 1974) whereas in protected shallow areas with poor circulation with fine sediment the hydrocarbons may remain unaltered for years (Blumer and Sass, 1972). Fauna and flora of habitats such as low energy marshes and mangroves may take upto several decades to recover to pre-spill conditions in terms of population and diversity (Thoraugh, 1991). In subtidal regions the hydrocarbon concentrations after spills remains many orders of magnitude lower than heavily oiled intertidal regions. However, in subtidal regions also the topography and the local hydrography are decisive factors, as is evident from the contrasts between the biological effects of the *Amoco Cadiz* spill which took place in a semi-enclosed bay with high

concentrations of fine particulate matter which facilitated the retention of hydrocarbons for a much longer period than in the *Exxon Valdez* spill which took place in Prince William Sound where the dilution and weathering were much faster. This is evidenced in the low concentrations of hydrocarbons measured after one year of the spill and barely detectable levels in the second year in the latter case (Lee & Page, 1997).

The backwaters as a whole is not in a critical state with reference to PHC pollution overall though within the harbour area the levels exceed that of 'healthy' limits in Station 4 and 7 and are high in Station 8 as well, especially during winter and premonsoon months. In addition values noted for sediment are on the higher side keeping in view the results from laboratory experiments in relation to toxicity to shrimps though conclusive interpretations are not possible since bioavailability of PHCs in the wild is a poorly understood phenomenon. Stations 4, 7 and 8 have comparatively higher values for sediment PHCs in addition to Stations 3.

Surface currents, dilution and evapouration were effective in removing PHCs after the spill as values in surface waters came to normal within two weeks. Also the effect of a substantial amount of rainfall in April might have effected dilution. However the rainfall was not sufficient to wash out the surface layer of sediment, rather it may have contributed to increased turbulence and resuspension of sediment causing higher values in subsurface waters upto the end of April. Persistence of high values in Station 4 even in May could be due to the baying effect. The oil spill occurred close to Station 6. Here the torque force created by the bifurcation of the channel creates heavy

turbulence and local currents. Oil from here may have been washed upstream to Mattancherry channel during high tides and trapped in the clayey sediment present there. The contribution from the fishing harbour results in high values for this station. Silting is higher in the Mattancherry channel as compared to the Ernakulam channel, with nearly a third of the deposition within the harbour occurring here, perhaps due to the weak current patterns prevalent. Sediments are also brought out to the barmouth and deposited in the outer harbour areas by river run-off (Gopinathan & Qasim, 1971). This may account for the comparatively high values observed in the Station 8.

The hydrography of the estuary is mainly controlled by the discharge of the Periyar in the north and the Muvattupuzha in the south and is governed by the tidal regimen, the monsoonal climate with four distinct seasons- the winter (Dec-Feb), premonsoon (Mar-May), monsoon (June-Aug) and postmonsoon (Sep-Nov) and manmade structures like the Thaneermookam bund. During the monsoon the estuary is turned into a freshwater water body except at the barmouth due to heavy discharge from the rivers. The post-monsoon period is a transition period. A strong salinity gradient develops during the premonsoon in the longitudinal direction along with the incursion of saline waters in the northern reaches resulting in a well stratified condition in this area. In contrast the southern parts are well=mixed in all seasons, due to the presence of the Thaneermookam barrage. In the premonsoon, river discharge is minimum and the influence of saline incursion maximum in the northern reaches. Maximum turbidity is also noticed during high tides in this period.

Organic carbon content follows the sediment distribution pattern in the estuary, with high values for both in the midreaches of the northern estuary in the present area of study. A seasonal maximum for the same is observed in the premonsoon months, resulting from the increased adsorption of organic compounds onto clay minerals under the influence of increasing salinity (Nair *et al*, 1995).

In the (upper) reaches of the estuary particulate matter (seston) originating from domestic sewage and industrial runoff, under the influence of tidal currents and monsoonal flow keeps the water relatively turbid (Sarala Devi, 1979).

The discontinuity in the vertical profiles of temperature and salinity during the monsoon and postmonsoon causes a stratification that results in maximum turbidity in the estuary. This contributes to the minimum settlement of detritus during this period. The converse is noticed when the estuary returns to a well mixed condition during the winter and premonsoon. (Nair, *et al.*, 1993). Tides in the estuary are of the mixed semi-diurnal type with a maximum range of 1-m. (Joseph & Kurup, 1987).

Mud and sandy mud predominate among the sediment type in the Ernakulam and Mattancherry channels whereas the sediment towards the barmouth is silty sand and sand. The sediments are very poorly sorted in general, platy and leptokurtic. Organic content varies from 0.24 to 6.15% and has been showing an increasing trend over the decades. Increased sediment load from the rivers, sewage channels and tidal activity causes siltation of the harbour (Veerayya & Murty, 1974; Serlathan, *et al.*, 1993). The sedimentation

pattern in the Cochin backwaters is complex. Stratification resulting in less dense river water at the surface and denser sea water in the bottom layers and heavy river discharge especially during the monsoons reflect in complicated sedimentation patterns in the channels. The sedimentation is 1 m per annum and is higher during the premosoon in the Mattancherry and Ernakulam channels during the pre and postmonsoons with the highest sedimentation taking place during the tide slack period. Increased sedimentation is seen in the approach channel during the monsoon due to changes in the circulation pattern (Gopinathan & Qasim, 1971).

Information on bioavailability of pollutants is essential to delineate cause and effect relationship between chemicals in the marine environment and observed biological abnormalities (Widdows *et al.*, 1982). Exposed habitats experience rapid weathering of spilled oiled whereas less exposed, sheltered ones typically exhibit a much slower pace of weathering (Saeur *et al.*, 1993). Whereas the results of episodic large spills is acute and obvious, the biological impact of chronic low level pollution is difficult to assess since subtle physiological effects can be masked by natural seasonal and spatial variability in marine populations (Vargo, 1981). A balance between uptake and depuration of xenobiotics exists In areas where contamination is limited to baseline levels. A baseline PAH content also is detected in organisms exposed to the least contaminated sites (Baumard *et al.*, 1998).

The toxicity of oil to individual organisms may be either physical or chemical. Acute physical toxicity is produced by smothering and clogging of respiratory surfaces, while the more relevant processes, especially in the case

of chronic oil pollution are the effects of the water soluble fraction, particularly the toxicity of polyaromatic hydrocarbons on organisms (Lehr & Jerina 1977, Bayne *et al.*, 1979, White, 1986). Lee and Page (1997) suggested three phases in episodic oil pollution with regards to its effect on animals: a) a toxicity phase where high hydrocarbon concentrations cause large scale mortality of flora and fauna b) followed by a phase where reduced but significant concentrations of hydrocarbons provide an environment conducive for opportunistic species which feed on microflora flourishing on hydrocarbons and c) an equilibrium phase where there is a return to normalcy and the reappearance of sensitive species..

Among polyaromatics, light aromatics or 2 - 3 ringed PAHs naphthalenes, fluorenes, phenanthrenes and anthracenes produce significantly higher acute toxicity in relation to heavier 4-7 ringed aromatics (chrysene to coronene). However, a significant number of high molecular weight PAHs and their derivatives (e.g. chrysene, benzo (a) pyrene) are proven carcinogens and mutagens, also producing immunotoxicity, reproductive toxicity and teratogenicity in humans and other organisms (Neff, 1979, Cosma & Garte, 1997). Heterocyclic compounds and their derivatives are currently being considered as more responsible than PAHs for toxicity of oil to aquatic organisms (Barron *et al.*, 1999).

Cochin estuary experiences continuous oil input associated with shipping, fishing vessel operations, transportation, urban run-off, accidental spills during tanker operations etc. Menon and Menon,(1998) are of the opinion that natural causes that control the distribution of petroleum

hydrocarbons in the surface and sub surface waters of the estuary are the estuarine circulation, oscillatory nature of tidal currents and dissolved and suspended load of waste. Basically the distribution of oil either in water or sediment depends on the availability of matrices, mainly organic matter onto which oil can get enmeshed (Menon *et. al.*, 2000).

REFERENCES

ABDALIi, F.,AL-NAJJAR,H. A., BAHLOUL, M., JACOB, P. G. and AL-GHADBAN, A. N., 1993. Pore diffusion transport of petroleum hydrocarbons within sediment columns in ROPME sea area. Presented at the scientific workshop on the results of the Mt. Mitchell open sea cruise Kuwait, Arabian Gulf, 24-28 Jan 1993.

ACKMAN, R. G. and NOBLE, D., 1973. Steam distillation: A simple technique for recovery of petroleum hydrocarbons from tainted fish. *J. Fish. Res. Board Can.* 30: 711-714.

AJITH, J. K., 1996. Strait dynamics of tropical tidal inlets. Ph. D. thesis, Cochin University of Science and Technology, Cochin, India.

AL-LIAIBI, S. S. and AL-OMRAN, L., 1995. Petroleum hydrocarbons in offshore sediments from the Gulf,. *Mar. Pollut. Bull.* 32, 65-69.

AL-LIAIBI, S. S. and GHAZI, S. J., 1997. Hydrocarbon distribution in sediments of the open sea of the Arabian Gulf following the 1991 Gulf War Oil Spill. *Mar.Pollut.Bull.* 34 (11): 941-948.

AL_SAAD, H. T. and AL_TIMARI,A. A., 1993. Sources of hydrocarbons and fatty acids in sediment from Hor Al-Hammar Marsh, Shatt Al-Arab, and North-west Arabian Gulf. *Mar. Pollut. Bull.* 26 (10): 559-564.

ANIRUDHAN, T. S., BALCHAND, A. N., NAIR, S. M. and NIMBISAN, P. N. K., 1987.Distribution pattern of salinity and silicon and their interrelationship in Cochin backwaters. Proc. Natl. Sem. Estuarine Manage., Trivandrum; 26-31 pp.

ANON, 1996. Oil Spill in Cochin Harbour. *The Indian Express*, 19.04.1996, p.1.

BALCHAND, A. N., NAMBISAN, P. N. K., and NAIR, S. M., 1991. River inputs into ocean systems: role pf synoptically synergistic modulation processes in estuaries. Proc. IUGGXX Gen. Assembly – IAPSO Symp., Vienna: 181 pp.

BALCI, A., 1993. Dissolved and dispersed petroleum hydrocarbons in the Eastern Aegean Sea. *Mar. Pollut. Bull.* 26 (4): 222-223.

BARRON, M. G., PODRABSKY T., OGLE S. and RICKER, R. W., 1999. Are aromatic hydrocarbons the primary determinants of petroleum toxicity to aquatic organisms? Aquat. Toxicol. 46: 253-268.

BAUMARD, P., BUDZINSKI, H. and GARRIGUES, P., 1998. PAHs in Arachon Bay, France: Origin and Biomonitoring with caged Organisms. *Mar. Pollut. Bull.* 36 (8): 577-586.

BAYNE,B. L., MOORE, M. N., WIDDOWS, J., LIVINGSTON, D. R. and SALKEID, P., 1979 Measurement of the responses of individuals to environmental stress and pollution. Studies with bivalve molluscs. *Phil. Trans. R. Soc. Lond.* B. 286, 563-581.

BLUMER and SASS, S., 1972. Oil pollution persistence and degradation of spilled fuel oil. *Science*, 176:1120-1122.

BROWN, L. R., PABST, G. S. and LIGHTS M., 1978. A rapid field method for detecting oil in sediments. *Mar. Pollut. Bull.* 9 (3): 81-82.

CANEVARI, G. P., 1969. General Dispersant Theory. In: Proc. Joint. Conference on Prevention and Control of oil spilled. A. P. I, Washington D. C., pp.171-177.

CHRISTENSEN, J. H., HANSEN, A., ANDERSEN, O. and MORTENSEN, J.,2000. Development and application of a hyphenated analytical and chemometric method in petroleum characterisiation, identification and degradation studies. In: Oil &

Hydrocarbon Spills II: Modelling, Analysis and Control. Rodriguez G. R. and Brebbia C. A (eds.) WIT Press, Southampton, U. K., p.189-198 .

CLARK,R. C. and BROWN,D. W., 1977. Petroleum properties and analyses in biotic and abiotic systems. In: Effects of petroleum on Arctic and subarctic marine environments and organisms. Vol I: Nature and fate of petroleum. Malins D. C. (ed.) Academic Press, New York. p. 1-90.

CLARK,R. C. and MACLEOD,W. D. 1977. Inputs, transport mechanisms and observed concentrations of petroleum in the marine environment. In: Effects of petroleum on Arctic and subarctic marine environments and organisms. Vol I: Nature and fate if petroleum. Malins D. C. (ed.) Academic Press, New York.p. 91-224.

COSMA,G. N. and GARTE,S. J., 1996. Human Gene Inducibility: A marker of exposure and susceptibility to environmental toxicants. In: Molecular Environmental Biology. Seymour J. Garte (ed.) pp225-245.

CRIPPS, G. C., 1992. Natural and anthropogenic hydrocarbons in the Antarctic marine environment. *Mar. Pollut. Bull.* 25 (9-12): 266-273.

CRIPPS, G. C., 1993. Baseline levels of hydrocarbons in seawater of the Southern Ocean: Natural variability and regional patterns. *Mar. Pollut. Bull.* 24 (2): 109-114.

DONKIN,P. and EVANS,S. V., 1984. Application of steam distillation in the determination of petroleum hydrocarbons in water and mussels (*Mytilus edulis*) from dosing experiments with crude oil. *Anal. Chimi. Acta* , 156 : 207-219.

EHRHARDT, M. and BLUMER, M., 1972. The source identification of marine hydrocarbons by gas chromatography. *Environ. Pollut.* 3: 179-194.

EMARA , H. I ., 1990 Oil pollution in the Southern Arabian Gulf and Gulf of Oman. *Mar Poll Bull,* 21(8) : 399-401.

FARRINGTON, J. W. and TRIPP, B. W., 1975. A comparison of analysis methods for hydrocarbons in surface sediments. In: Marine chemistry in the coastal environment. *Am. Chem. Soc. Symp. Ser.* 18 : 267-284.

FARRINGTON, J. W., TEAL, J. M. and PARKER, P. L., 1976. Petroleum hydrocarbons. In: Strategies for marine pollution monitoring. Goldberg E. D. (ed.), Wiley- Interscience, New York, p. 3-34.

FAY, J. A. 1971. Physical processes in the spread of oil on a water surface. In: Proceedings of 1971 Joint Conference on Prevention and Control of oil spills, American Petroleum Institute, Washington D. C. p.463-467.

*FONDEKAR, S. P., TOPGI, R. S. and NORONHA, R. J., 1980. *Indian J. Mar. Sci.* 9:286

FOWLER, S. W., 1988. Coastal baseline studies of pollutants in Bahrain, UAE and Oman. In: Proc. of Symp. on Regional Marine Pollution Monitoring and Research Programmes. Regional Organisation for the Protection of the Marine Environment, Kuwait. , ROPME/GC-4/2, p.158-180.

GESAMP, 1993. Impact of oil and related chemicals and wastes on the marine environment, GESAMP Reports and Studies. No.50: 180 pp.

GEYER, R. A., 1980. In: Marine Environmental Pollution, 1: Hydrocarbons Geyer R. A. (ed.). Elsevier Oceanographic Series. Elsevier Scientific Publishing. Co., Amsterdam, p. 12.

GIGER, W. and BLUMER, M., 1974. Polyaromatic hydrocarbons in the environment : Isolation and characterisation by chromatography, visible, ultraviolet and mass spectrometry. *Anal. Chem.* 46: 1663-1671.

GOPALAN, U. K., VENGAYIL, D. T., UDAYAVARMA, P., and KRISHNANKUTTY, M., 1983. The shrinking backwaters of Kerala. *J. mar. biol. Ass. India* 25: 131-141.

GOPINATHAN, C. P. and QASIM, S. Z., 1971. Silting in navigational channels of the Cochin harbour area. *J. mar. biol. Ass. India* 13: 14-26.

GUNDLACH, E. R., McCAIN, J. C. and FADLALLAH, Y. H., 1993. Distribution of oil along the Saudi Arabian coastline (May/June 1991) as a result of the Gulf War oil spills. *Mar. Pollut. Bull.* 27: 93-96.

* Not referred to in original

HANSEN, A. B., CHRISTENSEN, J. H., AVNSKJOLD, J., ANDERSEN, I. and RASMUSSEN C. A., 2000. Identification of oil spills by GC/MS fingerprinting in relation to the Danish maritime oil spill response. In: Oil & Hydrocarbon Spills II: Modelling, Analysis and Control. Rodriguez G. R. and Brebbia C. A (eds.) WIT Press, Southampton, U. K., p. 200-207.

HEINRICHSON D., 1990. Our common seas: Coasts in Crisis. Earthscan Publ., London, U.K. 184 pp.

IMO, 1990. Petroleum in the marine Environment . Document MEPC 30/!INF. 13 submitted by the United States: International Maritime Organisation, London.

INGOLE,S. A., KADAM, A. N.,MAYADEO, M. S. and DHADDKE, P. M., 1995 Petroleum hydrocarbons in intertidal ecosystem along the Bombay coast. *Indian J. Mar. Sci.*, 24: 116-118.

IOC (INTER GOVERNMENTAL OCEANOGRAPHIC COMMISSION), 1982. The determination of petroleum hydrocarbons in sediments. Manuals and Guides No. 11, UNESCO, Paris, pp.38.

IOC (INTER GOVERNMENTAL OCEANOGRAPHIC COMMISSION), 1984. Manual for monitoring oil and dissolved/dispersed petroleum hydrocarbons in marine waters and on beaches. Manuals and Guides No. 13, UNESCO, Paris, pp. 35.

JELTES, R, and DEN TONKELSAR W. A. M., 1972. Gas chromatography versus infra-red spectrometry for determination of mineral oil dissolved in water. *Water. Res.* 6: 271-278.

JOHNSTON, R., 1984. Oil pollution and its management. In: Marine Ecology, Vol. V, Part 3. Kinne, O. (ed.) . Wiley- Inter science Publication. New York, Toronto, Singapore, 1432 pp.

JOSEPH, J. and KURUP, P. G. 1987. Tidal response and circulation of the Cochin estuary. In: Proc. Natl. Sem. Estuarine Management.1987. Trivandrum, p. 88-92.

JOSEPH, J. and KURUP, P. G. 1989. Volume transport and estuarine features at Cochin

inlet. *Mahasagar* 22: 165-172.

KADAM, A. N., 1987. Petroleum hydrocarbons in surface sediments in Kandla creek. *Mahasagar.* 20(4): 243-248.

KADAM, A.N. and BHANGALE, V. P., 1993. Petroleum hydrocarbons in northwest coastal waters of India. *Indian J. Mar. Sci.* 22 : 227-228.

LAW, R. J, KLUNGSOYR, J. and FRERIKS, I. L., 1997. The QUASIMEME Laboratory Performance Study of Polycyclic aromatic hydrocarbons (PAH): Assessment of the first three rounds, 1994-1995. *Mar. Pollut. Bull.* 35 (1-6): 64-77.

LAW, R. J. AND BISCAYA, J. L., 1994. Polycyclic aromatic hydrocarbons (PAH) - Problems and progress in sampling, Analysis and Interpretation. *Mar. Pollut. Bull.* 29 (4 & 5) 235 - 241.

LAW, R. J., 1981. Hydrocarbon concentration in water and sediments from UK Marine Waters, determined by Fluorescence Spectroscopy. *Mar. Pollut. Bull.*,12(5): 153-157.

LEE, R.F., 1980. Processes affecting the fate of oil in the sea. In Marine environmental pollution : Vol I, Hydrocarbons, Geyer, R.F. (ed.) , Elsevier, Amsterdam, pp 337.

LEE. R. F, and PAGE, D. S., 1997. Petroleum hydrocarbons and their effects in subtidal regions after major oil spills. *Mar. Pollut. Bull.* 34 (11): 928-940.

LEHR, R. E and JERINA, D. M., 1977. Metabolic activation of polycyclic hydrocarbons. *Arch. Toxicol.*, 39: 71-80.

LEVY, E. M., 1972. The identification of petroleum products in the marine environment by absorption spectrophotometry. *Water. Res.* 6: 57-69.

MACCUBBIN, A. E., BLACK, P., TRZECIAK, L. *and* BLACK, J. J., *1985.* Evidence for polynuclear aromatic hydrocarbons in the diet of bottom feeding fish. *Bull. Environ. Contam. Toxicol.* , 34 : 876-882.

MACKAY, D., JOY, M. and PATERSON, S., 1983. A quantitative water, air, sediment interaction (QWASI) fugacity model for describing the fate of chemicals in lakes. *Chemosphere*, 12: 981-997.

MARCHAND, M., 1981. *Amoco Cadiz*. Bilan du colloque sur les consequences d'une pollution accidentelle par hydrocarbures. Publication due Centre National pour l' Exploration des Oceans (CNEXO) , Paris. Rapports Scientifiques et Techniques, No. 4, 86 pp

MASON, R. P., 1987. A comparison of fluoroscence and GC for the determination of petroleum hydrocarbons in mussels. *Mar. Pollut. Bull.* , 18 (10) : 528-533.

McAULIFFE, C. D., 1966.Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin and aromatic hydrocarbons. *J. Phys. Chem.*, 70:1267-1275.

MENON, N. N. and MENON, N. R., 1998. Status of oil contamination in the Cochin estuary – Presented at the International Conference on Environmental Sciences (ICES) 1998 at RRL, Trivandrum, dec 2-4, 1998.

MENON, N. N. and MENON, N. R., 1999. Uptake of polycyclic aromatic hydrocarbons from suspended oil bound sediments by the marine bivalve *Sunetta scripta*. *Aquat. Toxicol.* 45: 63-69.

MENON, N. N., BALCHAND, A. N. and MENON, N. R., 2000. Hydrobiology of the Cochin backwater system – a review. *Hydrobiologia*, 430: 149-183.

MILLE G., RIVET L., JAWAD A. I. and BERTRANDE J. C., 1992. Hydrocarbon distributions in low polluted surface sediments from Kuwait, Bahrain and Oman coastal zones (before the Gulf War). *Mar. Pollut. Bull* 24 (12): 522-624.

MOHAN, C. P. and PRAKASH, R. R., 1998. Concentration of petroleum hydrocarbons in bivalve *Mytilopsis sallei* and in the harbour waters of Vishakapatnam, east coast of India. *Indian J Mar. Sci.*, vol 27, pp 496-498.

NAIR, C. K.., BALCHAND, A. N. and CHACKO, J., 1993. Sediment characteristics in relation

to changing hydrography of Cochin estuary. *Ind. J. Mar. Sci.* 22: 33-36.

NAIR, V. T., CHACKO, J. and CHANDRAMOHANKUMAR, N., 1995. Distribution of petroleum hydrocarbons in the sediment of the Cochin estuary, south west coast of India. *Indian J Mar Sci* 24: 240-242.

NATIONAL ACADEMY OF SCIENCES, 1975. Petroleum in the marine environment, Washington D.C., 107 pp.

NEFF, J. M., 1979. Polycyclic aromatic hydrocarbons in the aquatic environment : sources, fates and biological effects. Applied Sc. Publ. Ltd., London. pp 262.

PENDOLEY, K., 1992. Hydrocarbons in Rowley shelf (Western Australia) oysters and sediments. *Mar. Pollut. Bull.* 24 (4): 210-213.

PHILIP, R. P., SMALLWOOD, B. and ALLEN, J., 2000. Correlation of weathered and unweathered oil samples using the carbon isotopic composition of individual components in the crude oil. In: *Oil & Hydrocarbon Spills II: Modelling, Analysis and Control. Rodriguez G. R. and Brebbia C. A (eds.) WIT Press, Southampton, U. K., p. 211-219.*

RAMAMURTHY,V. D., 1991. Effects of oil pollution on bio ecology and fisheries on certain enclosed coastal regions of the Arabian sea. *Mar. Pollut. Bull.* 23: 239-245.

RAMAMRITHAM, C. P. and JAYARAMAN, R., 1963. Some aspects of the hydrographical conditions of the backwaters around Willingdon Island (Cochin). *J. Mar. Biol. Ass. India*, 5(2): 170-177.

RASHID, M.A., 1974. Degeneration of Bunker C oil under different coastal environments of Chedabucko Bay, Nova Scotia, *Estuarine Coastal Mar. Sci.* , 2: 137-144.

READMAN,J. W., PRESTON,M. R. and MANTOURA,R. F. C., 1986 An integrated technique to quantify sewage, oil and PAH pollution in estuarine and coastal environments. *Mar. Pollutn. Bull.* 47 (7): 298-308.

SARLA DEVI, K., 1989. Temporal and spatial variations in particulate matter, particulate organic carbon and attenuation coefficient in Cochin backwaters. *Ind.J.Mar.Sci.*18: 242-245.

SARLA DEVI, K., VENUGOPAL, P., REMANI, K.N., LALITHA, S. and UNNITHAN, R.V., 1979. Hydrographic features and water quality of Cochin backwaters in relation to industrial pollution. *Ind. J. Mar. Sci.* 8: 141-145.

SAUER, T. C., BROWN J. S., BOEHM,P. D., AURAND,D. V.,MICHEL,J. and HAYES,M. O. ,1994. Hydrocarbon source identification and weathering characterization of intertidal and subtidal sediments along the Saudi Arabian coast after the Gulf War Oil Spill. *Mar. Pollut, Bull*, 27: 117-134.

SEN GUPTA, R., QASIM Z., FONDEKAR, S. P. and TOPGI, R. S., 1990. Dissolved petroleum hydrocarbons in some regions of the Northern Indian Ocean. *Mar Pollut. Bull* 2: 65-68.

SEN GUPTA, R., FONDEKAR,S. P. and ALAGARASAMY, R.,1993. State of oil pollution in the Northern Arabian Sea after the 1991 Gulf oil spill. *Mar. Pollut. Bull.* 27; 85-91.

SERLATHAN, P., MEENAKSHIKUTTY, N. R., ASHRAF K. V. and PADMALAL, D. 1993. Sediment and organic carbon distributions in the Cochin harbour area. *Indian J. Mar. Sci.* 22: 252-255.

THORHAUG, A., 1991. Oil spills in the tropics and sub tropics. In : Pollution in Tropical Aquatic Systems. Connel, D. W. and Hawker, D. W. (eds.) CRC Press Inc. , Boca Raton, Florida.

UNNITHAN, R. V., VIJAYAN, M., RADHAKRISHNAN, E. V. and RAMANI, K. N., 1977. Incidence of fish mortality from industrial pollution in Cochin backwaters. *Ind. J. Mar. Sci.* 6(1): 81-83.

VARGO, S. L., 1981. The effects of low chronic concentrations of No.2 fuel Oil on the physiology of temperate estuarine zooplankton community in the MERL-Microcosms. In: Biological Monitoring of Marine Pollutants. Verberg F. J (ed.) Academic Press Inc.New York, pp.295

VEERAYYA,M. and MURTY, P. S. N., 1974. Studies on the sediments of Vembanad Lake, Kerala State: Part III- Distribution & Interpretation of Bottom Sediments. *Ind. J. Mar. Sci.* 3: 16-27.

WAKEHAM, S. G., 1977. Synchronous fluorescent spectroscopy and its applications in indigenous and petroleum derived hydrocarbons in lacustrine sediments. *Environ. Sci. Technol.* 11: 272-276.

WANG, Z. and FINGAS, M., 1997. Development in the analysis of petroleum hydrocarbons in oils, petroleum products and oil – spill- related environmental samples by gas chromatography. J. Chromatography A, 774: 51-78.

WARNER, J. S., 1975. Determination of sulphur-containing petroleum components in marine samples. In: Proceedings of 1975 Conference on Prevention and Control of Oil Pollution. American Petroleum Instt. Washington D. C., p. 97-101.

WHITE, K. L., 1986. An overview of immunotoxcology and carcinogenic polyaromatic hydrocarbons. *Environ. Carcin. Rev.*, 2:163-202.

WIDDOWS, J., BAKKE, T., BAYNE, B. L. , DONKIN, P. , LIVINGSTONE, D. R., LOWE, D. M. , MOORE, M. N. , EVANS, S. W. and MOORE, S. L., 1982. Response of *Mytilus edulis* to the water accommodated fraction of North Sea oil. *Mar. Biol.*, 67 : 15 - 31.

ZHIJIE,F. and ZHANG,Y. E., 1988. Levels and trends of oil pollution in the Bohai Sea. *Mar. Pollut. Bull.* 19 (11): 572-578.

ZYLSTRA, G. J., 1994, Molecular analysis of aromatic hydrocarbons degradation In: Molecular Environmental Biology. S. J. Garte (ed.) CRC Press inc.83-115.256 pp.

SUMMARY

This thesis has been arranged in five chapters viz. physiological reposes, incidental pathology, histopathology, ultrastructure and environmental load of petroleum hydrocarbons in the Cochin estuary. A general introduction that precedes the chapters gives an overview of oil pollution and the objectives of this study..

Results of experiments with juveniles and subadults of the penaeid shrimp *Metapenaeus dobsoni* (Miers), a commercially important benthic species, subjected to lethal and sublethal toxicity tests are presented in Chapter 1. Analysis of these results revealed that the toxicity of the water-accommodated fraction of Bombay High Crude was dose-, time-, size- and moult stage-dependant. Oxygen consumption and accumulation of petroleum hydrocarbons in the tissue of the shrimps when exposed to sublethal doses were investigated to gain an insight into the sublethal physiological stress responses. The increased rate of oxygen consumption was found to correlate with increased tissue load of hydrocarbons in the lower doses and lower periods of exposure. An inverse relationship was seen when the dose and time were high. Suppression of moulting in shrimps exposed to higher concentrations of hydrocarbons was noticed. Repeated incidence of pathological infestations took place in 13-15% of shrimps (size 30-35 mm) exposed to 1 ppm and 5-8% of shrimps exposed to 4 ppm beyond 18 days in a 30-days moulting pattern

study. Curiously enough the shrimps exposed to 8 ppm as also those maintained under control conditions did not show any signs of disease. This phenomenon is discussed in detail in the following chapter.

Chapter 2 is a detailed analysis of the disease afflicting the shrimps mentioned in the previous chapter. This pathogen caused the formation of dark lesions on the abdominal segments and occasionally the carapace of the shrimps accompanied with opacity of musculature. Isolation of the causative factor, a chitinoclastic *Vibrio* sp. was accomplished by using microbiology techniques. Kochs Postulate was successfully proved to establish the pathogenicity of the isolated bacterium. The pathogen was found to be virulent when injected into the shrimp at a dilution of even 100 cells/ml. Mortality, disease manifestation and moulting as a response to the induction of the pathogen into body of the shrimp was quantified and found to be statistically significant at 1% level in terms of the first two phenomena. The *Vibrio* sp. was presumed to be an opportunistic pathogen as no such condition was recorded in the shrimp maintained under control conditions. Chronic exposure of shrimps to low sublethal doses of petroleum hydrocarbons was found to render them susceptible to vibriosis. The environmental ramifications of the same have been mentioned.

The chapter on histopathology presents and discusses the results of the exposure of juveniles of penaeid shrimp *M. dobsoni* (30-35 mm) in the intermoult stage to lethal and sublethal doses of water accommodated fraction of Bombay High Crude, which resulted in histopathological changes in the hepatopancreas

and gills. Comparisons with the histology of starved and normal hepatopancreatic tissue and normal gill tissue were also made. Six plates with micrographs taken under a light microscope have been included. The deterioration of the hepatopancreatic tubules was found to be dose- and time-dependent under sublethal toxic conditions. Cytorrhexis in the proximal tubules, proliferation of R-cells, proliferation of haemocytes, reduction in tubule width due to shrinkage of the tubules, detachment of the *tunica propria*, obliteration of the tubule lumen by cellular debris and delaminated cells, reduction in epithelial height due to atrophy of cells, increase in epithelial height due to vacuolation and storage of lipid, proliferation of B-cells, vacoulation of E-cells, reduction in the number of E-cells and proliferation of connective tissue were the twelve parameters studied. These changes are presumed to be related to a reduction in feed intake, assimilation and excretion of high caloried faeces due to the deterioration of the tubules. In the gill tissue there was varied degree of necrosis of the gill filaments. Necrosis of haemocytes, proliferation of haemocytes, distention of pillar cells, shrinkage of pillar process cells and sloughing off filaments were the notable changes. The histopathological parameters studied were quantified and subjected to statistical analysis using Wilcoxon signed rank test, Spearmans rank correlation, analysis of variance and discriminant function analysis.

The histopathological changes observed in light microscopic studies were further investigated by fine structure analysis and the results are presented and discussed in the chapter on ultrastructure. Twelve plates of electron micrographs,

depicting the changes to cell organelles within the cells of the hepatopancreatic tubules and the filaments and cuticle of the gills, are included. The stress responses at fine structure level include disruption of cell membranes and organelles, presence of large lipid droplets, marked pyknosis of the nucleii, proliferation of endoplasmic reticulum, distortion and disruption of microvilli and proliferation in and distortion of mitochondria in the R-cells of the hepatopancreas. The changes appeared to be dose and time related. In the gill tissue, atrophy of cuticle and filament cells, formation of melanised plaques and coagulated haemolymph appeared to be dose related. Proliferation and necrosis of haemocytes and accumulation of petroleum hydrocarbons as large lipid droplets was also recorded. Clearly the hepatocytes and gill filaments are seen to be actively involved in the breakdown and expulsion of PHCs from the shrimp. Toxic effects of PHCs on subcellular organization are also evident. The light microscopic and fine structure findings correlated well with the rate of oxygen consumption and the PHC load accumulated by the shrimps during the course of the experiments.

The fifth chapter presents the results of the monitoring of the seasonal and spatial distribution of petroleum hydrocarbons in the water and sediment of Cochin estuary during the premonsoon, winter and post monsoon periods and discusses the findings. The Cochin harbour and the adjoining backwater are moderately contaminated with petroleum hydrocarbons with surface water values for dissolved and dispersed petroleum hydrocarbons ranging from 2.8 – 298.46

μg/l, subsurface values ranging from 1.12 - 573.93 μg/l and sediment values ranging from 0.67 – 652.96 μg/g. Contamination can be directly linked with anthropogenic use of the harbour and backwaters which may be directly related to shipping/berthing activities and transport of crude oil and its derivatives. The recovery of the harbour from an event of episodic pollution was found to be rapid and this was attributed to the peculiar circulation pattern prevalent in this estuary. The status of the estuary with regards to petroleum hydrocarbon contamination remains below the critical level though in pockets the values recorded for sediments remains high, which predicts deleterious effects on benthic communities and species.

All findings in this thesis are supported by literature, the relevant ones among which have been listed at the end of each chapter in alphabetical order.

****