INVESTIGATIONS ON THE EFFECTS OF PETROLEUM HYDROCARBONS ON THE MARINE BIVALVES *PERNA VIRIDIS* (LINNAEUS) AND *SUNETTA SCRIPTA* (LINNE')

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By
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APRIL 1997

To my loving husband

CERTIFICATE

This is to certify that the thesis entitled "**Investigations on the Effects of Petroleum Hydrocarbons on the Marine Bivalves *Perna viridis* (Linnaeus) and *Sunetta scripta* (Linne')**", is an authentic record of the research work carried out by Ms. Nandini Menon, N., under my scientific supervision and guidance in the School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment 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.

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Cochin 16
April 1997

DECLARATION

I hereby declare that this thesis entitled "**Investigations on the Effects of Petroleum Hydrocarbons on the Marine Bivalves *Perna viridis* (Linnaeus) and *Sunetta scripta* (Linne')**", 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, Cochin and that this has not previously formed the basis of the award of any degree, diploma or associateship in any University.

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Nandini Menon. N

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NANDINI MENON. N.

CONTENTS

PREFACE

Episodal pollution of coastal and open ocean by oil tanker disasters has generated enormous interest among students of science, scientists, policy makers and journalists on oil pollution. The development of rational, effective and economical strategies to solve the problems of contaminated environment will depend greatly on our ability to predict remedial actions that will improve environmental quality and how these changed conditions will affect aquatic organisms. A forerunner of the above situation is the realisation of harmful effects of oil to aquatic biota. Considerable volume of literature has been published on the effects of oil on marine animals which come essentially under two categories: (1) assessment of faunistic and floristic populations of polluted beaches and (2) mass mortality and other deleterious effects of water accommodated fractions of oil on pelagic fauna. Laboratory studies succeeded the above type of work and have resulted in the accumulation of data on lethal toxicity, sublethal toxicity, effect of growth on microalgae, effects on activity, feeding and metabolism and ultimately evidences on disruptions in cellular structure and activities. To what an extent the information gathered from laboratory studies becomes applicable in evolving effective strategies to improve environmental quality remains to be seen.

The present investigation was planned and carried out keeping the above objectives in mind and hence contained two separate experimental protocols viz., field studies and laboratory studies. Attempts have been made to couple these two by a set of experiments to analyse the role played by animals' feeding strategy on contaminated sediments and resultant variations at organic and organismic level. It is fervently hoped that the present study will throw some light on the issues in question.

GENERAL INTRODUCTION

The fate of oil in the marine environment has been studied extensively in both qualitative and quantitative details over the past twenty years. Ecological impacts of oil are also now better understood, biological effects have been measured and some toxicological patterns have been identified. Concern for chronic sublethal effects caused by spills in low energy shallow coastal waters and shorelines is increasing. Methodology for the analysis for hydrocarbons in seawater, sediment and biological tissues have been perfected. It is now understood that reproductive, developmental and behavioural processes are very sensitive to exposure to hydrocarbons. Generally, young life stages are more sensitive than adults. Many juvenile crustaceans and molluscs are more sensitive than juvenile fish. It is well established that different oil types vary in their toxicities and acute toxicity is largely due to components of water accommodated fractions and dependent upon exact conditions and duration of exposure to them.

Effects of oil can be in two basic ways: (1) by directly affecting the exposed organisms' overall health and survival (2) by contaminating natural resources which ultimately humans may consume. Studies conducted in the laboratories mainly centre around employing a variety of

marine organisms to find out toxic effects. Exposure to complex mixtures of oil often results in reactions difficult to explain since the water accommodated fractions of oil will contain aliphatics and aromatics along with non hydrocarbons. However, toxic effects of oil with respect to a particular component has relatively limited utility and significance and hence normally laboratory studies are carried out using water-oil mixtures prepared employing approved methods.

Presence of petroleum hydrocarbons (PHC) in marine and estuarine waters can be ascertained only by monitoring the coastal waters and estuaries. Large quantities of oil get settled at the bottom of the coastal waters and estuaries by various physical and biological processes. The quantity of PHC available in the sediments of oil polluted areas are always much higher than those found in the upper lying waters. Since the oil on sediments is mainly bound by adsorptive processes than by way of absorptive function, these fractions are rendered easily accessible both physically and biologically. Chronic pollution of estuaries due to berthing of vessels, tanker operations etc. have been reported in the literature. The water accommodated oil fractions from the oil slick get transported to the sediments by sorption on suspended solids. This can result in both horizontal and vertical diffusion of oil in the estuarine and coastal marine waters. Constant sinking of oil contaminated particles in estuarine and coastal

marine waters ensures a constant supply to these fractions. Absence of physical processes which result in vertical mixing of water could make sheltered areas of the coast and estuaries get highly polluted by oil. Further, normally tanker operations and berthing operations are constant phenomena in sheltered seas and coastal regions. Processes such as dissolution, advection, evaporation and sorption on suspended solids ensure constant presence of oil in sheltered bays and estuaries.

Studies on the toxicity of oil should therefore contain broader aspects of quantitative structure - activity relationships, analyses of critical factors controlling persistence, exposure, uptake, bioaccumulation and toxicity and metabolism of hydrocarbons. Further, measurement of a wide range of toxic responses by conducting experiments employing oil at sublethal levels, experiments with oil contaminated sediments on life and activity etc. in the laboratory will give a correct picture on the bioavailability and toxicity of sediment bound hydrocarbons.

Regarding the relationship between PHC contamination and the well being of marine animals, it has been proved beyond doubt that in sufficient concentrations, this can cause pathologies in several organ systems and it has been opined that prolonged exposure could lead to even severe tissue

damage. Majority of these pathological changes could be duplicated by exposure to other toxicants also. However, certain types of neoplasias occurring in bivalves in oil contaminated areas have been proved inconclusive. Coexistence of different types of stressors in the coastal environment, clear association of pathologies in fish and shellfish with petroleum contamination is often not possible. It is also known that on chronic exposure to oil, principal enzymatic degradative pathways are induced. Therefore only laboratory exposure of marine animals selected from "clean" sites could help to follow the history of exposure. This is mainly because of the fact that one substrate may influence the favoured metabolic route of another in the principal enzymatic degradative pathways.

From a review of information available on the effects of oil on marine organisms, it has become amply clear that no concerted effort has been made in tropics to delineate fine structural damages or modifications of cardinal organs like gills, digestive tubules etc. of bivalves on chronic exposure to oil.

With the above broad aspects in mind, the present work was planned and carried out. The protocols included experiments to study sublethal effects of oil on certain physiological parameters, behavioural aspects and structural morphology of tissues and cells.

CHAPTER 1

STATUS OF OIL CONTAMINATION IN THE COCHIN BACKWATERS

1.1 INTRODUCTION

Monitoring for the presence and distribution of oil in the natural environment is an integral part of oil pollution research. In many cases, natural disasters accompanying major oil tanker mishaps have amply proved the significance of monitoring strategies to adopt countermeasures. As a matter of fact, consciousness of oil pollution among scientists and policy makers has come out only as a post mortem reaction of episodal oil pollution. Monumental examples are Torrey Canyon (1967), Amoco Cadiz (1978), Exxon Valdez (1989), Braer (1993) etc. The literature that accumulated after the first two major mishaps has provided considerable information on the effects of oil pollution on the standing crop of coastal waters. It was also provided that regaining normalcy was a time consuming process and at many instances the damages caused to intertidal communities were irreparable.

Evidences gathered on the effects of crude received by natural aquatic systems on account of major tanker disasters show that the effects are mainly physical and seldom chemical. The reasons for the latter are mainly because of lack of clearcut documented evidence on the direct effects of crude or its derivatives on the life and activity of marine plants and animals. In this context, gathering information on

the distribution and abundance of those chemical fractions of oil present in natural waters subject to chronic discharge of insignificant quantities of refined oil components become meaningful with reference to monitoring strategies. It is necessary that the presence of oil fractions in estuaries and coastal waters is detected by a close watch of these components by regular sampling as is advocated in the case of other major toxicants such as heavy metals, organic wastes, radioactive nuclides etc. In this context, it was felt that any investigation on oil pollution of the Cochin backwaters should have a monitoring component. Accordingly, the petroleum hydrocarbons in water and sediments from selected stations were monitored for a period of 2 years. The information gathered and presented in this section is the only documental evidence available on the distribution of oil based on the internationally accepted chrysene equivalents.

1.2 REVIEW OF LITERATURE

Oil pollution as it is popularly understood, arises from the exploration, extraction, stabilization, transport, storage and refining of crude oil and thereafter, in the subsequent manufacture and handling of products. (Johnston, 1984). It has been generally agreed that there has been no evidence of irrevocable damage to marine resources on a broad oceanic scale (NRC, 1985) from either major spills or other

chronic sources of petroleum hydrocarbons (PHC). The best current estimate is that 2.35 million tonnes of PHC/yr, enter the marine environment from various sources (GESAMP, 1993).

The deleterious effects of oil pollution, to a large extent, rests on the fact that substantial quantities of hydrocarbons are introduced in limited areas over short periods of time. Among the many sources, wrecks of oil-tankers and blow-outs of off-shore wells often receive special attention in this context. Although such catastrophic inputs have received a great deal of study, they make relatively small contribution to the total amount of hydrocarbons entering the ocean each year (Clarke and MacLeod, 1974). The more continuous inputs, such as those associated with normal transportation, urban run-off and discharges and off-shore oil production, make the most significant contribution to the total PHC burden entering the ocean (GESAMP, 1993). Because they occur close to the shore and often in a confined area, such as ports, their environmental damage to the immediate vicinity can be considerable. A study conducted by the U.S. National Academy of Science in 1990 concluded that the estimate of oil entering the sea from marine transportation activities has been reduced from 1.47 million tonnes in 1981 to 0.57 million tonnes in 1989 (IMO, 1990).

Crude petroleum and its refined products are extremely complex mixtures of many thousands of organic

compounds. All hydrocarbons are, to a certain degree, soluble in water. The aqueous solubility depends on the chemical nature and molecular weight of the components. Therefore, the fraction of an oil which, upon release on the surface of a water body, dissolves in it has a composition different from that of the original oil, with an enrichment of the more water soluble low molecular weight aliphatic and aromatic components (Shiu *et al.*, 1990). Studies have suggested that the toxicity of an oil is due primarily to the water soluble fraction (Rice *et al.*, 1977). The light aromatics (benzene $\longrightarrow$ naphthalene) are considered to be one of the most immediately toxic components of petroleum other than the carcinogenic polycyclic aromatic hydrocarbons (PAH). Behaviour and distribution of PAH in the environment and therefore, the effects on biological systems vary due to differences in molecular weight. The low molecular weight PAH i.e., 2-3 ring aromatics (naphthalenes, fluorenes, phenanthrenes and anthracenes) have significant acute toxicity to aquatic organisms, whereas the high molecular weight PAH i.e., 4-7 ring aromatics (chrysene-coronene) do not (Neff, 1979).

Physical, chemical and biological fates of hydrocarbons from spilled oils are better understood in qualitative and quantitative terms now, when compared to twenty years ago. On the occurrence of a spill, volatile components evaporate, at rates depending upon temperature,

vapour pressure and mass transport conditions, leaving behind a residue which may become dense enough to sink. (Payne and Phillips, 1985). It is rather difficult in many cases to ascertain the actual concentration of PHC in the aqueous phase. Studies of the processes and effects after the grounding of the tanker Amoco Cadiz in Brittany by Marchand (1981) has shown that *ca.* 30% of the spiled oil evaporated, roughly 14% dissolved in water, 8% was incorporated into sediments and 28% was washed up on the shore. Recent research on the polar fractions of dissolved oil residues has shown the presence of large numbers of photo-oxygenated derivatives of aromatic hydrocarbons (Ehrhardt, 1987 (b) & Ehrhardt *et al.*, 1990), but their toxicity to marine organisms is largely unknown.

In open bodies of water, currents and diffusion can decrease concentrations of contaminants rapidly. Except for wild life which frequents both coastal and open seas, biological concerns largely focus on shallow, near shore areas and coastlines. There is some evidence at spill sites of long-term effects on populations and communities. Habitats like low-energy marshes and mangroves require decades to return to their pre-spill condition of population numbers, species diversity and habitat quality (Thorhaug, 1991), while others recover relatively quickly, i.e., within months to one or two years (Baker, *et al.*, 1991 (a), (b)). The effects of both

chronic discharges and accidental releases of PHC have been studied with a wide variety of organisms and ecosystems and biological processes.

Persian gulf is probably the most oiled area in the world, especially in the wake of Iran-Iraq war and the Gulf war in 1991, releasing about 6-8 million barrels of oil from Kuwait oil fields into the Northern gulf (Sci.Amer., Oct. 1991). Studies have estimated an oil budget of 30,000 tonnes for the western gulf coastal waters (EI Samra, 1989); generally, the levels of hydrocarbons in the water column were not exceptionally high compared with other areas of the world, being 4.4 - 63 $\mu g\ l^{-1}$ (Emara, 1990)

Gupta *et al.*, (1989) have described the presence of surface oil, tar accumulations and distribution along Indian beaches. Oil slicks are very common in the Arabian sea and northern Indian ocean, mostly from shipping. Hinrichsen (1990) estimated that *ca.* 5 million tonnes of oil enters the Arabian sea each year, while the Bay of Bengal gets 400,000 tonnes. Surface waters of many harbours have such high concentrations (*ca.* 100 $\mu g\ l^{-1}$) of hydrocarbon in the water. Within the EEZ along the west coast of India, dissolved PHC values appear to be rather consistent i.e., 1-26 $\mu g\ l^{-1}$ in the depth range of 0-20 m (Anon., 1990). Data collected over the northern Indian ocean for the last one decade indicate an

apparent reduction in oil pollution due to reduction in oil transport in this area.

The Vembanad lake which is the largest (235 sq.km) among the backwaters of Kerala, runs almost parallel to the coast extending from Alleppey in the south to Munambam in the north between lat. $9^{o}28'$ and $10^{o}10'N$ and longitudes $76^{o}13'$ and $76^{o}31'E$. In this lake there are a number of small islands like the Willingdon Island, Pathiramanal, Vallarpadam etc. The Cochin backwaters which form the northward extension of the Vembanad lake have characteristics of a typical tropical estuary (Ramamritham and Jayaraman, 1963). The Cochin harbour entrance ($9^{o}58'N$ and $76^{o}15'E$) and another inlet near Azhikode connect the estuary with the Arabian sea. A third opening near Thuravur is seasonal and remains closed except during monsoon season. The Cochin harbour has a dredged approach channel of about 10.5 km long and Ernakulam channel (5 km) and Mattancherry channel (4 km) on either side of the Willingdon island which are the entrance and berthing places of normal and super tankers. The shipping channels, tanker berths and the small islands in this estuarine region have significant influence on the circulation in the estuary.

In recent years, considerable changes have been brought about in the water quality of this lake due to urbanization, industrialisation and harbour development

activities. Tanker and other fishing vessel operations in the Cochin estuary add large quantities of PHC into the water and sediment. The oil terminal located in the estuary has no reception facility for oily ballast from ships. This coupled with the poor operational practices of the harbour authorities of Cochin result in frequent spillages of oil and accidents in the estuary. Spilling of naphtha and flushing bilge washings from ships into the channel caused fire twice resulting in loss of property and human lives (Sen Gupta, 1991, 1992). These urge the need for monitoring the estuary for oil pollution.

Study conducted by Badawy and Al-Harthy (1991) to establish baseline levels of PHC in the Omani coastal waters reveal that current levels of PHC at these sites are not exceptionally high (8.69-14.44 $\mu g\ l^{-1}$) and are within the range as reported in other areas of the world oceans. River inputs and the non-treated urban effluent sewage were considered the two major sources of PHC pollution in the western Mediterranean coastal waters (Marchand *et al.*, 1988). Durrani and Siddiqui (1990) are of the opinion that even though considerable quantities of PHC are discharged into the coastal waters of Karachi by way of exploitation, refining etc the water characteristics like oxygenation, light-temperature availability, strong tidal currents etc discouraged the persistence of oil in water thereby reducing the oil

pollution. Probably this was the reason why large scale discharge of untreated crude to this area during the Gulf war did not result in any drastic ecological catastrophe (Anon.). Estimation of PHC in seawater as a part of environmental monitoring has been adopted by many workers (Boylan and Tripp, 1971; Keizer and Gordon, 1973; Burns and Villeneuve, 1982; IOC, 1984; Klungsoyr *et al.*, 1988; Cripps, 1992).

Analyses of sediment samples can provide baseline values before activities leading to the discharge of pollutants starts, results of an oil spill, chronic seepage or pollution, atmosphere fallout over time and the fate of HCs associated with sinking particles (IOC, 1982). In a monitoring programme of the Southern Baltic sea, the highest HC concentration were found in fine sediments which occurred either inshore or in deep offshore basins (Law and Andreulewicz, 1983). The ubiquity of PAH in marine sediments has been well established (Laflamme and Hites, 1978; Martel *et al.*, 1986). Rowland and Robson (1990) reviewed the occurrence of acyclic isoprenoid HCs (C_{20}, C_{25} and C_{30}) in sediments. However, the compounds were found to disappear rapidly in older sediments possibly due to biodegradation and reaction with sedimentary sulphur. The presence of PAH in marine sediments corresponds either to high temperature fossil combustions or oil activities like tanker operations, accidental spills, direct discharges etc. Once deposited in

sediments, PAH are less subjected to photo-oxidation and biodegradation and tend to accumulate to high concentrations. Thus Atwood *et al.*, (1987), Botello *et al.*, (1991), Ram and Kadam (1991), Pendoley (1992) have used sediment PAH assemblages as indices of the rate of imput of PAH to aquatic environments. In spite of the fact that Bombay coast is contaminated by PHC originated from various sources, a recent study by Ingole *et al.*, (1995) on the PHC content in intertidal sediment and water samples along the Bombay coast revealed that the concentrations in sediment were negligible and uniform. The authors had attributed the reason to the sediment characteristics and strong tidal action. With respect to PHC concentration in seawater, only Worli area appeared to be contaminated (4.9 - 153.8 ug l^{-1}) due to inputs through water.

The bioaccumulation of contaminants by *Platichthys flesus*, *Carcinus maenas*, *Mytilus edulis*, *Littorina littorea* and *Nucula tenuis* was investigated by Bakke *et al.* (1988) for the GEEP workshop by transplanting the organisms to four indoor mesocosms with different concentrations of copper and WAF of diesel oil in seawater. The common mussel is considered one of the best biological markers in defining water pollution. To evaluate seawater quality along the Italian coast, Amodio Cocchieri *et al.* (1993) estimated the levels of metals and PAH in marine organisms. The majority of animals

analysed showed low level of contamination by metals and PAH, values not different from their open sea counterparts, whereas only in *Mytilus galloprovincialis*, the tissue load of PAH and cadmium indicated moderate pollution.

There is considerable controversy about which parameters to measure in assessing petroleum contamination and which methods to apply to the task. Ultra violet fluorescence spectroscopy (UVF) has been widely recommended as an analytical tool for the determination of total hydrocarbons in seawater due to its rapidity, reproducibility, ease of use and its high sensitivity to aromatic hydrocarbons (Law *et al.*, 1987; Ehrhardt and Petrick, 1989). It was shown in a number of intercalibration exercises (IOC/UNEP Workshop, Bermuda, 1984; IOC/UNEP River Input Workshop, Bangkok, 1986) to yield comparable data with a precision of ±30% (Law *et al.*, 1988 ; Ehrhardt *et al.*, 1991). However, UVF can, at best only provide information concerning the classes of compounds present whereas chromatographic methods can provide the concentration of individual compounds, especially when coupled with mass spectrometry (Mason, 1987). The results of an inter-laboratory comparison of tissue load of oil in mussels show that significant correlations exist between the aliphatic and aromatic values analysed by GC and UVF (Broman and Ganning, 1985).

It was concluded that for fluorescence analysis, measurement should be made at an optimum excitation and emission wavelength with quantification against a synthetic standard which has an overall fluorescence spectrum similar to that of the samples to be analysed (Farrington *et al.*, 1982; Friocourt *et al.*, 1985). Hence chrysene was selected as the standard for the present study. The quantitative measurement is made by measuring emission intensity at 360 nm with excitation set at 310 nm. These wavelengths have been determined to be acceptable for measuring the fluorescence of several crude and residual fuel oils (IOC, 1984; Law *et al.*, 1987; Farrington *et al.*, 1988; Ram and Kadam, 1991, Kadam and Bhangale, 1993).

Picer and Picer (1993) suggested three modifications of simple spectrofluorometry in estimating PHC levels in seawater. (i) by alumina cleaning (ii) by addition of standards and (iii) by diluting to minimize quenching. But statistically significant differences were noticed only in higher concentration ranges where dilution and standard addition modifications yielded higher results.

1.3 AREA OF STUDY

The present study was confined to the Cochin harbour area of Vembanad estuary located at $10^{o}56'N$-$10^{o}59'N$ lat and

$76^{o}14'E$ - $76^{o}18'E$ long. The area of study encompassed the two channels on either side of the Willingdon Island, the Mattancherry channel and Ernakulam Channel which join with the approach channels and make connection to the sea. The channels are maintained at a depth of 10-13m for the passage of ships by frequent dredging. The depth of this estuary otherwise ranged from 2-8m.

The area of investigation and the station locations are indicated in fig.1. The stations were fixed so as to represent fairly the polluted areas in the estuary as follows.

1. **Station 1** - Marine Science Jetty - adjacent to the main land. Depth 2m.

2. **Station 2** - Oil terminal - located in the Ernakulam channel near the southern oil tanker jetty. Depth 11m.

3. **Station 3** - Backwater buoy No.4 - located in the Ernakulam Channel off Dufferin point in the Willingdon Island. Depth 11m.

4. **Station 4** - Mattancherry channel - is located in the Mattancherry channel south of the Willingdon Island. Depth 12m.

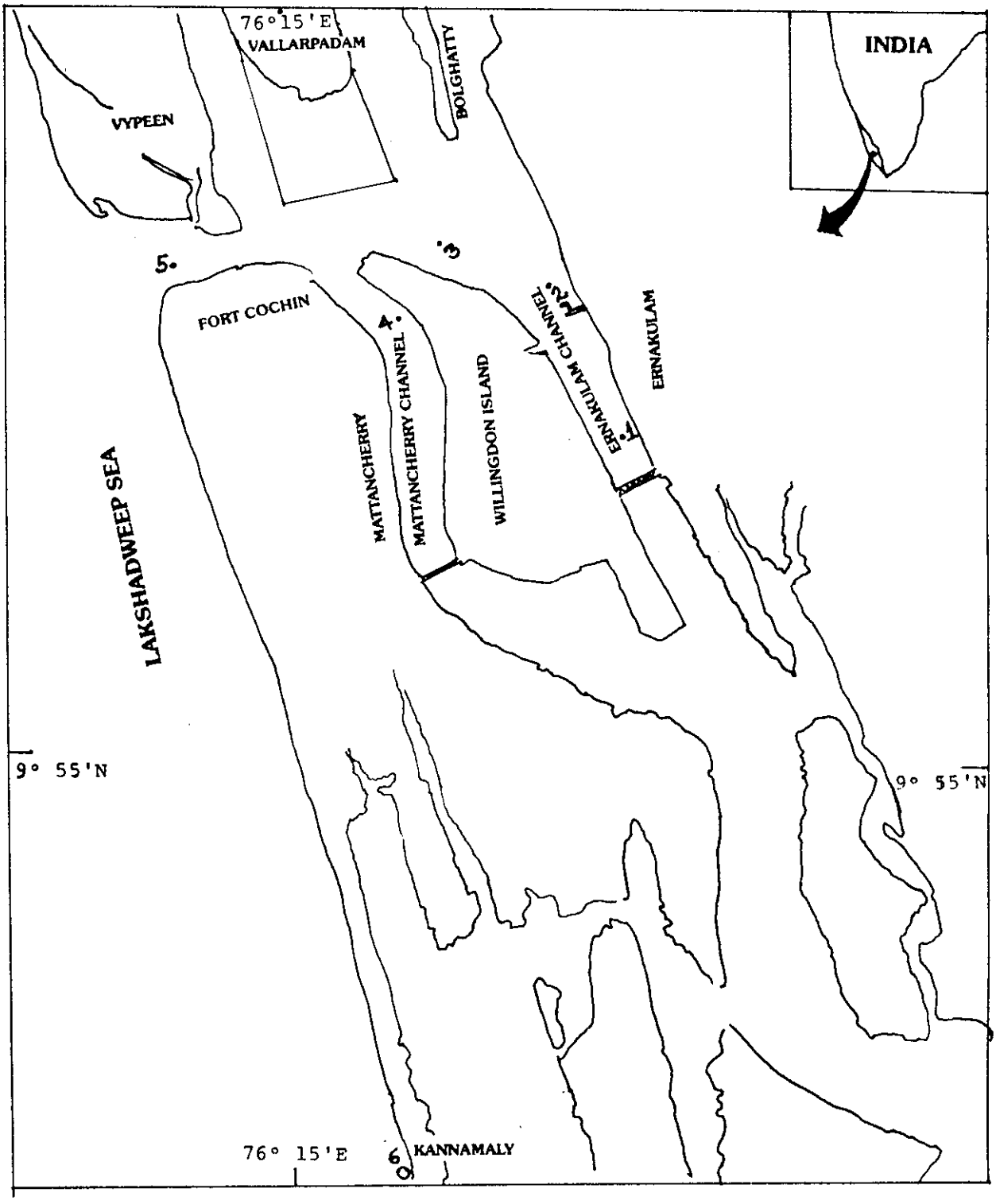


Fig. I

Map of Cochin estuary showing location of stations

5. **Station 5** - Bar mouth - located in the approach channel near the estuarine mouth. Depth 12-13m.

6. **Station 6** - Kannamali (reference station) - An open sea site located at $9^{o}53'N$ and $76^{o}16'E$. Depth 4m.

1.4 MATERIALS AND METHODS

The PHC content of the waters of the stations 1 to 5 and that of station 6 (as stated above) was monitored for a period of 21 months.

1.4.1 COLLECTION OF WATER SAMPLES AND SEDIMENT

Samples of surface water, subsurface water and sediment were collected once in every fortnight, for a period of 21 months from April '92 to December '93. Sampling of surface water was done using a pre-cleaned stainless steel bucket. Subsurface samples from a depth of about 3m were collected using a sub-surface water sampler.

Sediment samples were collected by a van Veen grab. Triplicate sub-samples of approximately 100g were taken carefully from the surface layer of the sediment collected. Macrobenthos from the samples were removed before the sub-

samples were wrapped in Aluminium foil and stored in deep freezer at a constant temperature of -20^{o}C.

1.4.2 ANALYSIS OF SAMPLES

1.4.2.1 Seawater

500 ml of seawater was taken in a separatory funnel and the oil extracted with 25ml. n-Hexane (HPLC grade). The procedure was repeated to effect maximum extraction. The pooled concentrate was chemically dried using anhydrous sodium sulphate and made up to 50ml by addition of Hexane (IOC, 1984). The concentration of PHC was determined against a standard of chrysene, using fluorescence Spectrophotometer (Hitachi-Model F-3010) at wavelengths 310nm (EX) and 360nm (EM).

1.4.2.2 Sediment

Wet sediment samples of 50g each were digested for 2 hours with 50ml of 0.5N Methanolic KOH (HPLC grade). The resultant organic phase was extracted twice with 25ml n-Hexane (HPLC grade). The combined extracts were dehydrated using anhydrous sodium sulphate and subjected to a clean-up procedure using activated Alumina Columns (2g, 10cm). The hydrocarbons were diluted with n-Hexane and made-up to a final volume of 25ml each (IOC, 1982). The concentration of PHC is

expressed in chrysene equivalents using the fluorescene spectrophotometer (EX-310nm, EM-360nm).

1.4.2.3 Preparation of Chrysene Standard

A stock solution of chrysene was prepared by dissolving 1.0mg chrysene in 100ml n-Hexane (HPLC grade). This was allowed to stand overnight before dilution to ensure complete dissolution (IOC, 1984). A range of standard solutions of chrysene were prepared from the standard stock. The fluorescent intensity of standards relative to n-Hexane were read using the fluorescence spectrophotometer. The concentrations of PHC in the various samples were calculated employing the calibration graph prepared following the above method.

1.5 RESULTS AND DISCUSSION

The information available from literature on the distribution pattern of PHC in coastal and oceanic waters has indicated that a close monitoring of the distribution of this component in tropical estuaries is an important pre-requisite for any study of the toxic or pollutional effects of PHC. As a follow-up of this concept, five different sites distributed within the Cochin estuary and a relatively clean location in the open sea were selected to monitor the PHC concentration of

surface, subsurface waters and sediments. The most conspicuous information gathered based on this study, from the spatial and temporal point of view was the drastic variations in the load of PHC (Tables I, III and V and fig.II, III and IV). The following account gives a general picture of the distribution pattern of PHC.

Station 1 located in the Ernakulam Channel, near to the Marine Science Jetty, recorded all through highest concentration of PHC. This region which is relatively shallow is situated at a point flanked on both sides by land protrusions and tanker berths. Evidently, this resulted in a baying effect. Normally during the monsoon season, large quantities of floating aquatic weeds, mainly, *Eicchornia*, tend to get trapped at this station indicating strong eddying effect. Robinson (1983) has opined that during flood tide effect of bottom friction acts more strongly which in turn exerts a torque at certain shallow areas located at the lateral sides of the estuary. Similar effects were reported by Joseph and Kurup (1990) also. Statistical analysis by two way ANOVA also proves that among the different stations studied, maximum values for PHC were obtained at this station with a significant difference at 0.1% level in the PHC content of surface waters, subsurface waters and sediment (Tables II, IV and VI). The quantitative relationship in the distribution of PHC between the sediment and subsurface waters is clearly indicated by the sediment load and subsurface load recorded in

TABLE I :

Concentration of PHC ($\mu g\ l^{-1}$ - chrysene equivalents) in surface waters of six different stations located in the Cochin estuary.

Period		Stations and locations					
		Marine Sciences Jetty	Oil Terminal	Backwater Buoy No.4	Mattancherry channel	Barmouth	Kannamali (Reference station)
1992	APR	762.56	82.86	168.13	460.25	166.37	21.98
	MAY	587.80	118.05	310.24	293.66	405.35	20.23
	JUN	704.67	71.02	215.59	237.01	163.74	19.21
	JUL	798.35	54.70	158.92	142.18	418.86	16.04
	AUG	460.17	54.62	108.92	134.27	189.01	22.34
	SEPT	529.56	112.27	157.17	246.48	156.73	22.60
	OCT	586.05	142.26	249.45	324.65	159.96	24.75
	NOV	769.33	282.46	218.22	522.38	295.44	22.52
	DEC	797.83	137.70	222.17	419.82	322.62	22.88
1993	JAN	623.25	107.95	410.43	451.24	270.77	23.04
	FEB	816.24	N.D	198.59	286.13	195.42	21.68
	MAR	773.70	82.92	204.19	357.03	208.84	21.60
	APR	702.12	72.26	129.89	167.53	137.70	21.38
	MAY	381.40	189.03	224.90	629.05	238.67	22.16
	JUN	354.72	98.58	129.71	163.22	147.62	20.23
	JUL	332.70	173.39	137.68	208.14	121.12	20.05
	AUG	421.93	153.14	179.72	283.91	220.95	21.64
	SEPT	517.64	147.26	193.85	410.71	232.09	22.52
	OCT	638.34	167.71	129.71	343.94	208.84	22.42
	NOV	663.86	147.26	218.22	296.57	194.37	22.08
	DEC	717.11	157.17	226.99	338.94	281.05	22.68

N.D. - Not determined

TABLE II

Two way ANOVA showing relationships between stations and seasons with respect to PHC content in surface waters.

Source	ss	df	ms	F
Total	4581503	120	78149.50	7.97**
Seasons	156299	2	663354.20	67.63***
Stations	3316771	5	9809.14	
Error	1108433	113		

** $p \leq 0.01$

*** $P \leq 0.001$

Season's average		
Pre-monsoon	Monsoon	Post monsoon
114.35	128.38	160.65

Station's average	
Station 1	573.76
Station 2	127.6
Station 3	199.7
Station 4	320.0
Station 5	225.52
Station 6	21.71

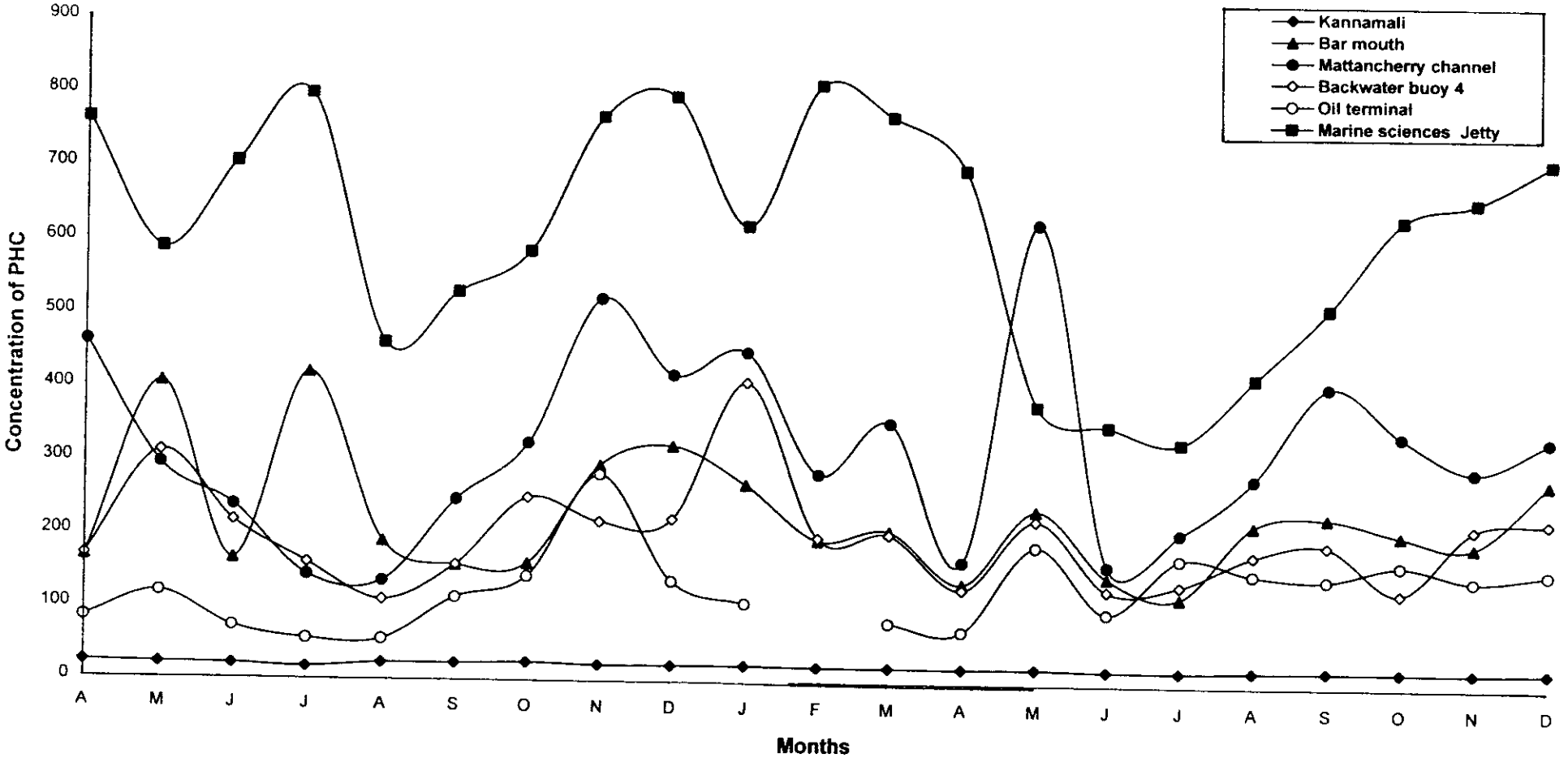


Fig. II

Concentration of PHC (ug l^{-1} - chrysene equivalents) in surface waters of six different stations located in the Cochin estuary.

TABLE III :

Concentration of PHC ($\mu g\ l^{-1}$ - chrysene equivalents) in subsurface waters of six different stations located in the Cochin estuary.

Period		Stations and locations					
		Marine Sciences Jetty	Oil Terminal	Backwater Buoy No.4	Mattancherry channel	Barmouth	Kannamali (Reference station)
1992	APR	571.66	111.81	305.86	283.92	398.32	22.08
	MAY	464.65	82.34	187.43	444.91	152.34	21.82
	JUN	696.14	65.40	203.75	218.32	157.96	21.46
	JUL	788.25	50.22	145.93	129.01	411.65	21.19
	AUG	448.50	46.19	104.96	124.21	157.52	21.64
	SEPT	522.00	105.91	150.07	237.09	144.81	20.67
	OCT	575.81	137.46	232.37	314.01	147.26	20.83
	NOV	761.56	274.55	208.92	507.88	281.13	20.93
	DEC	790.98	123.65	208.56	384.81	301.38	22.60
1993	JAN	610.08	91.81	373.59	415.69	239.54	22.86
	FEB	699.65	430.02	185.32	245.80	174.80	21.64
	MAR	130.56	111.89	177.43	319.27	173.31	21.28
	APR	678.69	60.24	105.87	148.30	118.83	21.38
	MAY	365.86	165.14	197.42	619.92	227.95	22.24
	JUN	340.95	81.64	114.62	142.70	115.32	20.23
	JUL	134.45	91.03	104.96	127.43	122.17	19.97
	AUG	398.58	135.41	142.42	259.55	184.80	20.31
	SEPT	461.65	87.34	96.37	372.63	197.86	20.83
	OCT	618.95	70.84	123.65	284.88	177.39	21.28
	NOV	636.60	128.65	195.60	268.74	177.17	22.42
	DEC	709.24	137.42	187.87	304.90	284.80	22.86

TABLE IV

Two way ANOVA showing relationships between stations and seasons with respect to PHC content in sub surface waters.

Source	ss	df	ms	F
Total	4869866	125		
Seasons	182166	2	91083.00	8.11***
Stations	3362121	5	672424.20	59.86***
Error	1325579	118	11233.72	

***$P \leq 0.001$

Season's average		
Pre-monsoon	Monsoon	Post monsoon
235.25	183.02	272.50

Station's average	
Station 1	543.19
Station 2	123.24
Station 3	178.71
Station 4	293.10
Station 5	206.95
Station 6	21.43

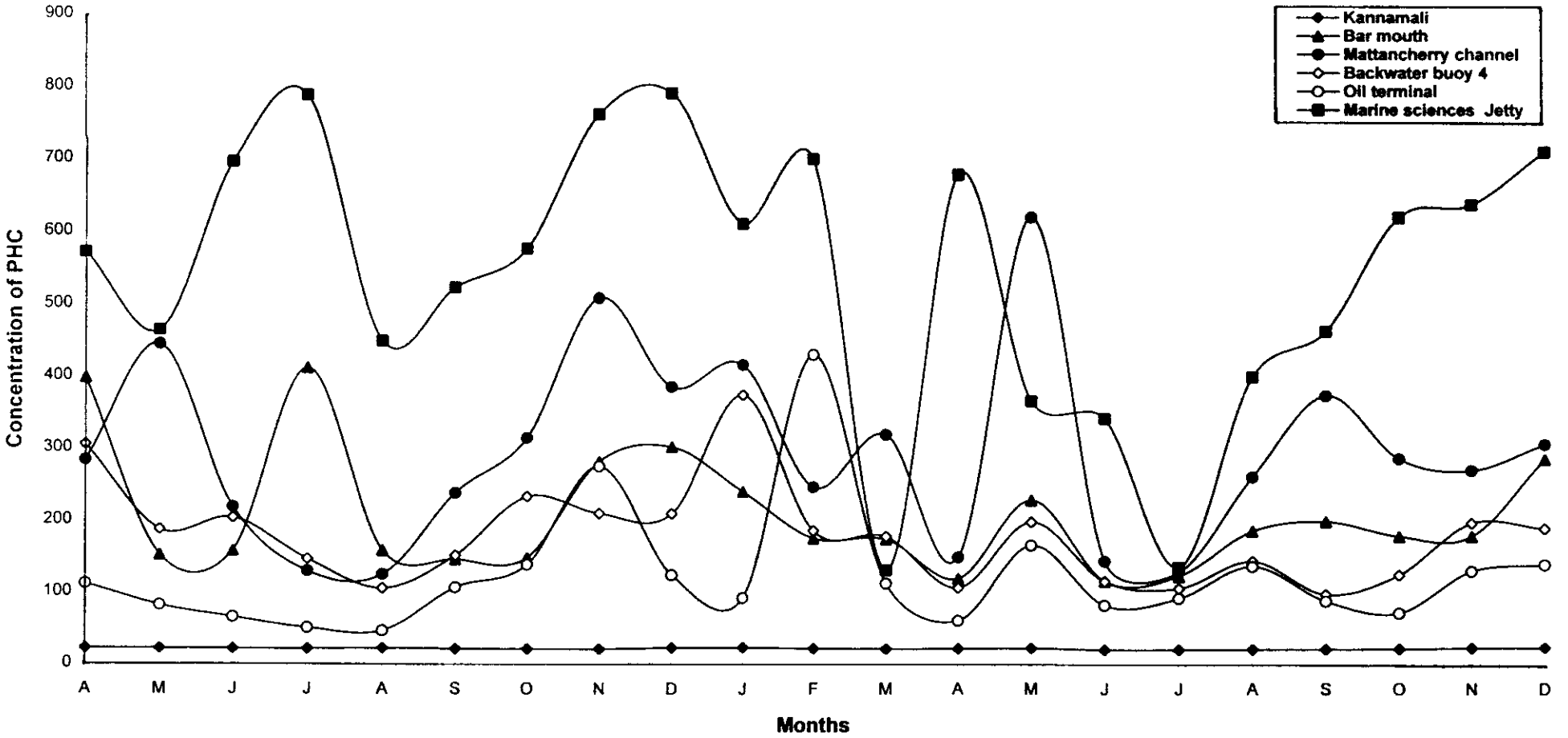


Fig. III

Concentration of PHC (ug l^{-1} - chrysene equivalents) in subsurface waters of six different stations located in the Cochin estuary.

TABLE V :

Concentration of PHC ($\mu g\ g^{-1}$ - wet wt - chrysene equivalents) in sediments of six different stations located in the Cochin estuary.

Period		Stations and locations					
		Marine Sciences Jetty	Oil Terminal	Backwater Buoy No.4	Mattancherry channel	Barmouth	Kannamali (Reference station)
1992	APR	184.09	65.50	46.91	166.75	84.63	24.01
	MAY	192.50	65.58	61.46	130.42	72.42	24.45
	JUN	206.84	73.41	63.49	133.05	63.13	23.38
	JUL	126.14	55.06	37.25	105.87	46.19	23.04
	AUG	145.41	37.70	46.73	69.17	52.35	24.01
	SEPT	167.77	41.45	85.93	98.14	42.43	22.68
	OCT	142.34	85.65	105.87	122.87	81.66	24.53
	NOV	197.44	78.71	55.40	105.87	69.87	22.96
	DEC	222.27	89.89	46.39	125.78	96.63	23.82
1993	JAN	237.46	96.29	56.12	98.56	81.22	24.89
	FEB	125.08	48.21	32.52	94.36	51.04	24.69
	MAR	145.85	55.86	37.43	96.55	54.20	24.27
	APR	237.19	86.11	47.17	114.38	63.93	23.20
	MAY	245.06	68.57	43.92	127.26	74.27	22.78
	JUN	185.60	64.98	39.78	106.13	53.13	21.64
	JUL	143.40	48.13	34.52	83.12	42.17	20.83
	AUG	139.19	47.43	40.69	118.23	67.50	23.48
	SEPT	123.41	62.25	37.86	105.87	47.69	24.61
	OCT	152.36	63.59	38.66	97.33	47.49	24.97
	NOV	162.27	71.04	53.47	118.23	79.61	25.23
	DEC	162.61	74.27	54.72	129.73	79.97	25.75

TABLE VI

Two way ANOVA showing relationships between stations and seasons with respect to PHC content in sediments

Source	ss	df	ms	F
Total	357143	125		
Seasons	7163	2	3581.5	8.17**
Stations	298220	5	59644.0	135.97***
Error	51760	118	438.64	

** $P < 0.01$

*** $P < 0.001$

Season's average		
Pre-monsoon	Monsoon	Post monsoon
85.06	72.29	89.43

Station's average	
Station 1	173.52
Station 2	65.71
Station 3	50.76
Station 4	111.76
Station 5	64.33
Station 6	23.81

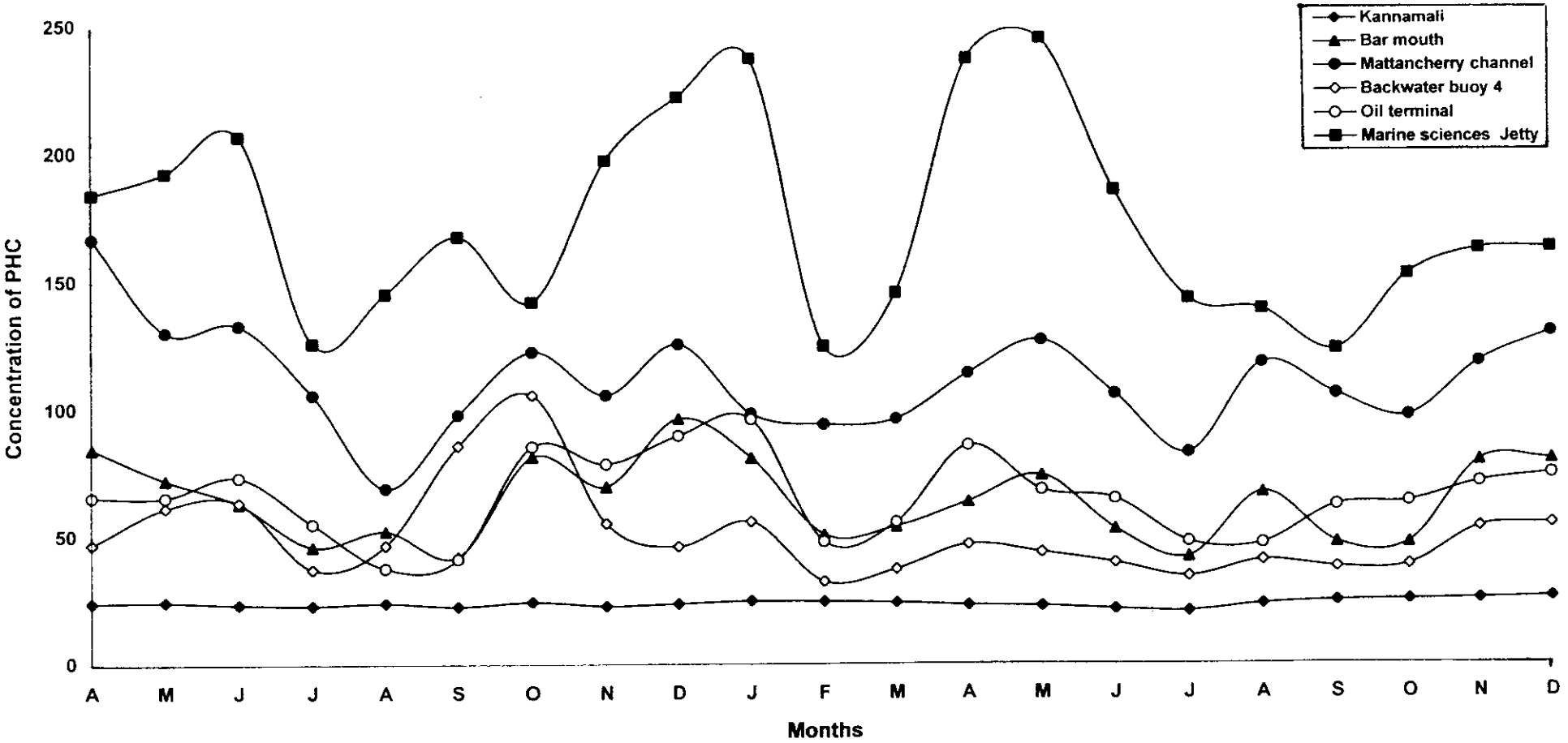


Fig. IV

Concentration of PHC (ug g^{-1} wet wt. - chrysene equivalents) in sediments of six different stations located in the Cochin estuary.

the present instance. It has to be assumed that the source of oil to the bottom sediment of the present sampling localities was through the overlying water. Lee (1980) has recognised the bottom sediments as the ultimate sink for undegraded oil. Since episodal pollution resulted from crude oil-carrying tanker disasters has been a rarity in this part of the Indian Seas, it is assumed that the oil that has entered the waters of Cochin estuary is the water phase of dissolved oil or that reaching by related processes. It is known that higher weight aromatics and aliphatics in water adsorb on to suspended particles because of their low water solubility and lower weight hydrocarbons and more polar oil components remain dissolved in the water (Lee, 1980). Cochin estuary has a very high quantity of suspended solids contributed by disintegrating plant material derived from floating weeds, materials of terrigenous origin brought by rain water that drain into the backwater through the major rivers and tributaries. These particles carry hydrocarbons to the bottom. Studies on the sedimentary characteristics of the Cochin estuary have shown that the waters contain very high quantities of humic acids derived from vegetation and detritus which remain in suspension in the water for a considerable period. A scanning electron microscopic study of detrital particles has revealed that rough surface of detrital particles with bacteria fastened by mucoid like pads and fibrillar appendages form excellent matrix for the adsorption

of hydrocarbons. Further, clays and fine suspended sediments in seawater are excellent surfaces for adsorption of dispersed oil globules. Estuaries and coastal waters harbour suspended solids in varying quantities. The coastal waters and estuaries can have considerable load of suspended solids. The wave breaking zone essentially will have high concentrations. Similarly, the sediment load of the estuaries show temporal and spatial variations. Downward transit of hydrocarbon from water to the bottom can be very rapid in such estuaries where rate of sedimentation is high. Cochin backwater is known for its high rate of sedimentation. Periodical dredging of the shipping channels of this estuary is necessitated by the heavy rate of sedimentation. The turbidity of this area ranged from 40-100 mg l^{-1} (Rasheed & Balchand, 1995).

Natural waters contain substances such as humic acids, fulvic acids and other degradative products of biological materials. Cochin backwater is known to contain considerable quantities of humic acids, lipids, proteins and different plant pigments. The studies conducted have also shown that the composition of these components in the sediments show seasonal trends and that the post monsoon and pre-monsoon periods support high quantities of these fractions. (Nayar, 1992). The high PHC content of the sediments during this period substantiates the relationship between organic matter content of the sediment and its ability

to harbour PHC. The removal of dissolved organic matter from seawater was found to result in a 50-99% decrease in the amounts of certain components of PHC. Because of the low aqueous solution and hydrophobic character of PHC, adsorption to particulate material and solid substances in water must be the main factors influencing PHC availability in sediments and the overlying waters.

The PHC content in the surface waters at Marine Sciences Jetty was maximum in Feb. 93 (816.24 $\mu g\ l^{-1}$) whereas in subsurface waters, maximum PHC content was noticed in December '92 (790.98 $\mu g\ l^{-1}$). Sediment oil load was highest in Dec. 92 (222.27 $\mu g\ g^{-1}$ wet wt) and Jan '93 (239.46 $\mu g\ g^{-1}$ wet wt). The leaching of PHC from the sediment to the overlying water column may be the reason for this. But it has been reported that leaching or biological activity in the sediments may return only a small fraction of the sedimented PAH to the water column (Neff, 1979, Rand & Petrocelli, 1985). The increased traffic of oil tankers during the post monsoon months in the estuary may also contribute to the high PAH load at this site.

The dissolved/dispersed PHC content was minimum in July '93 (332.7 $\mu g\ l^{-1}$ - surface water, 134.45 $\mu g\ l^{-1}$ - subsurface water) at Marine Sciences Jetty. But on the contrary, the highest export of petroleum products from the

nearby oil terminal was in July '93 (102.429 M.T.). A drop was noticed in the PHC content of sediment also in July '93. Low PHC values were noted not only at Marine Sciences Jetty but also at all other stations, except oil terminal, in July '93. This evidently should be owing to very heavy monsoon flushing of the estuary.

Therefore it has to be assumed that normal traffic of crude and refined petroleum products in this part of the harbour does not affect the PHC concentration of this area and episodal pollution such as flushing out of bilge waters, leak in the pipe line overlying the water etc must be the causative factors for high PHC pollution. Station 2 located at the oil terminal jetty (O.T) expressed low level of oil pollution throughout the monitoring period, in spite of the constant crude and refined oil pumping operations at this site. The peculiar circulation pattern and the influence of tidal currents in this region may be attributed to this low level of PHC. The concentration of dissolved PHC in the surface and subsurface waters at this site did not show much variation throughout the monitoring period. A rise in the PHC content in surface waters was noted in July '93 (173.39 $\mu g\ l^{-1}$) which coincided with the increased oil export that month. The dissolved PHC level in surface waters was maximum in November '92 (282.46 $\mu g\ l^{-1}$) with a corresponding hike in the PHC content of subsurface waters (274.55 $\mu g\ l^{-1}$). At this site the

sediment PHC was highest in January '93 (96.29 $\mu g\ g^{-1}$ wet wt.) and in subsurface waters the PHC content was maximum in February '93 (430.02 $\mu g\ l^{-1}$). "Indian Express" reports the presence of a mass of oil found drifting off the south tanker berth of Kochi port on 11.2.92 afternoon. The oil was identified as diesel. Factors like increased traffic during the non-monsoon months, reduced riverine discharge, rich organic load of the sediment (Nayar, 1992) and oscillation of tidal currents (Joseph and Balchand, 1994) influence enhanced sedimentation of suspended particles with adsorbed PHC.

The monsoon months, especially July and August recorded the lowest PHC concentration at the oil terminal (values - surface water -54.70 $\mu g\ l^{-1}$ and 54.62 $\mu g\ l^{-1}$, subsurface water -50.22 $\mu g\ l^{-1}$ and 46.19 $\mu g\ l^{-1}$ and sediment - 37.70 $\mu g\ g^{-1}$ wet wt.) The reason for this could be the wide - spread flushing of the estuary by heavy discharge of fresh water. Further, the resident time of terrigenous sediment which is on the coarser side is short and sediments contain less organic matter during the monsoon months (Nair *et al.*, 1993). The relatively short-lived fresh water regime of the estuary at the tanker berth area is virtually devoid of oil.

The third station is near the backwater buoy No,4 situated in the Ernakulam channel, close to the Willingdon Island. Moderate, but almost consistent levels of PHC

contamination was observed throughout the monitoring period. The PHC content in both water and sediment of this site was slightly higher than that of oil terminal but less than that of Marine Science Jetty and Mattancherry channel. Here also, the post-monsoon month of January '93 recorded the highest PHC concentration in surface and subsurface waters (410.43 $\mu g\ 1^{-1}$ and 373.50 $\mu g\ 1^{-1}$ respectively).

Muddy sediments predominate in the Ernakulam and Mattancherry channels (Seralathan *et al.*, 1993). Even though these fine particles flocculate quickly, depending on the oscillation of the energy levels like variation in the fresh water discharge, spring and neap tides etc., they get suspended to the water column above to form consolidated static suspensions. It has already been noted by Lewis (1975) that elevated particulate PAH is generally accompanied by elevated soluble PAH concentrations. Joseph and Kurup (1990) have pointed out that even though during post-monsoon riverine discharge is negligible into the estuary, the discharge rate of Muvattupuzha river was more during January to May. The fresh water content in the Ernakulam channel is slightly higher than the Mattancherry channel, indicating greater influence of fresh water discharge on the Ernakulam channel which brings with it waste materials including industrial pollutants.

Sediments at this site showed highest values in October 1992 (105.87 $\mu g\ g^{-1}$ wet wt.). The least quantity of volume transport of water across Cochin inlet is found during neap flood in October (Joseph and Kurup, 1989). During this period the estuary is in a partially mixed condition. In this condition the fine-sized particles brought along with the salt wedge during the monsoon, settle down and cover the channel forming a clayey bed. This results in a consequent increase in the level of sediment adsorbed PHC.

In surface water, low level of PHC at station No.3 was recorded in August '92 (108. 92 $\mu g\ 1^{-1}$). Corresponding low level in sub-surface waters was noticed in August '92 (104.96 $\mu g\ 1^{-1}$) and in September '93 (96.37 $\mu g\ 1^{-1}$). Karl Banse (1968) has reported lower mean sea levels during monsoon months for several stations in the West Coast of India. This was corroborated by Udaya Varma *et al.*(1981) in their study on the tidal influence on the seasonal variation in current and salinity around Willingdon Island, which will aid the quicker flushing of the estuarine water through the bar mouth resulting in low PHC levels in the channel.

In the case of sediments, even though low values of PHC were noted in July 1992 (37.25 $\mu g\ g^{-1}$ wet wt.) and July '93 (34,52 $\mu g\ g^{-1}$ wet wt.), the lowest level of PHC was noted in February '93 (32.52 $\mu g\ g^{-1}$ wet wt.). However, the reason for this cannot be explained.

Station 4 located in the Mattancherry channel south of the Willingdon Island is about 12 m deep. Being a navigational channel, it is constantly traversed by ships and ferries and subjected to periodic dredging. Such disturbances along with the peculiar circulation pattern of the estuary keeps Mattancherry channel in the second place with respect to oil pollution in the present monitoring study. Dissolved PHC concentration in the surface and subsurface waters was maximum in May '93 (629.05 $\mu g\ l^{-1}$ and 619.92 $\mu g\ l^{-1}$ respectively).

It has been noticed by Udaya Varma *et al.* (1981) and Joseph and Kurup (1989) that thorough mixing of the estuarine waters occur during premonsoon months owing to the flow pattern prevalent during the period. This may be one of the causative factors for the high turbidity of the surface waters during hot premonsoon months. This factor is found to influence replenishment of PHC in the estuarine bottom with supply from surface waters.

Maximum PHC concentration in the sediments at this site was recorded in April '92 (166.75 $\mu g\ g^{-1}$ wet wt. Premonsoon period in the estuary is marked by a uniformly high salinity (34×10^{-3}) and high temperature (30-32^{o}C) (Gopinathan and Qasim, 1971; Joseph and Kurup, 1989). In the Mattancherry channel, silty sediment with small amount of clay is present during premonsoon months (Sundaresan, 1991).

Increased salinity enhances the rate of flocculation of the sedimentary material and their sedimentation. This may cause a corresponding rise in the level of adsorbed PHC in sediments.

Minimum amount of PHC in the water and sediment of Mattancherry channel was noted in Aug '92 (134.27 $\mu g\ l^{-1}$ surface water, 124.21 $\mu g\ l^{-1}$ subsurface water, 69.17 $\mu g\ g^{-1}$ wet wt. - sediment). The flow pattern during monsoon is the combined effect of tidal influence and fresh water influx. The presence of a salt wedge and the higher values of circulation parameters of the estuary assist in the effective flushing of oil-spilled surface and sub-surface waters from the Mattancherry channel. Even though the channel showed silty sediment with clay, probably associated with the salt wedge in this region (Sundaresan, 1991), the high turbid condition in the monsoon season caused by the river discharge and interaction with tidal currents considerably reduced the siltation rate of sediments, thus lowering the sediment PHC values.

Station 5 marked as the bar mouth (BM) is about 12.8 m deep and is located in the estuarine mouth along the approach channel. During the monsoon season the waters of this station received high dissolved PHC whereas the sediment PHC was high only during the post monsoon season. Reduction

in the rate of deposition of PHC laden sediment during the monsoon months, lowered the PHC values of the surface sediment. Maximum PHC content in the surface and subsurface waters were recorded in July '92 (418-86 μg 1 $^{-1}$ and 411.65 μg 1 $^{-1}$). Hydrographic characteristics and tidal prism studies at Cochin bar mouth by Rama Raju *et al.* (1979) highlight the outward surface flow from the estuary to the near shore coastal waters during the monsoon season.

Sediment PHC showed maximum value in December '92 at the bar mouth (96-63 μg g^{-1} wet wt.). The presence of clay in the bottom sediment at the Cochin barmouth region during the onset of post monsoon has been reported by Sundaresan (1991). It may be pointed out here that a steady increase in the salinity of estuarine waters beginning from the latter half of September could have resulted in increased rate of flocculation and subsequent increase in PHC laden sediment resulting in enhanced PHC values in the sediment. Minimum PHC content in the sediment was noticed in July '93 (42.17 μg g^{-1} wet wt.). In monsoon months, the churning action of the waves transport coarse sediments towards the approach channel from south-west direction (Sundaresan, 1991) - PHC has a lower tendency to adsorb on to coarse fractions of sediments. Low value of dissolved PHC at this site was reported in April '93 (137.70 μg 1 $^{-1}$ -surface, 118.83 μg 1 $^{-1}$ - sub surface waters).

Station 6 is located in a relatively clean area and is treated as the reference site. The PHC values in both water and sediment were negligible throughout the monitoring period. Analysis using two way ANOVA also confirms this.

There is neither a general pattern nor the presence of a gradient for the PHC concentration of the different stations studied. Since it is assumed that hydrographic and hydrological features of estuary will have some influence in estimation and availability of PHC in the region, the year was divided into three seasons taking monsoon as the most important factor influencing the buff.

Basically, distribution of oil either in water or in sediments depends on the availability of matrices on to which the oil can get enmeshed. Therefore, presence of dissolved organic matter in water or presence of organic matter in the sediment controls the distribution of PHC in these two components. Since the coastal and estuarine sediments are organic rich, they can harbour higher quantities of PHC. Seawater contains comparatively less quantities of organic matter in dissolved form. Therefore at any point of time the seawater would contain low quantities of organically bound PHC unless otherwise there is an episodal pollution like accidental spillage or chronic seepage from moored vessels. This makes a comparison of the distribution of PHC in water

and sediment meaningless, unless this is complemented by determination of the organic load of these environments (Menon, pers. comm.). Notwithstanding this, the division of year based on monsoon was done with a view to giving importance to the fresh water run off *vis-a-vis* tidal influence which in turn can affect the distribution of organic matter in water and sediments. The sediments often contained concentrations of PHC of higher levels than the overlying water. Sedimentary PHC tend to be quite persistent and accumulate to high concentrations. For all aromatic hydrocarbons, except the most soluble low molecular weight aromatics, partitioning between the aqueous and sedimentary phases greatly favour the sedimentary phase. Mc Groddy *et al.* (1996) based on their study on Boston harbour sediments also claim that only a fraction of the total sedimentary PAH is available for equilibrium partitioning with the aqueous layer. Because of the favourable partition coefficients and greater persistence of sedimentary PHC than PHC in solution, the sediments nearly always contain concentrations of PHC greater by a factor of thousand or more than the overlying water (Neff, 1979).

Monsoon season is the period of maximum fresh water influx in the estuary. The tidal range, dilution and flushing characteristics of this estuary have great influence on the transportation, dispersion and dilution of natural materials

and pollutants (Joseph and Kurup, 1989). This effective flushing results in low values of PHC in water at all stations, except barmouth, where accumulation of wastes from the estuary occur. Station 1 (MSJ) being very shallow, experiences least tidal influence and this together with the mooring of boats may be the reason for comparatively higher PHC values at this site even during monsoon. Further, flood and ebb currents are comparatively high in the Ernakulam channel than in the Mattancherry channel (Udaya Varma *et al.*, 1981) which may be attributed to the comparatively low levels of PHC in the Ernakulam channel than Mattancherry channel. During the monsoon season, finer bed materials from the upper reaches move towards the lower estuarine mouth causing a reduction in PHC concentrations in the sediments at the different stations.

During the post monsoon season, the estuarine circulation is mainly controlled by the tidal currents. The oil content in the surface and sub-surface waters during this fair weather season is found to be high at all stations. This was consequent to the increased traffic, fishery operations and meagre riverine discharge which prevents effective flushing of estuarine water. It has to be assumed that the resident time of WAF of oil within the estuary is higher during the nonmonsoon months which in turn would influence the distribution pattern. According to Boehm and Quinn (1973),

substances such as humic acid, fulvic acids and other degradative products of biological materials in the water may act as PAH solubilisers. High amount of humic acid in the surface and subsurface waters of the Cochin estuary during post and premonsoon have been reported by Nayar (1992). High values of trace metals in the Cochin estuary due to their complexation/binding to the humic substances have also been reported by many workers (Paul and Pillai, 1983).

The tidal currents have a dominant effect over the rate of transport as well as the settling rate of the sediments (Adams *et al.*, 1990). The oscillatory currents at tidal inlets have a predominant effect on the distribution of suspended solids (Joseph and Balchand, 1994) and probably this leads to the presence of varying proportions of PHC in the sediments at various locations of the estuary. Nayar *et al.*, (1995) have also reported variation in sediment PHC with the change in characteristics of the sampling sites in the Cochin estuary. According to them, low values observed during monsoon (291 $\mu g\ g^{-1}$ dry wt.) could be due to flushing of the surface sediments by the run off. High values during non-monsoon season (372 $\mu g\ g^{-1}$ dry wt.) was attributed to anthropogentic activities and the increase in pH and salinity.

Premonsoon season was a period of meagre riverine discharge into this estuarine region (Joseph and Kurup, 1990) and the circulation during this period is mainly driven by

tidal currents. The estuary is in well mixed condition during this season. Highest velocity of ebb and flood currents are found at the surface layers (Joseph and Kurup, 1989) which may flush out the oil laden surface waters towards the lower estuarine regions. Further, the increase in salinity during this season may reduce the solubility of PHC in seawater owing to salting out (Rossi & Neff, 1978). But this season is also marked by an increase in temperature which increases the aqueous solubility of PHC. The construction of Thanneermukkam bund at the southern part of the Vembanad lake to prevent salt water intrusion during premonsoon has considerably reduced the sand content in the Ernakulam and Mattancherry channels (Seralathan *et al.*, 1993). The accumulation of fine sediments due to the supply of fine materials from the sea through tidal currents (Veerayya and Murthy, 1974) and deposition of suspended load result in the elevation of the sediment PHC levels during this season. Statistical analysis using two way ANOVA indicates significant difference in the PHC content between seasons. In the case of PHC of surface waters, the significance was at 1% level with post monsoon values significantly higher followed by monsoon and pre-monsoon. Subsurface waters-maximum values were observed in post monsoon ($p \leq 0.001$) followed by premonsoon and monsoon. Sediment PHC values were also significantly higher during post monsoon ($p \leq 0.01$).

From the above features it can be concluded that the concentration and distribution of PHC in the surface and subsurface waters and the bottom sediments of this estuary is influenced by the estuarine circulation and the oscillatory nature of tidal currents. Further, the variability in PHC concentration with respect to seasons is mainly attributed to the marine traffic and fishery operations together with the estuarine variability subjected to seasons. The apathy of the authorities during tanker operations leading to frequent leakages of crude oil and petroleum products (Sen Gupta, 1991, 1992; Indian Express 13-11-92 and 13-2-93) and discharge of ballast waters into the estuary often result in exceptionally high values at certain locations. Even though the Port authorities deny such charges, Idnian Express (13-2-93) reports that penalties were imposed on three merchant ships which were found to have discharged oil into the estuary in the past one year. Barring a short period of trawling ban during monsoon (22 days in '92, 23 days in 1993), mechanised fishing boats constantly traverse the estuarine region, discharging considerable amount of oil-laden bilge waters into the estuary. Steps taken to reduce such pollutional activities only can help in minimising the oil pollution of Cochin backwaters.

CHAPTER 2

EXPERIMENTS EMPLOYING TRANSPLANTS AND NATIVE BIVALVES

2.1 INTRODUCTION

Environmental contamination by a chemical pollutant is studied employing a monitoring strategy involving quantitative and qualitative measurements. Marine pollution, documented in terms of relative concentration of the contaminants in water and sediment alone is no longer acceptable to the scientific world. It is now widely accepted that bioassays form an integral part of a comprehensive approach to pollution assessment. Mc Intyre *et al.*(1978) argue that stress response has to be defined from a strictly biological point of view and only such responses that are reflected at the population level, have significance.

The need for developing convincing measures to scale biological responses resulting from physical and chemical disturbances has been recognised for some years and has prompted a great deal of research. Biological effects of pollutants can be manifested at sub-cellular, cellular and organismal levels of organization before manifesting at population, community or ecosystem levels (Capuzzo, 1981). Biological effects of PHC on marine organisms are dependent on their persistence and bioavailability, the ability of the organisms to accumulate and metabolize various hydrocarbons and the interference of hydrocarbons with normal metabolic processes that may alter an organism's chances of survival and

reproduction in the environment. Other environmental factors like temperature and salinity may influence bioavailability of the contaminant or modify the rate functions of the organisms (Varanasi *et al.*, 1985).

A condition like the presence of a chemical in the environment that induces an alteration in the physiological rate or in behavioural pattern of an animal in such a fashion that it exceeds the normal range of variability can be recognised as stress. Responses at these levels in the biological heirarchy occur over short time scales, from minutes to days, and they may be surprisingly sensitive. Measurement of physiological, energetic and growth responses can provide insight into the overall growth processes and also help in assessing rate of disruptions of the above by environmental stress and pollution. In addition, Donkin *et al.* (1989) are of the opinion that mussels and their physiological responses are of greater or equal sensitivity to environmental contaminants when compared to other aquatic animals.

2.2 REVIEW OF LITERATURE

Blumer (1969) was the first to suggest that exposures to PHC could interfere with chemoreception and this could result in altered behavioural patterns. Bayne *et al.* (1982) recommended research on "specific stress indices" that

could help identify causative agents affecting the health of mussels, resulting from pollutant bioaccumulation. A reduction in feeding rate of *Mytilus edulis* is known to occur at low and environmentally realistic concentration of PHC (30-40 $\mu g\ l^{-1}$) (Widdows *et al.*, 1984). Widdows *et al.* (1982, 1985) have also demonstrated a reduction in the efficiency of food absorption by *M. edulis* under PAH stress.

Rate of filtration has been suggested as a reliable sub-lethal toxicity index (Abel, 1976) for filter-feeding bivalves and being a non-destructive method can be carried out with ease. Effects of toxicants like hydrocarbon and chlorinated hydrocarbons on clearance rate of marine mussels have been worked out by D'Silva (1980); Reddy & Menon (1980) and Widdows *et al.* (1982). Environmental factors such as salinity, oxygen tension etc. are also known to influence the filtration rate (Bayne, 1975).

Axiak and George (1987) commenting on the observed reduction in the clearance rate in *Venus verrucosa* stated that the activities of the eulaterofrontal cirri were affected in such a way as to reduce their particle retention efficiencies. Long term exposure to PHC by *M. edulis* showed a significant inverse relationship between the mass-specific clearance rate and the concentration of aromatic hydrocarbon accumulated in the boby tissues (Widdows *et al.*, 1987). Behavioural and

physiological responses observed during short-term hydrocarbon exposure may be restored to control levels following transfer to uncontaminated seawater although recovery does not always occur immediately upon transfer (Capuzzo *et al.*, 1984). The inhibition of feeding probably results from the narcotic effect of hydrocarbons, particularly aromatic hydrocarbons, which may have a direct action on cilia and muscles (Johnson, 1977) and/or the nervous system which controls such activity (Hendry *et al.*, 1985). M. edulis exposed to 130 $\mu g\ l^{-1}$ diesel oil for 8 months showed a marked reduction in the feeding rate and a negative scope for growth, indicating the need to utilise body reserves in order to satisfy the animal's energy requirements (Widdows, *et al.*, 1985). This was confirmed by the observed 'degrowth' in body tissues which resulted in high mortality in this group. (Lowe and Pipe, 1987). Casalderrey *et al.* (1993) have also observed a decline in the rate of ingestion of algae by the fresh water rotifer *Brachiones calyciflorus* and the cladoceran *Daphnia magna* with increasing methylparathion concentrations.

Rate of oxygen consumption has been used as a valuable tool by many workers to assess stress, since it is an index of energy expenditure to meet the demands of environmental alterations (Prabhudeva and Menon, 1986 (a) ; Mohan *et al.* 1986 (a) and (b)). Particulate feeding marine animals remove as much as 13% oxygen from the seawater

passing over the respiratory surfaces although many others remove around 53% (Kinne, 1975). Compared to indices like growth rate, gonadal development and reproduction, a study of respiratory rate under toxicant stress is less sensitive although it is easy and quick.

Prabhudeva and Menon (1986 (a)) suggested that oxygen uptake is a product of three important factors, *viz.*, ventilation volume, the quantity of oxygen in water, the nature of respiratory pigments and surface area of the respiratory tissue (Menon, pers. comm.). Therefore, changes in oxygen uptake from the waters by the animal and variations in the amount of water propelled through the gills result in fluctuations in oxygen consumption, which indicates involvement of behaviour as well as physiological functions.

Exposure to hydrocarbons generally tends to increase respiratory rate (Bayne *et al.*, 1985). Likewise, there are interspecific variations in the rate of oxygen consumption. In a study of the relative respiration and feeding rates of oyster *Crassostrea virginica* and brackish water clam *Rangia cuneata* in the contaminated waters of Chesapeake Bay, Hartwell *et al.* (1991) demonstrated that *R. cuneata* has a lower respiratory rate than *C. virginica* when tested under the same conditions. The investigation by Correa and Garcia (1990) on the effects of benzene on the physiological responses of juvenile white mullet, *Mugil*

curema, has proved that benzene modifies the gaseous exchange and, therefore, has an influence on the respiratory rate. Low levels of PHC are known to enhance respiratory activities in several bivalves like *M. edulis* (Widdows *et al.*, 1982); *Astarte borealis* and *Mya truncata* (Hutcheson, 1982).

Recently, several researchers have suggested that changes in the physiology and/or behaviour of aquatic organisms, which can be collectively termed bio-indicators, can be used as an effective means of improving aquatic environmental monitoring strategies (Day and Kaushik, 1987; Janssen *et al.*, 1993; Casalderrey, *et al.*, 1993). These physiological indices, being the integration of several relatively inexpensive tests should provide a better assessment of stress from exposure to complex mixtures of xenobiotics in the environment than measurement of any single variable and, they may also serve as early-warning signals of population or community effects (Stein *et al*, 1992) A recent development in the field of environmental bioassays is the biomarker approach which provide early distress signals of exposure to bioavailable contaminants (Moore, 1990; Moore and Simpson, 1992). However, the simultaneous measurement of these cellular responses with organismal responses has been widely recommended for the accurate prediction of changes in the population and community structure (Agirregoikoa, *et al.*, 1991; Cajaraville *et al.*, 1993).

Information on bioavailability of pollutants is essential in our attempts to delineate cause and effect relationship between chemicals in the marine environment and observed biological abnormalities. Several field and laboratory studies have demonstrated a significant negative correlation between SFG and the concentration of specific aromatic hydrocarbons in the tissues of molluscs (*M. arenaria* by Gilfillan *et al.*, 1977; *M. edulis* by Widdows *et al.*, 1982; Stickle *et al.*, 1985; *Thais lima* by Stickle *et al.*, 1984).

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 PAH present at low concentrations in the ambient medium, food or sediments (Varanasi & Malins, 1979; Mc Leese and Burridge, 1987). The lipophilic character of the aromatic hydrocarbons facilitates their speedy accumulation in organisms. Several studies have been performed on the accumulation and release of PAH in solution by marine bivalves. Neff and Anderson (1981) have presented certain details on the uptake and release of PHC in oysters and clams. They found time dependent variations in the PHC concentrations in the tissues. Clams were found to accumulate less than oysters. Assigning influence of seasons on the distribution of PAH in the soft shell clam, *M. arenaria*, Mix and Schaffer (1983) found that PAH concentrations were lowest in the fall-winter and highest

during spring-summer period. In a study by Amodio-Cocchieri *et al.* (1993) to evaluate the level of industrial contamination in marine organisms in the Ionian sea, they concluded that the common mussel had the highest concentration of PAH in the tissues than other shellfishes and fishes. The uptake of aromatic hydrocarbons into the tissues of marine organisms was found to be directly related to the amount of hydrocarbons in WAF. Naphthalene is the most water soluble of the PAH tested, rendering it more readily bioavailable (Neff, 1979).

Studies on the bioaccumulation of xenobiotics from water has many limitations in that, many physical, chemical and biological factors act upon an oil spill in nature causing variations in the disposition and composition of PHC in the water and subsequently in the animal. So only in laboratory studies, where animals are exposed to unnaturally high concentrations of PHC, do we have a relative uptake of PHC in accordance with the toxicant level in water. In nature, pelagic species are the most affected by the WAF. Recently, the attention of scientists has been turned to bioaccumulation studies in pelagic fish, like salmon (Varanasi, 1992). One such study has shown that juvenile Chinook salmon (*Oncorhyncus tshawytscha*) from an urban estuary in Seattle, are exposed to considerably higher levels of aromatic hydrocarbons than are salmons from a rural estuary (Mc Cain *et al.*, 1990).

It has been proved that hydrocarbons reaching the surface of sea will be adsorbed on suspended particulates or incorporated into the faecal pellets of the biota and thus transported to, and retained in, bottom sediments. In the long term, the accumulation of these contaminants will result in concentrations in the bottom sediments to toxic or stressful levels (Sharp and Appan, 1982). The persistence of PAHs in sediments depends mainly on the sorption, volatilization, leaching and biological or chemical degradation processes, which may be influenced by a variety of environmental factors, like temperature (Bauer and Capone, 1985), organic content in the sediment (Wild *et al.*, 1991) etc.

Most PAH are relatively water insoluble and ultimately are deposited in sediments. In areas of major oil spills or in localized or shallow enclosed areas, sediments have shown to have high concentrations of PAH (Maccubbin *et al.*, 1985). Studies of the Amoco Cadiz spill reports a community "smothering", eliminating microphytic and macrobenthic communities (Le Campion - Alsumard *et al.*, 1984). The biological effects of drilling and discharges are usually confined primarily to the benthic environment (NRC, 1983).

Benthic organisms living in sediment and ingesting sediment particles may accumulate oil thereby resulting in high body burdens, and thus retrieve sediment associated contaminants to the food webs. Bender *et al.* (1988) in their

study on the relative accumulation of PAH in fishes and shellfish, calculated that oysters (*Crassostrea virginica*) accumulated about 3.7 times more total PAH than did clams (*M. mercenaria* and *R. cuneata*) at 25°C. Bio-accumulation via particle ingestion depends on the feeding rate and feeding selectivity of the organism, assimilation efficiency and the contaminant concentration of the ingested food particles (Lee *et al.*, 1990). Filter feeding benthic invertebrates such as clams may therefore serve as bio-indicators and integration of hydrophobic organic contaminants. The Asian clam *Potamocorbula amurensis* which filters large quantities of water and associated particulates is able to accumulate high concentrations of sediment bound PAH. Certain compounds like phenanthrene and acenaphthylene are strongly retained in lipid tissues of these organisms and probably are only slowly depurated (Pereira *et al.*, 1992).

Accumulation of PAH by benthic organisms from sediment may be attributed mainly to uptake of PAH contaminated interstitial water. It is proved that the source of PAH to the interstitial water is the PAH adsorbed on to particles. Since PAH has an inherent quality of associating with organic matter, particulate organic matter contain a good load of PAH. The partitioning of these hydrophobic organic contaminants between particulate and dissolved phases controls their environmental fate and distribution in aquatic systems.

In the Chesapeake Bay, plankton production and erosion of soils and near-shore sediments are the dominant sources of particles to the water column (Mc Lusky, 1989). Sediment resuspension plays a large role in supplying particles to the water column in shallow bays. There is an increasing need for laboratory exposure systems to study the effects of suspended particles introduced into the marine environment by ocean disposal operations like dumping sewage sludge or dredge spoil. A measure of assimilation efficiency of benthic animals with respect to ingested particles has been suggested to obtain information about the relative role of different accumulation routes. (Thomann *et al.*, 1992). *Diporeia sp.*, a benthic amphipod is an extremely selective feeder and can selectively ingest particles enriched in one hydrophobic contaminant over another relative to the particle's organic carbon content (Harkey *et al.*, 1994). Thus there is evidence that assimilation from ingested material can be a significant accumulation route for lipophilic compounds.

Secondary contamination of the environments already damaged by oil spills are mainly due to the oil trapped in sediments and by the oil which leaches out into the overlying water. This was apparent in Brittany coast where the bivalve *Tellina fabula* began to disappear several months after the Amoco Cadiz spill (Clark, 1982). Similar protracted damage has been claimed for the benthos of Buzzard's Bay, after the wreck of the barge Florida, though at this instance the

affected area was enlarged by the movement and resuspension of contaminated sediments. A study of hydrocarbon availability to the limpets (*Patella*) and clams (*Mya arenaria*) following the Amoco Cadiz spill showed that in contrast to *Patella* from rocky shore, tissue hydrocarbon level of *M. arenaria* inhabiting the sediments was above background level even ten months after the spill. In addition, the tissue hydrocarbon fluorescence spectra resembled more closely to those of either stranded Amoco Cadiz oil or sediment bound hydrocarbons. (Vandermeulen, 1982). A study by Varanasi *et al.* (1987) also implies that although the precise route of uptake i.e., direct uptake of particle bound contaminants or uptake of xenobiotics released into the sediment associated water could not be determined, it should be noted that the sediment acts as a major source of contaminants for English sole (*Parophrys vetulus*) and presumably other benthic fishes.

The presence of dissolved organic carbon affects the accumulation of organic xenobiotics by aquatic organisms, especially the more water insoluble compounds (Landrum *et al.*, 1987). Landrum (1988) found a seasonal pattern for the binding of BaP to organic carbon from lake water. The BaP was least bound in the spring in the great lakes when the dissolved organic carbon in the water column was dominated by products released from diatoms with a subsequent increase in the accumulation of xenobiotics by the amphipod, *Pontoporeia hoyi*.

Oviatt and Nixon (1975) reported that the material collected by sediment traps placed in Narragansett Bay was dominated by resuspended sediment particles, indicating that sediment resuspension may be a major contributor to particulate matter in the water column. Therefore, bivalves in polluted estuaries or near oceanic disposal sites may be chronically exposed to resuspended sediment contaminants. Partitioning models of the distribution of compounds between particulate and dissolved phases of seawater predict that the lower molecular weight compounds, which have higher water solubilities, would be more readily desorbed from the sediment into the dissolved phase (Mackay *et al.*, 1983). Pruell *et al.* (1987) exposed *M. edulis* to artifically resuspended sediments for 20 days and found that Benz(a)anthracene had the largest concentration factors for the PAHs that could be measured both in the dissolved and in the particulate phases of the dosing system.

2.3 MATERIALS AND METHODS

Biological impact of PHC was studied using the bivalve *Perna viridis* collected from Kannamali, an area with negligible PHC contamination. Around 100 specimens of *P. viridis* having shell length of 30 $\pm$ 2mm collected from the above site were transferred to large plastic cages. One cage each was kept suspended using artificial buoys at three stations, viz., Marine Sciences Jetty (Stn. 1), Oil terminal

(Stn. 2) and in the Mattancherry channel (Stn. 3). The Kannamali station which is being considered as the reference station (Stn. 4) received the fourth lot containing around 100 specimens kept in plastic cages.

Bioaccumulation of PHC by two sentinel organisms - *Perna viridis* and *Sunetta scripta*, and the associated physiological changes were monitored by laboratory experiments for a period of 15 days.

The bioavailability of PHC from artificially resuspended sediments to *S. scripta* was studied in the laboratory for 10 days.

2.3.1 TEST ANIMALS

2.3.1.1 ***Perna viridis (Linnaeus)***

The green mussel, *P. viridis* is an epifaunal bivalve mollusc inhabiting the rocky coasts and harbours. This commercially important species is abundant along the South west coast of India. Major recruitment of this species occurs around September. Animals of 30-35 mm length were collected from a unpolluted natural population from the unpolluted rocky shores of Kannamali.

2.3.1.2 ***Sunetta scripta (Linne')***

The marine mollusc *S. scripta* is an infaunal siphonate clam inhabiting intertidal region. They have

established comparatively large beds on the northern side of the Cochin barmouth. Animals of 30-35mm length were collected from the Fort Cochin area ($9^{o}28'$-$10^{o}00'$ & $76^{o}13'$ - $76^{o}11'E$).

The animals were brought to the laboratory in plastic bags containing seawater collected *in situ*.

2.3.2 WATER

The seawater used for experimental work was collected from an unpolluted area in the Arabian sea off Cochin. The water was stored in total darkness for about one week and the particulate fractions were allowed to settle. The seawater used for the experiment was filtered using fibre glass filter (length 32 cm, breadth 16 cm) containing glass wool and activated charcoal. The seawater used for the experiments (Salinity - 30 ± 2%., pH 8.2 ± 0.2) were aerated to saturation before use. The addition of toxicants did not cause any variations in pH and all sets of experiments were performed at room temperature (29 ± 2^{o}C)

2.3.3 LABORATORY CONDITIONING OF TEST ANIMALS

S. scripta was acclimated in large fibre glass tanks provided with clean sand (natural substratum of the animal). Well aerated seawater of 30 ± 2% salinity was used for maintaining the animals. The animals were allowed to acclimate for one week under laboratory conditions before

being used for experiments. Contamination by pseudo-faeces and metabolites were checked by daily renewal of water. Animals were fed daily on blue green algae, *Synechocystis salina*.

P. viridis were maintained in large fibre glass tanks containing well aerated seawater. They were allowed to acclimate to the same environmental and nutritive conditions as mentioned above.

2.3.4 TOXICANTS

2.3.4.1 **Water accommodated fractions**

Water accommodated fractions (WAFs) of Bombay high crude (BHC) oil were prepared daily, by vortex mixing of oil with seawater of required salinity at a ratio of 1:10 for 14h, in round perspex containers of 20 l capacity with bottom outlets. After stirring, the mixture was allowed to settle for a period of 10 minutes and WAF transferred for further separation into thoroughly cleaned separatory funnels of 2 l capacity for a period of 2 h. The WAF was transferred into clean beakers of 5 l capacity and this solution was treated as the 100% WAF. The concentration of the accommodated oil was estimated in ppm basis after extraction of oil from the WAF using n-Hexane.

50 ml of the WAF was transferred into a hexane cleaned beaker of 100 ml capacity and acidified with 1 ml concentrated HCl to bring the pH below 2. This was extracted using 15 ml n-Hexane (HPLC grade) by shaking in a separatory funnel for 2 minutes. The process was repeated twice. The combined hexane extract was freed from residual water by treatment with anydrous sodium sulphate (Sen Gupta *et al.*, 1980) and finally made upto 50 ml. The fluorescence intensity of the hexane extract was measured at 310 nm (EX) and 360 nm (EM) and the oil concentration in the 100% WAF was computed from the standard graph.

Calculated volumes of the WAF of the oil was then added to the test media to get the required PHC concentration. For *P. viridis*, the concentrations used were 1, 5 & 10 ppm whereas for *S. scripta*, they were 5, 15 and 50 ppm.

The actual quantities of PHC were estimated as per methods noted in Section 1.4.2.1.

2.3.4.2 **Sediment**

Sediment for the exposure system was collected off Marine Sciences Jetty using a van Veen grab. Organisms and shell debris were removed and a sediment slurry was produced by mixing 50 gm of sediment per litre of seawater. The finer fractions in the slurry was used for dosing by decanting the supernatant. The PHC content and total organic content of the

sediment were determined before starting the experiment. The organic content in sediments was determined using the method of Elwakeel and Riley (1957).

2.3.5 TOXICITY STUDIES

Sublethal toxicity studies were conducted for a period of 15 days using test animals exposed to the toxicants. Exposed animals were sampled from these experimental sites at 5 days intervals commencing from the 5th day and ending on the 15th day. The test solutions were never aerated during the period of exposure. Animals were fed with *Synechocystis salina* as well as artificial feeds.

2.3.5.1 Tissue load of PHC

PHC content in the mussel tissue was estimated by the method of Donkin and Evans (1984) with modification.

Extraction of PHC was conducted using a steam distillation apparatus. The pooled tissues of five mussels were homogenized and 3 g of the homogenized tissue was washed into a round bottomed flask containing 5 ml (4M) sodium hydroxide solution, 15 ml n-hexane (HPLC grade) and 50 ml distilled water. The total volume was made upto 250 ml. The mixture was saponified for 2 hours at a temperature of about

80°C and the resultant solution was neutralised by 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 was transferred to a clean vial. The vials were stoppered and stored in the deep freezer overnight. The n-hexane extracts were dried over anhydrous sodium sulphate and purified by activated alumina clean up columns. The PHC content of the samples were estimated using the fluorescence spectrophotometer (EX-310 nm, EM - 360 nm) and expressed as chrysene equivalents.

2.3.5.2 **Measurement of physiological indices**

2.3.5.2.1 Rate of oxygen consumption

Five animals from each station were kept in a conical flask of 2 l capacity containing 2 l of filtered seawater. The water columns of the flasks were sealed with inert liquid paraffin to prevent exchange of gas with atmosphere. Experiment was of 3 hours duration. Water samples were drawn at 45 min. intervals and the oxygen content of the water determined using the Winkler's method. After the experiment, the animals were dissected, soft tissues removed, cleaned in distilled water, dried at 70-80°C for a period of 48 hours and dry weights recorded to constancy. The rate of oxygen consumption was expressed as $\mu g\ O_2\ h^{-1}\ mg^{-1}$ (dry wt.)

2.3.5.2.2 Rate of filtration

Filtration rate was determined using an indirect method by monitoring the reduction in particle concentration at definite time intervals (Bayne *et al.*, 1985). Five animals from each station were introduced into beakers of 2 l capacity containing 2 l filtered seawater. The animals were allowed to recover from the handling shock. Then 25 ml of algal culture (*Synechocystis salina*) of known cell count was added to each beaker. The solution was allowed to mix thoroughly by gentle bubbling of air into the vessel without causing apparent disturbance to the animal. After thorough mixing, an initial sample of 10 ml was pipetted out. Experiment lasted for 2 hours during which water samples were drawn at 30 minutes intervals. The fluorescence intensity of the algae were measured using the fluorescence spectrophotometer (EX-268 nm, EM - 574 nm). This was converted into algal cell counts from the calibration graph with fluorescence intensity vs. cell count. Filtration rate was calculated in 10^8 cells h^{-1} using Quayle's equation (1948).

$$m = \frac{M}{nt} \log e \frac{C_o}{C_t}$$

where,

m - filtration rate ($l.h^{-1}$)

M - Volume of the test solution [seawater (l)]

n - number of animals per test vessel

t - time interval between sampling (h)

C_o - initial concentration of algal suspension (No. of algal cells)

C_t - final concentration of algal suspension (No. of algal cells)

This filtration rate was divided by the mean dry tissue weight of animals in one beaker, to get the filtration rate in X 10^8 cells h^{-1} mg^{-1} (dry wt.)

2.3.5.3 Experiment with suspended oil-borne sediments on uptake of PHC

Eventhough various test systems have been used to assess the effects of resuspended contaminated sediments on biota (Servizi and Martens, 1991; Cope *et al.*, 1996), the system devised by Schmidt-Dallmier *et al.* (1992) was employed with modifications in the present study. The experimental system (Fig. VIII) consisted of large fibre glass tubs with uniform layers of sand bed. 12 l seawater and 15 g oil contaminated sediment from *in situ* were added to the tubs. This sediment was left undisturbed in the control tanks whereas they were kept in suspension in the experimental tanks. The sediment particles were kept under suspension by churning the sediment constantly with a mechanical stirrer. The speed was regulated so as not to disturb the clams but to keep the sediments in suspension. Turbidity was determined

using Nephlo turbidometer. Constant turbidity values affirmed that sediments were maintained in suspension. Oil concentration of water column was measured on the first, fifth and tenth days. Organic content and PHC concentration of sediment were determined before and after the experiment. 24 animals were kept in each tub. The tanks were aerated. No suspended matter was supplied as food since it was felt that introduction of such particles would interfere with the suspended load of the experimental medium. Concentration was determined initially and on the fifth and the tenth days. Experiments were done in duplicate.

2.4 RESULTS

The present study has dealt with the changes in oxygen consumption and rate of clearance in relation to PHC load in the tissues of *P. viridis* and *S. scripta* exposed to different concentrations of oil borne particles. The results are presented in tables VII, VIII, IX and figures V, VI and VII.

2.4 *PERNA VIRIDIS*

2.4.1 **Activity studies on transplanted mussels**

Table VII and fig. V represent the rate of filtration and oxygen consumption in relation to PHC load of

TABLE VII

Perna viridis - transplanted: Average oxygen consumption (μg o_2 h^{-1} mg^{-1} dry wt.) and filtration (x 10^6 cells h^{-1} mg^{-1} dry wt.), in relation to body burden of PHC (μg g^{-1} wet wt.) along with respective standard deviations.

Sites	Parameter tested	Period of exposure (days)			
		15		30	
		Mean	S.D	Mean	S.D.
Kannamali (Reference site)	Body burden	1.38	± 0.31	1.48	± 0.51
	Oxygen consumption	4.68	± 1.07	4.52	± 0.58
	Filtration rate	1.62	± 0.74	1.06	± 0.16
Mattancherry channel	Body burden	6.87*	± 0.84	11.95*	± 1.90
	Oxygen consumption	3.11	± 0.89	2.31*	± 0.58
	Filtration rate	0.54	± 0.06	0.57*	± 0.09
Oil Terminal	Body burden	8.51*	± 1.66	14.11*	± 0.5
	Oxygen consumption	2.69	± 0.66	1.84*	± 0.29
	Filtration rate	0.54	± 0.44	0.41*	± 0.07
Marine Sciences jetty	Body burden	12.52*	± 0.74	18.19*	± 2.89
	Oxygen consumption	2.24	± 0.56	1.54*	± 0.25
	Filtration rate	0.31	± 0.10	0.21*	± 0.02

* $p < 0.05$

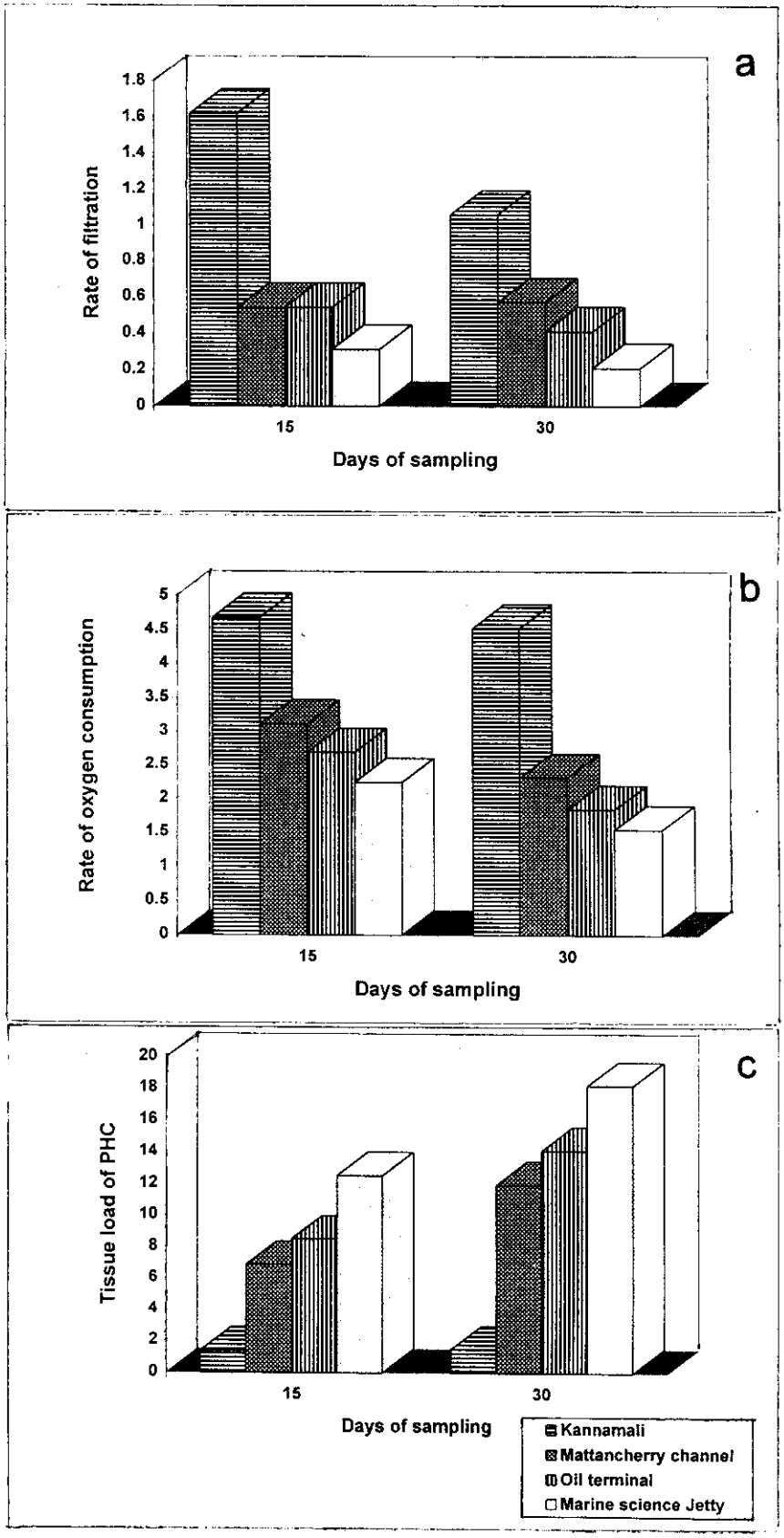


Fig. V

Transplanted *Perna viridis*: (a) rate of filtration ($x10^8$ cells h^{-1} mg^{-1} dry wt.) (b) Oxygen consumption (μg O_2 h^{-1} mg^{-1} dry wt.) and in relation to (c) tissue load of PHC (μg g^{-1} wet wt.)

P. viridis transplanted to heavily PHC polluted sites in Cochin backwaters.

The filtration rate and oxygen consumption were inversely proportional to increase in PHC concentration of the whole tissue. Tissue load of PHC of *P. viridis* showed a positive correlation with the pollutional status of the environment. Thus, animals exposed at Kannamali, the reference site, harboured minimum PHC in their tissues, the values being 1.38 and 1.48 $\mu g\ g^{-1}$ wet wt. on the 15th and 30th day respectively. Animals at the Marine Sciences Jetty accumulated the maximum quantity of oil in their tissues, i.e., 12.52 and 18.19 $\mu g\ g^{-1}$ wet wt. on the 15th and 30th day respectively. Similarly, the 15th and 30th days of exposure showed a similar trend of PHC accumulation in animals exposed at the other two stations.

2.4.1.2. Rate of oxygen consumption and filtration as a function of tissue load of PHC.

Trends in the rate of filtration and oxygen consumption in relation to tissue load of *P. viridis* exposed to 1, 5 and 10 ppm of WAF of Bombay high crude oil for 15 days were assessed and the results are presented in table VIII and fig. VI.

TABLE VIII

Perna viridis - Average oxygen consumption (μg o_2 h^{-1} mg^{-1} dry wt.) and filtration (ml h^{-1} mg^{-1} dry wt.) in relation to body burden of PHC (μg g^{-1} wet wt.), on exposure of the animals to sublethal and lethal concentrations of Bombay High crude oil at different periods.

Concentration of PHC (ppm)	Parameter tested	Period of exposure (days)		
		5 Mean S.D	10 Mean S.D.	15 Mean S.D.
Control	Body burden	0.472 ± 0.02	0.457 ± 0.06	0.387 ± 0.28
	Oxygen consumption	0.02 ± 0.006	0.012 ± 0.007	0.02 ± 0.006
	Filtration rate	1.82 ± 0.07	1.79 ± 0.08	1.72 ± 0.06
1	Body burden	3.563 ± 0.32	4.18 ± 0.14	6.16 ± 0.33
	Oxygen consumption	0.006 ± 0.003	0.005 ± 0.001	0.01 ± 0.002
	Filtration rate	1.484 ± 0.37	1.466 ± 0.424	1.604 ± 0.54
5	Body burden	2.89 ± 0.8	6.53 ± 0.41	9.93 ± 1.34
	Oxygen consumption	0.03 ± 0.01	0.02 ± 0.008	0.01 ± 0.002
	Filtration rate	5.14 ± 0.32	3.062 ± 0.17	5.836 ± 0.32
10	Body burden	2.84 ± 0.61	Mortality	Mortality
	Oxygen consumption	0.009 ± 0.002	"	"
	Filtration rate	1.472 ± 0.216	"	"

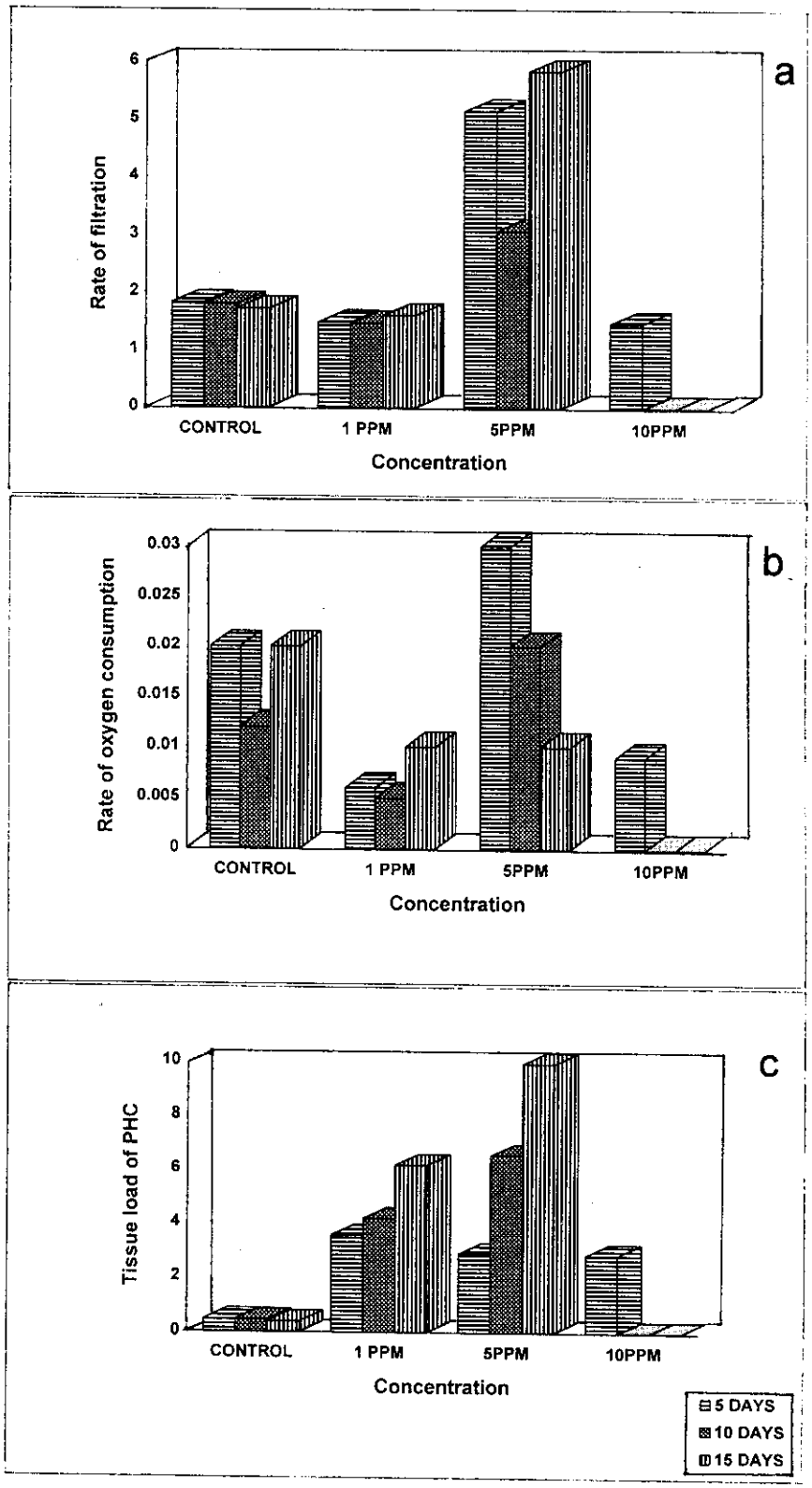


Fig. VI

Perna viridis: Rate of (a) filtration (ml h^{-1} mg^{-1} dry_{-1} wt.) in relation to (b) Oxygen consumption (μg O_2 h^{-1} mg^{-1} dry wt.) and (c) tissue load of PHC (μg g^{-1} wet wt.) on exposure to sublethal and lethal concentrations of BHC oil at different periods.

Exposure to 1 ppm BH crude WAF in the medium resulted in reduction in the oxygen consumption rate by *P. viridis*. After 10 days, the animals consumed still lower amount of oxygen, whereas after 15 days, it was enhanced slightly. The filtration efficiency of the mussels also reduced during the exposure period when compared with that of the control. The rate at which *P. viridis* accumulated PHC when exposed to 1 ppm of BHC WAF was more or less uniform from 0 to 15th day. The tissues contained the maximum PHC on the 15th day (6.16 $\mu g\ g^{-1}$ wet wt.)

An increase to 5 ppm of BH crude WAF in the test medium resulted in slight increase in the oxygen consumption rate by *P. viridis* than the control animals. But the rate of consumption decreased with the period of exposure, the highest rate being 0.03 $\mu g\ O_2\ h^{-1}\ mg^{-1}$ dry wt on the 5th day of exposure. Rate of clearance on the 5th day was 5.14 ml h^{-1} mg^{-1} dry wt, which was 2.8 times more than that of control. A reduction occured after 10 days and the animals were filtering 3.06 ml h^{-1} mg^{-1} dry wt., which was well above that of the control animals (1.79 ml h^{-1} mg^{-1} dry wt.) Curiously enough, the rate of filtration rose to 5.836 ml h^{-1} mg^{-1} dry wt by 15 days of exposure, which was 3.4 times more than that of control. Increase in the concentration of BHC WAF to 5 ppm resulted in enhanced rate of PHC uptake. The rate of uptake was more between the 5th and the 10th day, the tissue load

being 2.89 and 6.53 $\mu g\ g^{-1}$ wet wt. respectively. But during the early phase of the experiment (initial five days), the accumulation was at a lower rate when compared to those exposed to 1 ppm.

10 ppm of BHC WAF being the lethal concentration was found to retard the rate of oxygen consumption as well as filtration rate considerably. Animals survived for only 6 days in this concentration. On the 5th day, animals were consuming 0.009 $\mu g\ O_2\ h^{-1}\ mg^{-1}$ dry wt. which was 45% of the control. Increase in the concentration of BHC resulted in drastic reduction in filtration rate also. On the 5th day, they were filtering only 1.472 ml $h^{-1}\ mg^{-1}$ dry wt. The mussels accumulated PHC at a slow rate in this case. Thus, at the end of 5th day, the tissues contained only 2.84 $\mu g\ g^{-1}$ wet wt of PHC, whereas the moribund animals were found to have 8.12 $\mu g\ g^{-1}$ wet wt. of PHC in their tissues by the 6th day.

2.4.3 *SUNETTA SCRIPTA*

2.4.3.1 Rate of oxygen consumption and filtration as a function of tissue load of PHC.

The rate of ventilation and oxygen consumption by *S. scripta* in relation to the PHC load after pre-exposure to two sub-lethal (5 ppm, 15 ppm) and one lethal (50 ppm) concentrations of BHC WAF are given in table IX and fig. VII .

TABLE IX

Sunetta scripta - Average oxygen consumption (µg o_2 h^{-1} mg^{-1} dry wt.) and filtration (ml h^{-1} mg^{-1} dry wt.) in relation to body burden of PHC (µg g^{-1} wet wt.), on exposure of the animals to sublethal and lethal concentrations of Bombay High crude oil at different periods.

Concentration of PHC (ppm)	Parameter tested	Period of exposure (days)					
		5		10		15	
		Mean	S.D	Mean	S.D.	Mean	S.D.
Control	Body burden	3.53	± 0.31	3.56	± 1.53	3.12	± 0.56
	Oxygen consumption	1.447	± 0.98	1.361	± 0.055	1.379	± 0.05
	Filtration rate	0.355	± 0.06	0.307	± 0.009	0.339	± 0.047
5	Body burden	20.54	± 2.27	29.48	± 25.1	87.76	± 12.14
	Oxygen consumption	2.653	± 0.57	1.774	± 0.069	3.059	± 0.44
	Filtration rate	1.138	± 0.36	0.858	± 0.03	2.641	± 0.797
15	Body burden	15.78	± 1.87	58.96	± 18.27	24.56	± 7.73
	Oxygen consumption	1.576	± 0.52	1.994	± 0.17	2.611	± 0.037
	Filtration rate	1.046	± 0.14	2.073	± 0.22	1.889	± 0.73
50	Body burden	29.8	± 6.73	Mortality		Mortality	
	Oxygen consumption	1.009	± 0.098	"		"	
	Filtration rate	0.295	± 0.03	"		"	

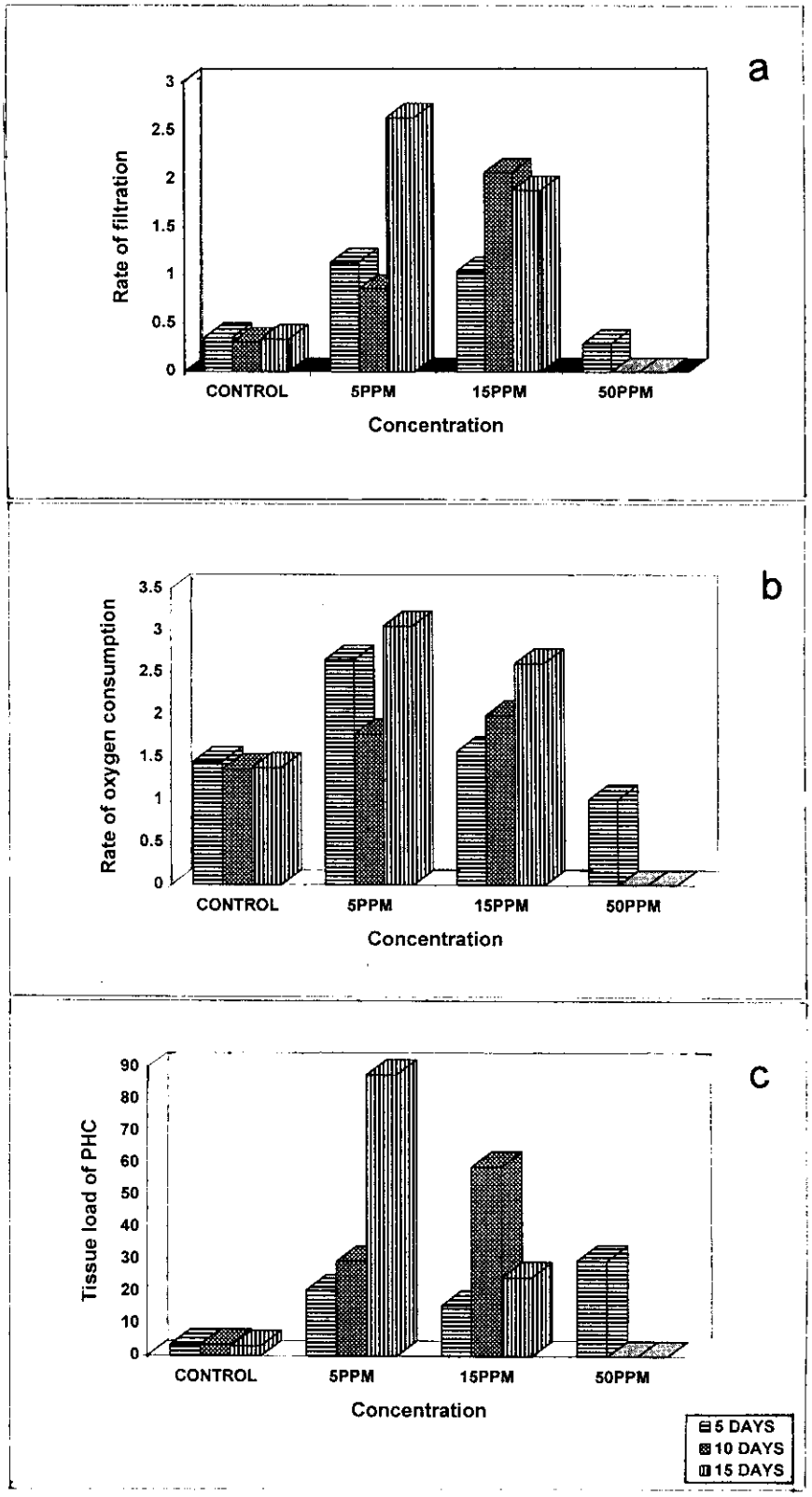


Fig. VII

Sunetta scripta: Rate of (a) filtration (ml h^{-1} mg^{-1} dry wt.) in relation to (b) Oxygen consumption (μg O_2 h^{-1} mg^{-1} dry wt.) and (c) tissue load of PHC (μg g^{-1} wet wt.) on exposure to sublethal and lethal concentrations of BHC oil at different periods.

The presence of 20 $\mu g\ g^{-1}$ of PHC in the tissue resulted in significant elevation in the filtration rate of *S. scripta* on the 5th day of exposure to 5 ppm. PHC. After 10 days, the animals filtered less quantity of water when the body burden was 29.5 $\mu g\ g^{-1}$. However this rate was higher than those of the control animals. Further exposure to the toxicant resulted in higher filtration rates. Thus, after 15 days, the filtration rate was 2.64 ml h^{-1} mg^{-1} dry wt, whereas on the 10th day it was only 0.857 ml h^{-1} mg^{-1} dry wt. The oxygen consumption also showed a significant increase on the 5th day of exposure by consuming 2.65 $\mu g\ O_2\ h^{-1}$ dry wt. which was almost double of that of the control. By the 10th day the oxygen consumption of BHC exposed animals decreased and the animals consumed only 30% more oxygen than that of control. The rate of consumption of oxygen again showed a steep rise after 15 days. During the early phase of the experiment, the quantity of PHC accumulated was more. Thus on the 5th day the tissue load of PHC was 20.54 $\mu g\ g^{-1}$ wet wt. whereas on the 10th day it was only 29.48 $\mu g\ g^{-1}$ wet wt. But the maximum uptake took place after 15 days, when the animals contained 87.76 $\mu g\ g^{-1}$ wet wt. PHC in their tissues.

Exposure of the animals to 15 ppm of BHC WAF also resulted in considerable increase in the filtration and oxygen consumption rates of *S. scripta*. The results obtained were of a comparable nature, the rate of oxygen consumption increasing with the increase in exposure period. On the 10th day of

exposure, the rate of filtration was almost double that of the rate of filtration on the 5th day, both being well above the control levels. The oxygen consumption rate showed a steady increase with the animals consuming maximum amount of oxygen, i.e., 2.611 $\mu g\ O_2\ h^{-1}\ mg^{-1}$ dry wt. on the 15th day of exposure. But the extension of the exposure period beyond 10 days resulted in a slight reduction in the ventilation rate. The percentage performance of the stressed animals to that of the control ones dropped from 6.7 times on the 10th day to 5.6 times on the 15th day of exposure. The animals accumulated PHC in their tissues at a very slow rate initially, that the tissue load of PHC was only 15.7 $\mu g\ g^{-1}$. wet wt. on the 5th day. Further exposure resulted in rapid uptake of PHC till the end of 10 days. Reduction in the PHC load recorded in the animals exposed for 15 days probably indicates an increase in the rate of depuration compared to that of accummulation. After 15 days, the tissue contained 24.56 $\mu g\ g^{-1}$ wet wt. of PHC.

On exposure to 50 ppm of BH crude WAF, the oxygen consumption and filtration rate lowered considerably. On the 5th day the oxygen consumption was 1.009 $\mu g\ O_2\ h^{-1}\ mg^{-1}$ dry wt (69.8% of control) and filtration rate was 0.294 ml $h^{-1}\ mg^{-1}$ dry wt which was 83% of that of the control. The amount of PHC accumulated in the tissues by 5 days was 29.8 $\mu g\ g^{-1}$ wet wt. of PHC. Even though the rate of uptake was not high, the

animals were not able to withstand the stress on basic metabolic functions. This led to the death of the experimental animals after 5 days of exposure.

2.4.3.2 Effect of suspended oil-borne sediments on the uptake of PHC

Table X and fig. IX give information on the rate of accumulation of PHC from artificially resuspended sediment by *S scripta*. The turbidity values as well as the PHC concentration of water of both control and test on 0, 5 and 10 days of sampling are also given. The determination of turbidity gives an idea about the quantity of sediment maintained in suspension. The control tank which contained contaminated sediment and maintained without disturbance, showed constant but minimal levels of turbidity - I NTU. In the case of the tank with suspended sediment, an initial high turbidity value of 27 NTU points to the high churning of sediment bringing almost all the particles into suspension. But by the 5th and 10th day of experimentation, the churning as well as the rate of suspension of the particles into the overlying water became stabilized. The turbidity value consequently dropped to 17 NTU which indicates that the heavier particles have settled to the bottom.

The quantity of PHC getting desorbed from the sediment into the overlying water is reflected in the PHC

TABLE X

Sunetta scripta - Turbidity (NTU) and PHC content in water column (µg l^{-1}), sediment (µg g^{-1} wet wt.) and tissues (µg g^{-1} wet wt.) of animals exposed to suspended oil borne sediments at different periods.

Period of exposure (days)	Parameter tested	Treatments	
		Control Mean ± S.D	Test Mean ± S.D.
0	Turbidity	1.5	27
	PHC content - water	4.518	6.126
	PHC content-sediment	9.925 ± 0.47	9.925 ± 0.2
	Body burden of PHC	0.114 ± 0.012	0.114 ± 0.012
5	Turbidity	1.0	17
	PHC content - water	4.718	7.812
	PHC content-sediment	N.D.	N.D.
	Body burden of PHC	4.463 ± 1.30	9.153 ± 2.54
10	Turbidity	1.0	17
	PHC content - water	5.378	11.526
	PHC content-sediment	7.697 ± 0.88	4.162 ± 0.19
	Body burden of PHC	13.346 ± 1.52	21.49 ± 1.75

N.D - Not determined

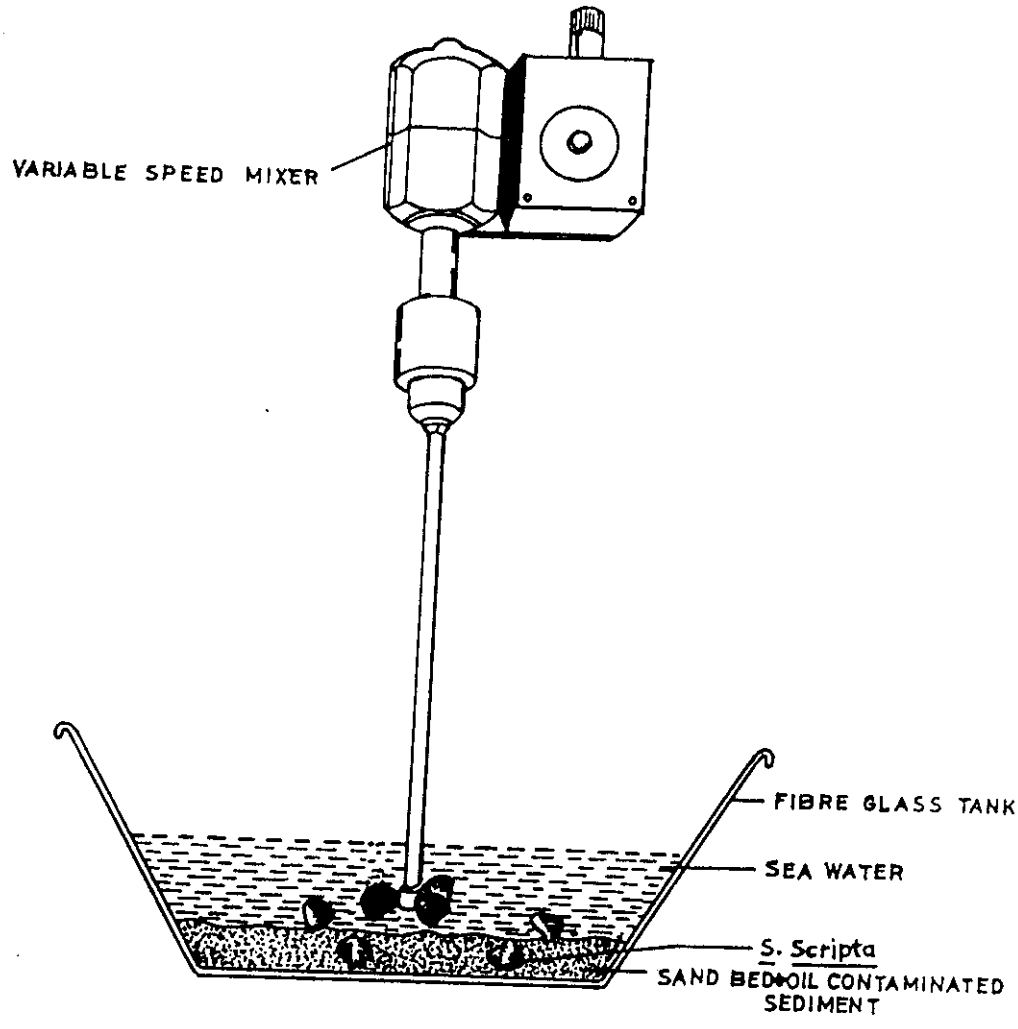


Fig. VIII

Experimental system developed for exposing *Sunetta scripta* to suspended oil contaminated sediments.

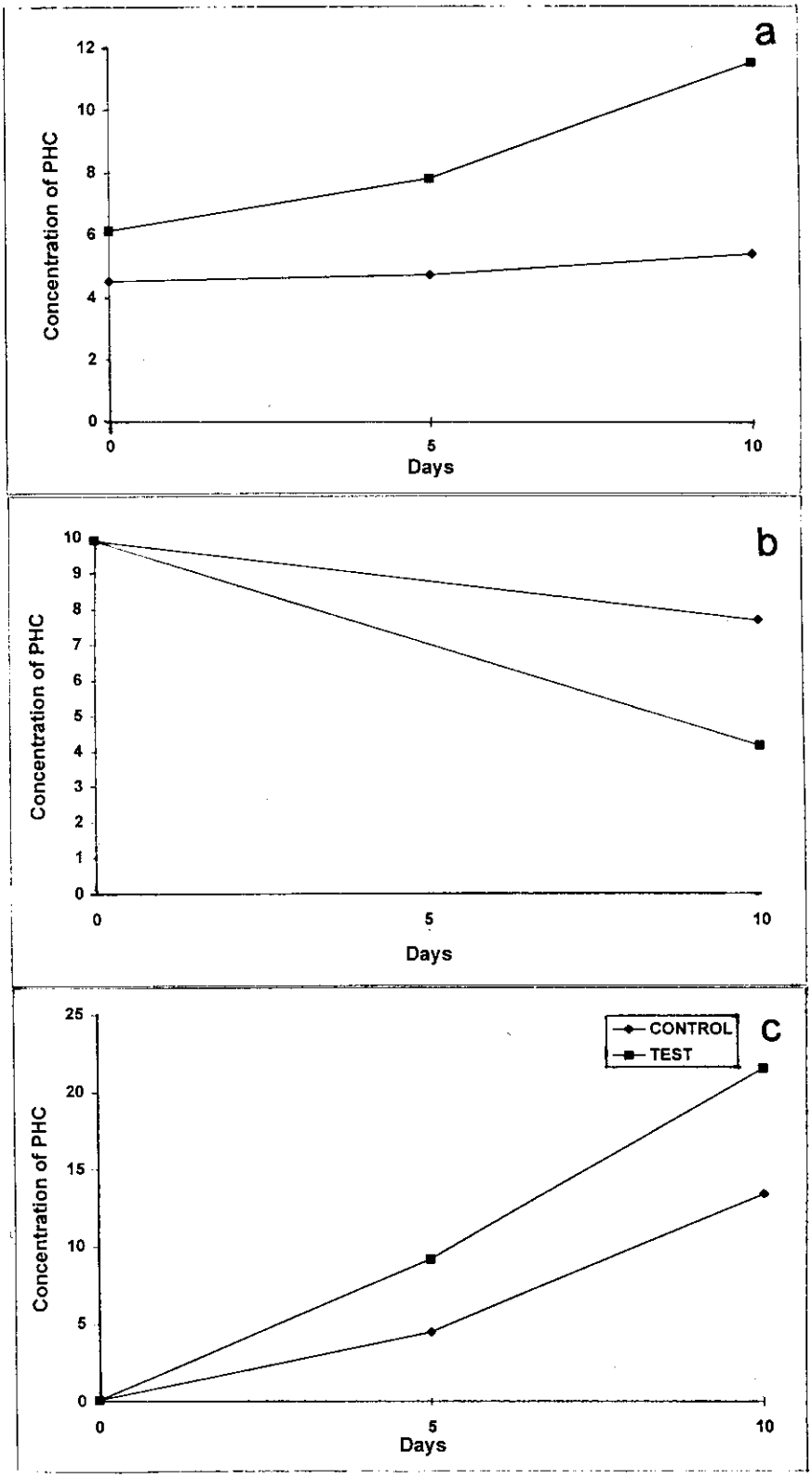


Fig. IX

Sunetta scripta: Concentration of PHC in (a) water column ($\mu g\ l^{-1}$), (b) sediment ($\mu g\ g^{-1}$ wet wt.) and (c) tissues ($\mu g\ g^{-1}$ wet wt.) of animals on exposure to suspended oil borne sediments at different periods.

concentration in the water column. On the day of starting the experiment, the water column in the control tank had a PHC content of 4.518 $\mu g\ l^{-1}$ whereas in the test tank, the dissolved/dispersed PHC content was slightly higher, 6.126 $\mu g\ l^{-1}$. By the 10th day of exposure, the dissolved PHC content in the control tank did not increase significantly whereas that in the test tank showed a significant rise ($p \leq 0.01$).

The uptake of PHC by *S. scripta* varied considerably in the test tank from that of the control tank. On the 5th and 10th days of exposures, the PHC accumulation in the tissues was almost double in the organisms in the test tank than those in the control tank. This significant increase ($p \leq 0.001$ - table XI) in the tissue load of PHC of *S. scripta* exposed to suspended sediment indicates the increased bioavailability of PHC from the resuspended system.

The PHC concentration in the sediment also reduced drastically in the suspension system. The oil contaminated sediment with an initial PHC content of 9.925 $\mu g\ g^{-1}$ wet wt. lost about 50% of its PHC content after 10 days of stirring. Analysis using two way ANOVA indicates a significant reduction in sediment PHC at 0.1% level. (Table XII). In the control tank, however, there was no significant reduction in the PHC content after 10 days, the value being 7.697 $\mu g\ g^{-1}$ wet wt.

TABLE XI

Sunetta scripta - Two way ANOVA showing variation in the tissue load of PHC between control and test animals in relation to the period of exposure to suspended oil borne sediments.

Source	ss	df	ms	F
Total	1821.2636	29		
Days	1556.4149	2	778.2075	181.151***
Treatments	153.1554	1	153.1554	35.652***
Error	111.6933	26	4.2959	
Day's mean	0	5	10	
	0.113	6.3652	17.426	
Treatment mean	Control	Test		
	0.113	6.3652		

*** $p \leq 0.001$

TABLE XII

Sunetta scripta - Two way ANOVA showing variation in the PHC concentration of sediment between control and test samples of the suspension system with duration of exposure.

Source	ss	df	ms	F
Total	83.2982	15		
Days	59.9070	1	59.9070	47.591 ***
Treatments	7.0265	1	7.0265	5.582*
Error	16.3647	13	1.2588	
Day's mean	0	10		
	9.92641	5.9290		
Treatment mean	Control	Test		
	9.0903	7.7649		

*** $p \leq 0.001$

* $p \leq 0.1$

2.5 DISCUSSION

The concept of assessing ventilation rate and O_2 consumption rate to explain toxicant stress arose out of the knowledge that metabolism and activity are interrelated. Variations in metabolic rate modify the scope for activity and the degree of activity affects metabolic rate. Usually, laboratory determinations of sublethal stress can delineate linear or nonlinear responses. Various concentrations of Hg, Cd, Zn and PHC had been demonstrated to create different types of sublethal effects (Baby, 1987). When a bivalve is exposed to a toxicant, reduction of contact with the medium can be brought about by the secretion of mucous or/and closure of shells. However reduction in the period of contact has to be limited since the animal must feed, exchange gases and defeacate.

In a number of field monitoring programmes, physiological components of the energy equation have been determined as an index of stress (Widdows, 1984; Varanasi, 1989). Although it is difficult to establish a cause-effect relationship in field studies because of numerous physical, chemical and biological factors that could influence the biological responses, in most cases, a decline is noted in the physiological condition of the population with increase in the level of anthropogenic stressor (Bayne, 1985). Likewise,

in the present field monitoring study also, the filtration and oxygen consumption rate were found to decrease with the increase in the body burden of PHC in *P. viridis*. The results also highlighted the importance of comparing the effects reflected in animals living in polluted sites with that of unpolluted appropriate reference sites. Kautsky *et al.* (1990) have demonstrated that in mussel transplant experiments over >1000 Km, the physiological responses and growth generally reflect environmental rather than genetic differences.

It has become apparent from the results obtained from the laboratory study that the trend in oxygen consumption and filtration rate can vary from linear to non-linear pattern. In the case of heavy metals it is proved that their presence can either elevate or depress the rate of oxygen uptake in marine bivalves. Normally, heavy metals are respiratory depressants (Mathew & Menon, 1983; Baby and Menon, 1986).

To understand the various factors that affect the rate of filtration, it is necessary to delineate the processes involved in filtration. The water enters the pallial cavity of the bivalve through the inhalant siphon before passing through the gill ostia or into the suprabranchial chamber. Such circulated water is expelled through a narrower exhalent siphon, located at the posterior end of the mantle. Both the

siphons possess velum, which can regulate the current flow. The bivalves are capable of controlling the effective area of lamellar contact with the water by means of mucus.

An enhanced rate of oxygen consumption or energy expenditure, appears to be a common response of molluscs (Widdows *et al.*, 1982); to low or moderate concentrations of PHC although it is not always apparent (Widdows *et al.*, 1985). In the present study, exposure to 1ppm of BHC WAF considerably reduced the rate of oxygen consumption by *P. viridis*. This may be due to the partial valve closure by the animals in the presence of the toxicant. Filtration rate remained almost constant throughout the exposure period by the animals exposed to the same concentration.

P. viridis increased the rate of oxygen consumption and filtration rate when exposed to 5 ppm of BHC WAF. Gilfillan *et al.* (1977) and Gilfillan (1980) have suggested that alterations in energetics and growth of bivalve populations might be related to burdens of aromatic compounds on the tissues. It has been noticed in the present study that the bioaccumulation of PHC by *P. viridis* exposed to 1ppm of BHC was more during the initial phase of experimentation than those exposed to 5 and 10 ppm. On the 5th day of exposure, the tissue concentration of PHC was 3.56 $\mu g\ g^{-1}$ wet wt. for the mussels exposed to 1 ppm whereas it was 2.89 $\mu g\ g^{-1}$ wet

wt. for those exposed to 5 and 10 ppm. This change in tissue load was correlated with the alterations in filtration and oxygen consumption. Thus there was a reduction in these responses in *P. viridis* exposed to 1 ppm and an enhancement in those exposed to 5 ppm. However, on the 15th day of exposure to 5 ppm, the mussels exhibited an erratic behaviour. The oxygen consumption was lowered which was associated with a sudden overshoot of filtration rate. The dye particles would have got enmeshed in the mucus secreted at a rapid rate by these animals. This would have caused a reduction of the dye concentration in the medium and thereby apparently increasing filtration rate.

The observed abrupt lowering of respiration and feeding at very high concentrations must be the result of the moribund state of the animals (Verriopoulos, *et al.*, 1986).

Body burden was found to be controlled by both external concentration and duration of exposure. Low external concentrations resulted in high body burden and low levels of oxygen consumption. Body burden, oxygen consumption and filtration rate are expected to show some relationship in the case of bivalves which are exposed to xenobiotics. However, in the present study, the body burden was found to show some semblance of relationship with concentration and time only when the animals were exposed to 5 ppm of PHC.

Further, increase in the tissue load is a cumulative effect and the variation in oxygen consumption and filtration rate need not be tissue load dependent if the load is not severe enough to hamper with the behavioural pattern of the animal.

Another explanation for the reduced rate of oxygen consumption and enhanced body load is that the trapped oil laden water in the mantle cavity triggered a passive entry of PHC into the body fluid. It is also likely that the anaesthetic effect of the straight chain hydrocarbons (<16C) virtually inactivated the cilia which are the most important organelles controlling ventilation. It is also likely that the highly aromatic hydrocarbon components present in the WAF resulted in increased body fluid flow. It should have also influenced the uptake of oil and oxygen consumption. It is propounded that the thin gill membranes, being semi-permeable, facilitates entry of PHC. This is passive diffusion and takes place across a concentration gradient. The lipid layer of simple biological membranes permits rapid diffusion of lipophilic organic molecules. Further it is opined that the rate of passive uptake at the gill tissue is proportional to the difference between chemical concentration in the water and that in the body fluid. The initial rate of diffusion remains constant if the external concentration also remains constant. Basically this is a simple bioconcentration model and it is

quite likely that this is the basis of bioaccumulation of hydrocarbons in the tissues of bivalves (Rand and Petrocelli, 1985).

Widdows and Donkin (1991) are of the opinion that it is unrealistic to examine the tissue concentration - response relationships for the $>10^4$ individual organic contaminants that enter the environment. Hermens (1986) and Donkin and Widdows (1990) have suggested the application of Quantitative structure-Activity Relationship (QSAR) approach which facilitates the prediction of toxicological properties of organic compounds from their chemical/structural properties, to overcome this problem. Oil is a complex mixture of structurally related organic compounds that form a single QSAR line and their mechanism of toxicity is predicted to be additive when present together (Hermens, 1986). Further, these toxicants induce effects via more than one mechanism of toxicity. Thus pentachlorophenol is both an uncoupler of oxidative phosphorylation, which results in enhanced oxygen consumption and also an inducer of narcosis which reduces ciliary feeding activity of the mussels (Widdows and Donkin, 1992).

An earlier study by transplantation in order to measure the physiological energetics in relation to chemical contaminants in *Arca zebra*, a bivalve which resembles *S. scripta* in structure, behaviour and habitat requirement along

a contamination gradient in Bermuda have led Widdows *et al.* (1990) to conclude that lower molecular wt. PAHs and alkanes with log $K_{ow} \approx 5.5$ are not only bioavailable from the water column, but their uptake kinetics are sufficiently rapid for them to accumulate in the tissues and reach near equilibrium with the water column within 11-12 days of exposure to a contaminated environment.

In *S. scripta*, the exposure to sublethal levels of BHC enhanced the rate of oxygen consumption. This enhancement may be due to the direct effects of PHC on metabolism. Hydrocarbons may increase respiration rate as a result of an uncoupling of oxidative phosphorylation or an increased flux through the glycolytic pathway.

Behavioural responses of an organism to contaminant stress may serve as mechanisms for detecting adverse contaminant concentrations, as well as for triggering adaptive mechanisms. Such adaptation may be the reason for increase in filtration rate towards the end of the exposure periods for both *P. viridis* and *S. scripta*.

The rate of filtration of *S. scripta* exposed to BHC was dose dependent. Under sublethal stress, it showed an elevated filtration rate at and below 15 ppm. The elevated activity may be due to the increased metabolic rate to

compensate the hydrocarbon stress, as explained by the increased oxygen consumption at these sublethal concentrations.

Another point brought about by the results, was the rapid decline in the body burden of PHC of *S. scripta* after 10 days of exposure. The PHC load in the tissues lowered from 58.96 $\mu g\ g^{-1}$ wet wt. on the 10th day of exposure to 24.56 $\mu g\ g^{-1}$ wet wt. on the 15th day of exposure. It has been reported that mussels may develop certain adaptative mechanisms that could neutralize in part the toxic effects of hydrocarbons, since planimetric parameters show certain recovery of digestive tubule cell condition after long exposure times (Cajaraville, 1992 (b)). But long term exposure is also associated with poor individual condition and with reduced somatic growth. It could be suggested that the extra demand of energy needed to develop adaptative mechanisms such as antioxidant detoxication processes might lead to significant changes in individual growth and condition (Cajaraville *et al.*, 1993).

Non-polar organic contaminants such as PAH and PCBs are bound to particles in the water and accumulate in sediments. By the action of tidal currents or storms, the less dense sediment particles are brought into suspension. Filter feeding animals which efficiently filter particles from

sea water could accumulate large quantities of these contaminants in their body tissues through these particles. Clams exposed to suspended particles rapidly accumulated more PHC than those in the control tank. Similarly, the dissolved PHC in the overlying water also increased considerably in the test tank, their only source of PHC being the contaminated sediment. Partitioning models by Chiou *et al.* (1977) and Mackay *et al* (1980) predict that the lower molecular weight compounds, which have higher water solubilities, could be more readily desorbed from the sediment into the dissolved phase. Sanford (1992) in his study has demonstrated using models that resuspended particles are transported between the water column and surficial sediments several times before being buried in the shallow waters of the Chesapeake Bay. Such activities increase the water column residence times of particle bound hydrocarbons (Baker *et al.*, 1991 (b)).

S. scripta, being a burrowing benthic bivalve feeds by sieving out particles from the water column just above the sediment-surface. This food collection by filtration is performed by the greatly enlarged ctenidia located on either side of the visceral mass. Powerful lateral cilia located on each side of the ctenidial filament and arranged in a continuous row create the inhalent and exhalent currents that enter and leave the inhalent and exhalent siphons respectively. This indicates that the PHC from the medium has

entered the animal through water as well as through the particles. A similar study was conducted by Vandermeulen (1982) on the bioavailability of hydrocarbons to the soft-shelled clam, *Mya arenaria* and *Patella sp.* inhabiting the low-energy mud flats, nine months after they were heavily contaminated by oil due to the Amoco Cadiz disaster. Vandermeulen (loc.cit) found that the tissue hydrocarbon fluorescence spectra resembled more closely those of water column samples rather than those of either stranded Amoco Cadiz oil or sediment-bound hydrocarbons. He is of the opinion that even though the mode of feeding is different in the two molluscs studied, *Patella*, by grazing on tidal-washed rock surfaces and *M. arenaria*, by sampling the water column above the sediment surface, the bioavailability route of the stranded hydrocarbons to these organisms is via water rather than through the sediments.

Bioaccumulation of xenobiotics from contaminated sediment or water depends mainly on the mode of feeding of the test animal. English sole (*Parophrys vetulus*) and other benthic fishes are able to take up particle bound xenobiotics either directly from the sediment or via food organisms which ingest particles containing xenobiotics. (Varanasi *et al.*, 1987). Exploring the reason for the inhibited growth of burrowing polychaete *A. brevis* exposed to sediments from contaminated sites in Puget sound, Varanasi (1993 (b)) found

that the animals had taken up chemicals all through their exposure to sediments.

Burns *et al.* (1990) are of the view that PAH bioaccumulation of most benthic invertebrates is from the aqueous phase rather than from the contaminated sediment particles. On the contrary, Pruell *et al.* (1987) argue that the PAH distribution in *M. edulis* exposed to resuspended contaminated sediments was similar to that found on the particles.

The use of sub-lethal effects significantly enhances our ability to test the toxic effects of xenobiotics. Based on the results in this chapter, it is evident that field studies rather than the contaminated levels in individuals are better in understanding the mechanisms of toxicity and how the animal responds to toxic insult.

CHAPTER 3

HISTOPATHOLOGY

3.1 INTRODUCTION

The study of structural damage of organs or tissues is an integral part of pollution toxicology. Organisms in general and aquatic ones in particular are easily susceptible to the toxic effects of environmental contaminants. A clear understanding of the cause and effect of such toxic reactions could be identified only with the help of histopathology. In respect of analysis of toxic effects, histopathology is more of a primary nature while physiology or biochemistry are supplementary.

Changes in rate functions at cellular level would manifest by alterations in the structure of cellular organelles. Such changes would ultimately reflect in the physiological processes of the animal (Moore, 1980). Depending on the nature of the toxicant, some of these cellular changes could be specific, whereas some could be of a general nature to toxicant stress (Moore, 1985).

Whilst there is considerable literature on morphometric changes associated with adverse environmental conditions in a wide range of fish, much of the quantitative histology on invertebrate response to stress has concentrated on reproductive and digestive cells of three groups - oysters, clams and mussels. However, Sindermann (1988), Calow

(1989 (a), (b)) and many other biologists believed these cellular indicators alone to be of poor ecological significance. Accordingly, Widdows (1985) proposed that measurements of cellular response should be carried out in conjunction with and should complement to measurements of organismic responses, which are readily interpretable as adverse or beneficial. It was with this intention that histopathology was included in the present investigation.

3.2 REVIEW OF LITERATURE

Xenobiotic induced cellular pathology reflects disturbances of structure and function at the organelle level of biological organization. When cells are stressed, they undergo a series of often irreversible biochemical and structural alterations. Cells are able to continue their existence following many types of sublethal injury by means of adaptive physiological responses. Examples of such adaptations in mammalian as well as other vertebrate and invertebrate cells include hypertrophy, atrophy, changes in the lipid contents, proliferation of smooth endoplasmic reticulum, increased lysosomal autophagy, aging,, neoplastic transformations and accumulation of materials such as lipofuscin (Trump and Arstila, 1975; Varanasi, 1989). Histopathological approaches are useful in providing a real

picture of the degree of such disturbances within the organ systems concerned.

Analysis of cell function and structure offer a means of identifying and characterizing adaptive responses or reactions to cell injury by PAH and other xenobiotics. In cell toxicology studies, absorption or intake, distribution, biotransformation and elimination of the toxicant are the key factors which influence its toxicokinetics. In the body of an aquatic organism, a toxic compound like PAH may be absorbed in the digestive apparatus through the skin or gills. It can also get distributed to fatty tissues like gonads. Most xenobiotics that enter the body undergo metabolic transformation, mainly in the liver. Thus field studies indicate strong correlation between levels of sediment PAHs and prevalence of liver lesions in *Parophrys vetulus* from several contaminated sites in Puget Sound (Myers *et al.*, 1990). The routes of elimination of these toxicants are via the kidneys and gills.

During the last decade, fish was used widely to indicate the degree of oil pollution. Woodward *et al.* (1983) and Prasad (1988) who studied the histopathological effects of crude oil on fishes at the light microscopic level observed that the changes associated commonly with the gills were lesions, oedema and mucous cell hyperplasia. A reduction in growth and high incidence of fin erosion were noted as

transient effects in fishes from the heavily contaminated waters of the Brittany coast after the wreck of Amoco Cadiz (Mc Intyre, 1982).

Pollutants constitute forms of environmental chemical stress and much of what we call "pathology" or "disease" is a consequence of actions of environmental stress. For example, bottom fish species from a number of chemically contaminated marine coastal areas were affected with pathological conditions associated with chemical contaminant exposure, especially liver lesions such as neoplasms and other lesions involved in the histogenesis of hepatic neoplasia (Malins *et al.*, 1988; Myers *et al.*, 1990). Therefore, certain pathological conditions like neoplasms, non-neoplastic proliferative lesions, specific degenerative or necrotic lesions, hydropic vacuolation of biliary or hepatocellular epithelial cells etc. are currently regarded as having utility as biomarkers of contaminant exposure effects (Moore *et al.*, 1989; Varanasi *et al.*, 1992 (a)). In a National Benthic surveillance project (Johnson *et al.*, 1992) to study fish histopathology and relationship between lesions and chemical contaminants, concentrations of high and low molecular weight aromatic hydrocarbon or their metabolites were measured in the sediments, stomach contents, liver and bile of *Pleuronectes americanus* to give a comprehensive picture of exposure and relationships between contaminant levels in these compartments

and idiopathic diseases. However, contaminant levels in liver and bile were considered to have the greatest relevance as toxicological risk factors, because they are most representative of the actual uptake of toxic compounds by fish. As Malins *et al.* (1984) comment, bottom-dwelling fish appear to be especially at risk from toxic chemicals, probably because of their close associations with and prolonged exposures to contaminated sediment.

Fletcher *et al.* (1982) noted a reduction in the size of the testes in male *P. americanus* exposed to oil sediments, the probable reason being the increase in metabolic demands of the fish by the oil, thereby requiring them to utilise the testes as an energy source.

Several workers have established the association between different cellular/tissuelar and organismic responses to pollution in mussels (Moore *et al.*, 1984; Viarengo *et al.*, 1984; Varanasi *et al.*, 1989; Cajaraville *et al.*, 1993). Xenobiotics exert negative effects on certain cellular and physiological functions, which may lead to disease either infectious or non-infectious (Mix, 1988) in shellfish. Bayne *et al.* (1980) give the following categories of histological changes that may be useful for monitoring pollution effects: hyaline degeneration of the collagenous connective tissue of

the gills, parasitic burden, production of mucus by gills, gonadal and haematopoietic neoplasms, granulocytomas, haemocytic infiltration of tissues and loss of synchrony in digestive tubules. Marine molluscs also contain a number of lysosome-rich tissues and PAHs are known to induce deleterious alterations in lysosomal structure and latency of lysosomal enzymes (Moore and Farrar, 1985; Moore, 1991). Histological analysis of mussels (*M. edulis*) from both mesocosm treatments and field sites indicate gamete degeneration of the germinal tissues (Lowe, 1988). The study also recorded a disturbance in lipid levels, resulting in alterations in digestive cell architecture.

Basically, there is a fundamental unity between the structural organization and various functions of cellular components. This has been well illustrated by studies on different organelles of various organisms which clearly show structural and related functional alterations when exposed to xenobiotics. Heavy metal accumulation stimulates lipid peroxidation process and formation of lipofuscin granules. These lipofuscin granules in turn, may trap toxic metals in a relatively stable form and may subsequently get eliminated by exocytosis of the residual bodies (Viarengo, 1985). Some effects such as destabilization by lysosomal membranes can be mechanistically linked to lysosomal enlargement and lipofuscin accumulation, both of which are indicative of autophagy.

Autophagy in turn is directly linked to digestive cell atrophy (Moore, 1988; Lowe and Pipe, 1994).

In bivalve molluscs, the digestive diverticula consist of blindly ending tubules which are linked to the stomach by a system of branched, partially ciliated ducts. The tubular epithelium contains two cell types - (i) acidophilic - columnar and vacuolated; and (ii) basophilic-pyramidal. The acidophilic cells are responsible for intracellular digestion of food and are usually referred to as digestive cells (Bayne, 1980). The basophilic cells are equipped for extensive protein synthesis(Owen, 1972).

Various investigations have concluded that cells of the digestive epithelium of bivalve and gastropod molluscs are important targets of the toxic action of different metallic and organic pollutants (Viarengo and Moore, 1982; Moore, 1988; Cajaraville *et al.*, 1993). Generally, thinning of digestive epithelium is observed in contaminated or stressed mussels (Couch, 1985). In the case of *M. edulis* exposed to diesel oil and copper mixture, Lowe (1988) reported pathologically enlarged heterogeneous secondary lysosomes, alterations in the architecture of pyramidal cells and thinning and erosion of epithelium in the digestive gland tubules. Severe tissue alterations including haemocytic diapedesis were reported in the digestive gland of *M. edulis* exposed to a mixture of

diesel oil and copper by Auffret (1988). Moore *et al.* (1982) observed significant reduction in the latency of lysosomal aryl sulphatase, β-glucuronidase and phosphatase from four polluted sites near Sullom Voe oil terminal. Digestive glands of molluscs are especially rich in lysosomes (Owen, 1972; Moore, 1982). Therefore the earliest detectable changes in many instances of xenobiotic induced cellular pathology are associated with these lysosomes (Slater, 1978). Further, aromatic hydrocarbons significantly reduced the protein level and thus disrupted the normal metabolism of the digestive gland of *M. edulis* (Viarengo and Moore, 1982). Malins *et al.* (1984) identified one or more lesions of the gills, hepatopancreas, midgut and antennal gland in virtually all the crabs from Everett Harbour. According to them, reason for this may be that these sites are portals of entry or absorption, storage and excretion of toxic chemicals.

Ray (1987) observed that in *M. edulis* exposed to Prudhoe Bay crude oil for 8-9 weeks, even though the haemocyte counts increased due to higher densities of granulocytes, their phagocytic response reduced. This was supported by the findings of Lowe (1995) on *P. viridis* transplanted to a contaminated site at Phuket oil Depot. He found a significant reduction in the dye retention capacity of the blood cell lysosomes indicative of a disturbance to the membrane structure.

Sunila (1988) correlated the occurrence of inflammatory reactions, ulcers and haemorrhages of the digestive tract and kidney lesions in *M. edulis* with polluted sites. According to Cajaraville *et al.* (1990), proliferation of basophilic cells in the digestive epithelium of petroleum hydrocarbon treated mussels constitutes an adaptation mechanism, possibly associated with the simultaneous loss of digestive cells after injury and with disintegration/ regeneration process of digestive tubules.

The basic structure of the gills of the lamellibranchs is described in the works of Paparo (1972) and Aiello and Sleigh (1972).

Atrophied epithelium, sloughing of cells and lesions as an ultimate tubular degeneration of gills have been reported by Sunila (1986) in mussels exposed to copper and cadmium. When *M. edulis* were exposed to copper and cadmium, connections between the tips of cilia and microvilli broke, cilia pairs got separated and the ciliary discs slided apart (Sunila and Lindstrom, 1985). Assessing the pathological changes in *P. indica* and *Donax incarnatus* on exposure to copper, cadmium and mercury, Mathew (1990) has opined that the inflammation and necrosis of the gill epithelia, sloughing off of epithelial cells etc. is a general phenomenon that could occur to mussels exposed to any kind of stress. He also found

that the metals had a synergistic effect when used in combination, causing greater damage of affected tissues.

Axiak and George (1987) discussed the energy budget of the clam *Venus verrucosa* in relation to the responses of ciliary activities on exposure to PHC. They found that the reduced pumping rates and interference with the normal beating activities of the lateral and eulaterofrontal cilia led to reduced clearance rates. On the other hand frontal ciliary activities were significantly accelerated, directing oil droplets to the mouth region as food particles. Berthou *et al.* (1987) found that even seven years after the Amoco Cadiz spill, the oysters *Crassostrea gigas* and *Ostrea edulis* showed minor damages (necrotic lesions) of the digestive tract, intestinal tissue and gills. Deformed gills with filaments fused and covered by a united epithelium were found in organisms found in the vicinity of an iron and steel factory in the Gulf of Finland (Sunila, 1987). Damages in mussels exposed to both 30 and 130 ppb diesel oil indicate a direct impairment of the reproductive processes and the implication is that reproductive capability would be reduced, both by degeneration of oocytes and reduction in ripe gametes, as well as by a reduction in the energy reserves available for gametogenesis as supplied by the connective tissue storage cells. (Lowe *et al.*, 1982; Lowe and Pipe, 1985).

In spite of all the structural damages caused by pollutants, marine bivalves survive and reproduce in heavily polluted waters. Membrane vesicles isolated from the gills, mantle and digestive gland of the marine mussel *M. galloprovincialis* indicated a *p*-glycoprotein mediated multi-xenobiotic resistance mechanism (Kurelec and Pivcevic, 1991) and a multi-drug resistance mechanism similar to that described in resistant tumour cell lines (Kurelec and Pivcevic, 1991). Kurelec (1992) and Waldmann *et al.* (1995) demonstrated the function of multi-xenobiotic resistance mechanism in pumping out anthropogenic toxic chemicals in aquatic organisms exposed to a polluted environment and indicated its role as part of a biological defence mechanism of organisms.

The number of circulating haemocytes in mussels has been shown to increase significantly under stressed conditions (Renwrantz, 1990). In *M. edulis*, an increase in total number of circulating blood cells was observed following exposure to fluoranthene (Coles *et al.*, 1994), whereas in some other bivalve species a reduction in total haemocyte numbers was noted following exposure to copper (Suresh and Mohandas, 1990). Since granulocytes play significant roles in phagocytosis, granuloma formation and internal defence in molluscs exposed to pollutants would need increased numbers of granulocytes to remove the overload of pollutants.

Statistical analysis of cellular damage have proved to be an important method in describing histological alterations due to pollutional effect (Widdows *et al.*, 1982; Cajaraville *et al.*, 1993). In a recent study, Pillai *et al.* (1997) calculated the extent of pathological damage to the digestive tubules and gills of *P. viridis* exposed to copper and mercury for four weeks, by employing rank analysis. Major structural damages considered were the reduction in height of epithelial cells of digestive tubules, presence of infiltrated haemocytes in the tubular lumen, dilation of branchial vein and presence of swollen endothelial cells of the gill filaments. The statistical results were found to substantiate the conclusions based on observations under light microscopy.

3.3 MATERIALS AND METHODS

This part of the thesis centred around delineating the histopathological effects on the gills and digestive diverticula of *P. viridis* and *S. scripta* exposed to both sublethal and lethal concentrations of WAF of Bombay high crude oil for a period of 15 days with periodical sampling on 5th, 10th and 15th day of exposure.

The details of the test animals, toxicant concentration and preparation of exposure medium, etc. have been given earlier in sections 2.3.1 to 2.3.4.1

3.3.1 PREPARATION OF MICROSLIDES

After termination of the exposure period, the animals were dissected out and digestive tubules and gills were washed in running water and immediately fixed in the Bouin's fixative for 24 h. The following time schedule was used to make paraffin wax blocks for histological studies.

1. Washed overnight in running water.
2. The tissues were treated with saturated solution of lithium carbonate in 70% alcohol to remove yellow colour of picric acid.
3. After softening, the tissues were stored in fresh 70% alcohol. In this stage, the tissues can be stored till further processing.
4. Tissues dehydrated by transferring them sequentially to 80%, 90% and 95% alcohol - 2 h each.
5. Transferred to absolute alcohol (2 changes) for 1 h each.
6. Placed the tissues in 1:1 mixture of absolute alcohol and xylene for 30 min.
7. Cleared in xylene until the tissues became translucent.
8. Tissues transferred to a mixture of xylene and paraffin wax and left overnight.

9. Infiltered the tissues in 2-3 changes of molten paraffin wax of melting point 60-62^{o}C for 1 h each.

10. Embedded in paraffin wax of melting point 60-62^{o}C.

The blocks were trimmed and sections of 5 μ m thickness were cut with a rotary microtome.

3.3.2 STAINING TECHNIQUE FOLLOWED WITH TRIPLE MALLORY STAIN

1. Deparaffinised and hydrated slides to water. As $HgCl_2$ is absent from fixative, sections treated with saturated aqueous mercuric chloride (mordant) plus 5% glacial acetic acid for 10 mins.

2. Washed, treated with Lugol's Iodine and Sodium thiosulphate.

3. Washed and rinsed in distilled water.

4. Stained in Mallory I: 15 sec.

5. Rinsed in distilled water : 10 sec.

6. Treated in phosphomolybdic acid : 1-5 min.

7. Stained in Mallory II : 2 min.

8. Rinsed in distilled water.

9. Differentiated aniline blue in 90% alcohol: 5 sec.

10. Dehydrated in absolute alcohol (2 changes): 1 min. each.

11. Cleared in xylene.

12. Mounted in D.P.X.

3.3.3 STATISTICAL TESTS EMPLOYED

3.3.3.1 **Wilcoxon signed rank test**

To confirm that the enlargement of cells has taken place as a consequence of PHC pollution, this statistical analysis was carried out using the following methodology.

The test consisted in taking count of the number of cells within 1 mm^2 area in eight different fields. Since the area considered in all was the same, the decrease in number of cells could be considered as evidence of enlargement. The statistical test used in this connection is the *Wilcoxon signed rank test* for paired observations. Since the two samples under comparison could not be viewed as independent the hypothesis considered is that there is no difference in the number of cells per unit area before and after exposure to PHC. The alternative hypothesis is that the average number of cells per unit area is less after exposure to PHC. The readings at individual fields are compared to see whether there is evidence of decrease in the number of cells after implementation of pollution.

The 'r' value is the sum of the rank of negative differences. If the hypothesis of no differences in the number of cells is true, then the sum of positive ranks and negative ranks would 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.

3.3.2.2 **Rank correlation**

To analyse the histological status of tissues by assessing the degeneration undergone by the tissues due to the effect of PHC, a method of rank correlation was employed. The statistical problem is to test whether there is an increase in the degree of degeneration of tissues due to the effect of PHC. Various ranks - 0 (nil), 1 (low), 2 (medium) and 3 (high) are given to the different stages of degeneration based on visual and micrometer measurements. Since it is unlikely to observe an increase in degeneration in the absence of pollutants, positive correlation between the detailed ranks was tested. The hypotheses considered were (1) there is no tendency for the PHC dosed tissues to undergo degeneration - against the alternative that (2) there is some tendency to

increase the degree of degeneration i.e., to increase positive correlation.

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

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

where n = number of observations in sample

d = difference in rank

For samples of size 12, the hypothesis of positive correlation is supported by $r \geq 0.5035$.

The indices selected for the assessment of cell damage and tissue damage following rank correlation method has got limitations. The various parameters selected are supposed to give an overall picture of the health of the cell. Absence of a few of these indicators while examining the affected tissues can result in arriving at conclusions contradicting the actual nature of damage occurring to the tissues.

3.4 RESULTS

The gills and gastric diverticula (digestive tubules) of animals exposed to various concentrations of

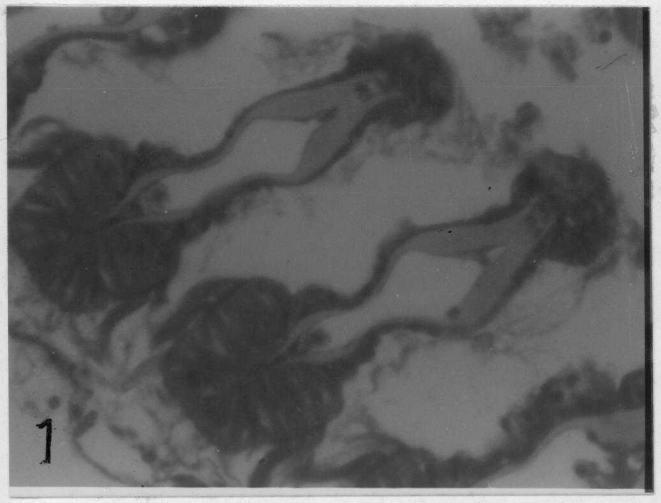
Bombay High crude oil have been subjected to a thorough histological study to assess morphological variations, if any. The ratio of disturbances in the morphology was assessed by comparing the photomicrographs with those of control animals.

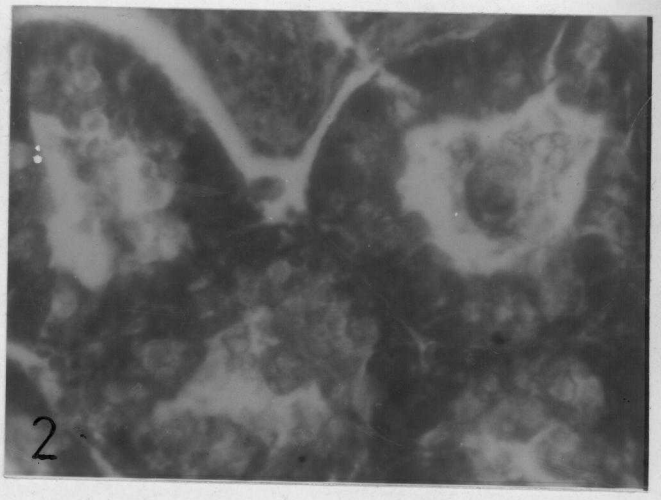
Many species of fish and shellfish often depict species specific differences in gross anatomical and histological characteristics. Therefore in experimental situations, use of normal control animals are indispensable for the diagnosis of specific lesions and their etiology.

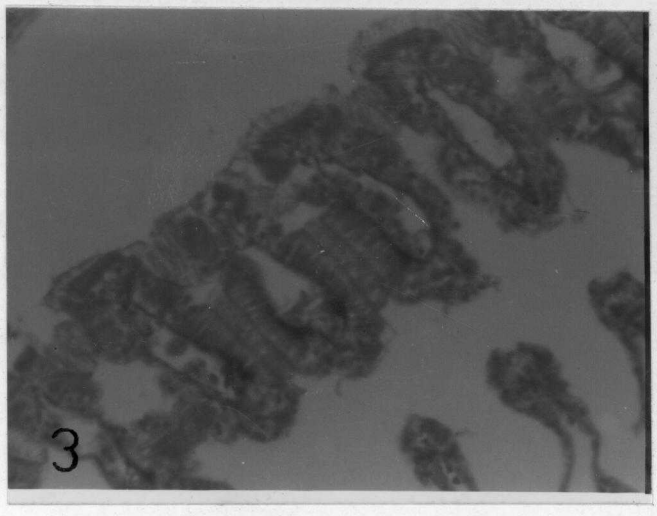
3.4.1 *PERNA VIRIDIS*

Photomicrographs 1 and 2 show the structure of the gill filaments and digestive tubules of control animals.

The gills consist of four pairs of demibranchs which separate the pallial cavity into inhalant and exhalent chambers throughout its length. Each demibranch comprises of two lamellae, one ascending and one descending. Interlamellar blood vessels connect ascending and descending lamellae. Individual lamellae are formed of rows of ciliated filaments, which are joined to each other by ciliary interfilamentar junctions. A branchial vein runs through a filament, the epithelium of which is formed of several cell types, either ciliated or non-ciliated. Innermost in the branchial vein are flattened epithelial cells that lie beneath the supporting







Perna viridis : C.S. of gill filaments and digestive tubules of animals maintained under control conditions.

Photomicrograph 1 : Gill filaments x 200

Photomicrograph 2 : Digestive tubules with ingested food material in the lumen x 200

Photomicrograph 3 : Gill filaments showing interfilamentar ciliary connections x 100.

chitinous rod which has a strong affinity for aniline dyes and turns blue on staining. The two rows of muscles *viz.*, the frontal and the abfrontal, maintain the structural integrity of the gill. The frontal end of the filament is composed of columnar frontal cells with frontal cilia. Adjacent to these cells lie the large ciliated laterofrontal cells. The post laterofrontal cells are non-ciliated. A row of lateral cells bear the lateral cilia. Small, non-ciliated post lateral cells are gradually replaced by the squamous endothelial layer of the branchial vein. Where the interfilamentar junction is present, the endothelial cells are replaced by ciliary discs. The abfrontal end of the filament consists of abfrontal cells that asymmetrically bear the abfrontal cilia. Beneath the ciliated abfrontal cells are seen the mucus glands.

The gills of the control animals maintain a normal structure as described above. The interfilamentar ciliary junctions which lock the filaments to each other at regular distances could be seen in the photomicrograph 3. A few haemocytes are seen migrated into the vein as well as among endothelial cells. However, the presence of granulocytes in the vein and among endothelial cells are considered as a sign of inflammatory reaction (Sunila, 1986).

In bivalves, intracellular digestion occurs in the digestive cells. The digestive diverticula consist of

blindly ending tubules linked to the stomach by a system of partially ciliated main ducts and non-ciliated secondary ducts. Tubules are more or less circular in cross-section, surrounded by a sheath of collagen fibres, and an external system of smooth muscle fibres forming a meshwork. Occasionally, the basal regions of the cells lining the tubules are markedly indented by the underlying muscle fibres, suggesting that the latter are contracted. The tubular epithelium contains two types of cells (1) the larger acidophilic or digestive cells which are columnar and vacuolar (2) the basophil cells which are pyramidal and flagellated, occurring in three or four well defined groups occupying crypts which extend the length of the tubule.

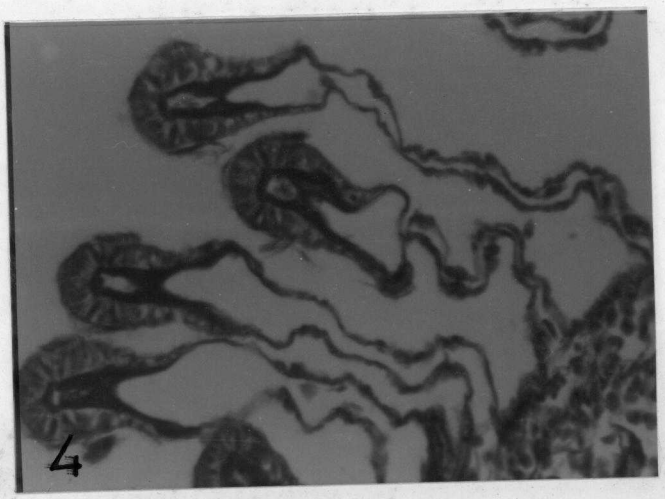
The digestive tubules of control animals showed a normal structure with food granules in the lumen. The tubules seemed to be in the absorptive phase. High percentages of absorptive digestive tubules seem to reflect a good nutritional condition of the mussels.

Five days of exposure to 1 ppm of PHC, the gills depicted enlargement resulting in less number of gill filaments occupying unit area (Photomicrograph 4) The chitinous rods were denuded of abfrontal cells as a result of detachment. Lateral cilia of the gills remained intact whereas cilia of other cells were found considerably

distorted. The branchial vein was dilated and endothelial cells were swollen. The interlamellar space was found to be filled with haemocyte like granules. Collagenous layer of the digestive tubules had disintegrated. Increased vacuolation has resulted in the enlargement of the epithelial cells.

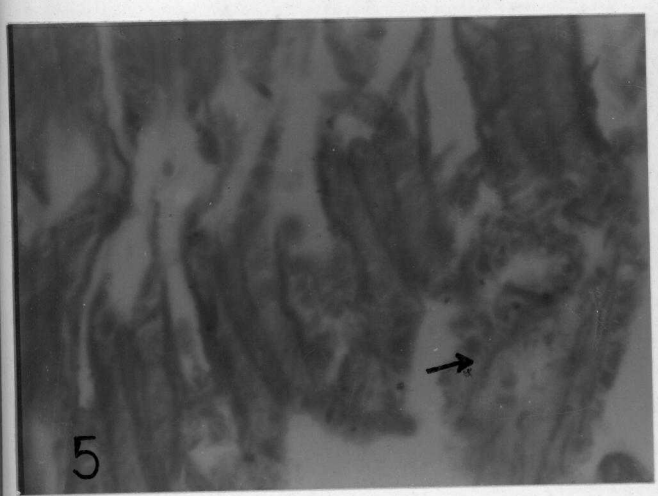
Exposure for 10 days resulted in the following damages. The chitinous rod was broken at many places due to damage of abfrontal and frontal muscles, this probably has resulted in dilation of branchial veins also. All cilia were found ruffled in the frontal region. Endothelial cells were swollen and vacuolated. Detached abfrontal cells resulted in the development of a space at the proximal end of the gill filament and this space was interspersed with haemocytes (Photomicrograph 5). Vacuolation of the acidophilic cells was the major damage that occurred to the digestive tubules of *P. viridis* exposed to 1 ppm oil after ten days. The collagenous layer was found to be detached, disrupted and dislocated. There was marginal increase in the number of acidophilic cells (photomicrograph 6).

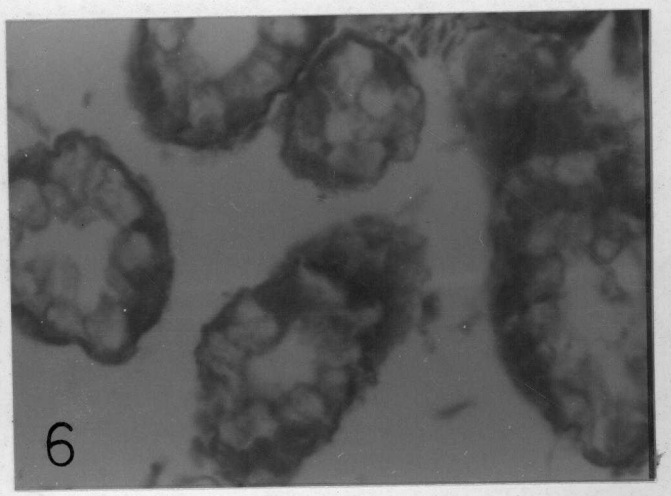
Basically the nature of damages occurred to gills and digestive tubules of the animals exposed to 1 ppm of oil for a period of 15 days was the same. However, the extent of damage was much more than that occurred after 10 days. The branchial vein was dilated considerably. The chitinous rods of



Perna viridis : C.S. of gill filaments of animals exposed to 1 ppm PHC for 5 days.

Photomicrograph 4 : Gill filaments showing enlargement x 100





Perna viridis : C.S. of gills and digestive tubules of animals exposed to 1 ppm PHC for 10 days

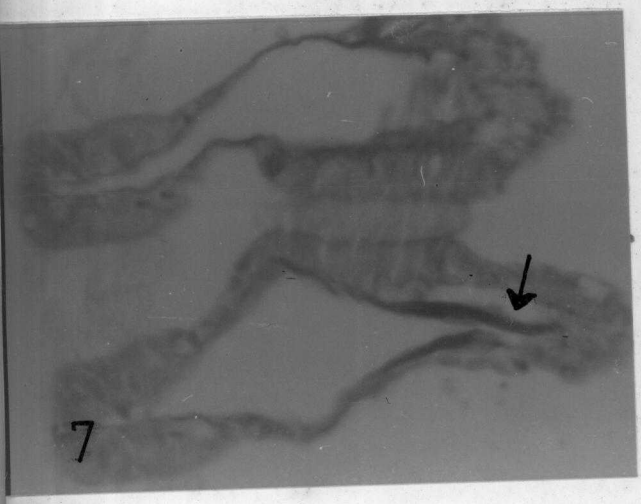
Photomicrograph 5 : Gill filaments showing damaged abfrontal cells (arrow) x 200.

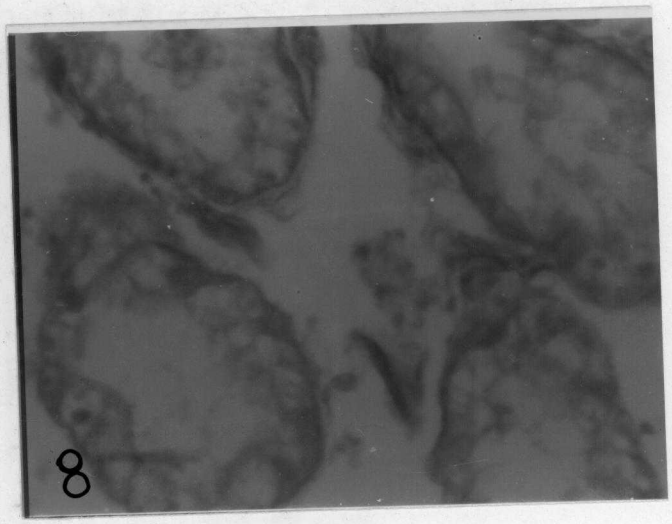
Photomicrograph 6 : Digestive tubules x 200.

the gills were broken at both the ends. Highly vacuolated endothelial cells was found loosely attached to the chitinous rod (photomicrograph 7). Dilation of digestive tubules, heavy vacuolation of cells, atrophy of different digestive cells resulting in sloughing off of cells were the conspicuous features of damages of digestive tubules (photomicrograph 8).

Five days of exposure to 5 ppm PHC, led to enlargement and slight vacuolation of both epithelial and endothelial cells, giving an impression that the gill filaments have enlarged in size. Frontal cells bearing cilia were highly distorted and branchial vein dilated. The gill filaments looked disjointed due to tearing off of tissues at the interfilamentar junction. Vacuolation was high in digestive tubules. Atrophy of cells noticed. The lumen of the digestive tubules was sometimes occupied by dislodged vacuolated cells.

There was an increase in the number of haemocytes in the gill filaments after ten days of exposure. Sloughed off endothelial and abfrontal cells were visible (photomicrograph 9), The digestive tubules were found enlarged when compared with that of control. Presence of wandering haemocytes in the surrounding connective tissue probably indicates internal bleeding due to blood vessel rupture (photomicrograph 10). Heavy vacuolation of the epithelial cells resulted in the

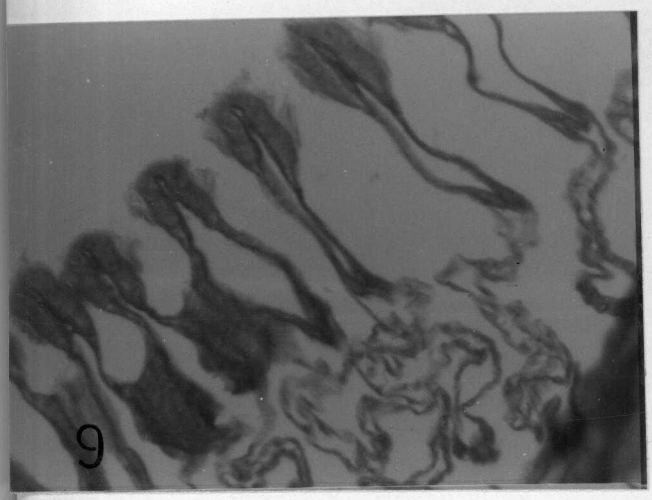


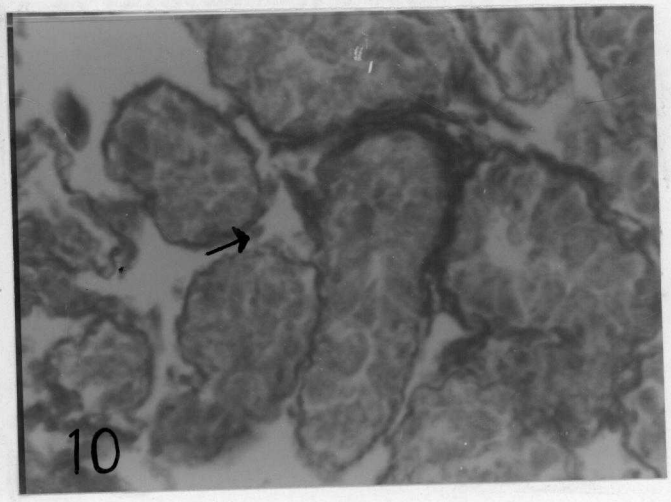


Perna viridis : C.S. of gill filaments and digestive tubules of animals exposed to 1 ppm PHC for 15 days.

Photomicrograph 7 : Arrow indicating detachment of endothelial cells from chitinous rod x 200

Photomicrograph 8 : Digestive tubules with atrophied and sloughed off cells x 200

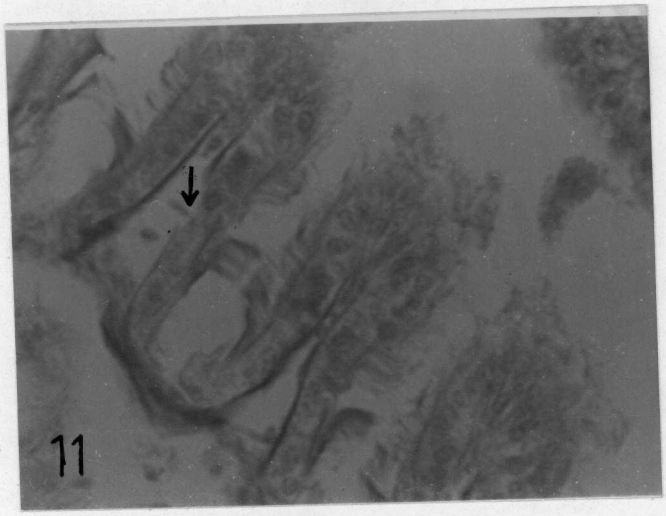


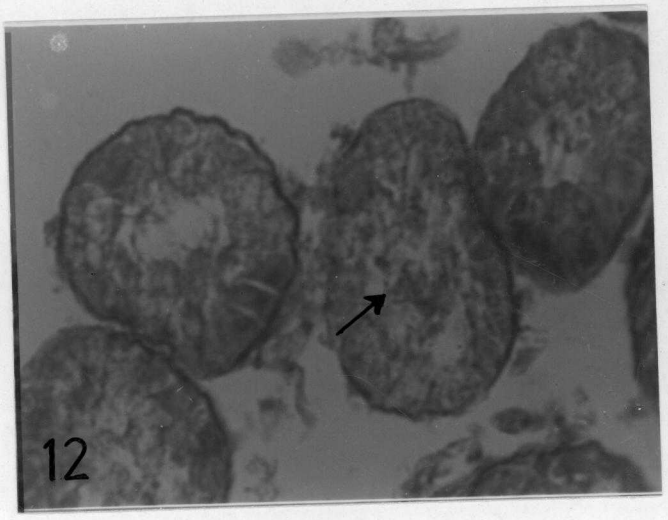


Perna viridis : C.S. of gill filaments and digestive tubules of animals exposed to 5 ppm for 10 days

Photomicrograph 9 : Gill filaments x 100

Photomicrograph 10 : Arrow indicating wandering haemocytes x 200





Perna viridis : C.S. of gill filaments and digestive tubules of animals exposed to 10 ppm PHC for 5 days

Photomicrograph 11 : Gill filaments with broken chitinous rod (arrow) x 200

Photomicrograph 12 : Digestive tubule with obliterated lumen (arrow) x 200

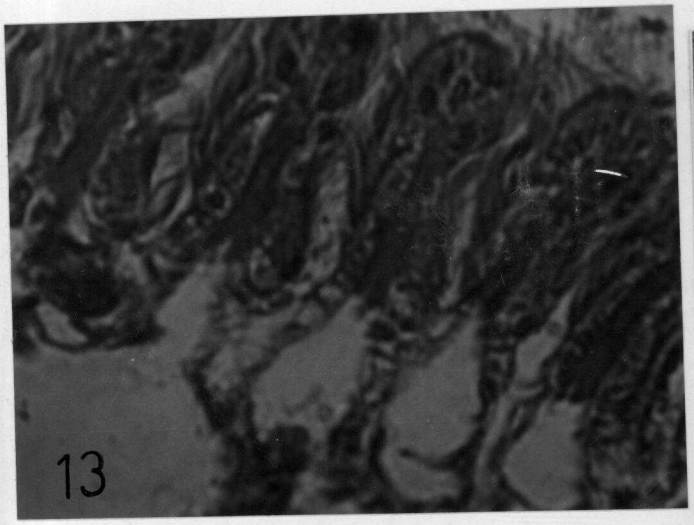
obliteration of the lumen of the digestive tubules. Sloughed off cells were also seen in the lumen.

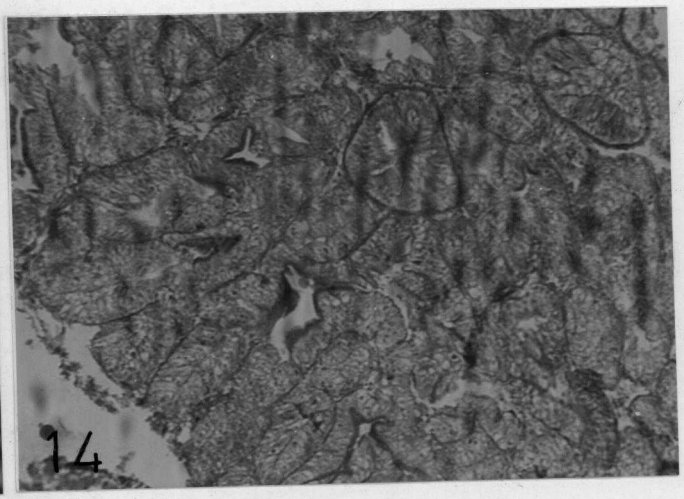
Apart from all the pathological indications, the animals exposed for fifteen days showed further cytological disturbance. This included partial disintegration of the collagenous layer of digestive tubules, migration of basal nuclei to the apex of the cell etc.

Enlargement of gills and digestive tubules, distortion of frontal cilia and infiltration of haemocytes into the laminar spaces were the immediate indications of exposure to 10 ppm for 5 days (Photomicrograph 11). Chitinous rod was found broken at many places. The lumen of the digestive tubules was found loaded with degenerated and sloughed off epithelial cells (photomicrograph 12).

3.4.2 *SUNETTA SCRIPTA*

The gills and digestive tubules of the control animals of *S. scripta* (photomicrograph 13 and 14) showed similarity in basic structure to that of *P. viridis*. The digestive tubules did not have much luminar space. The gill filaments were, however, longer than those of *P. viridis*.

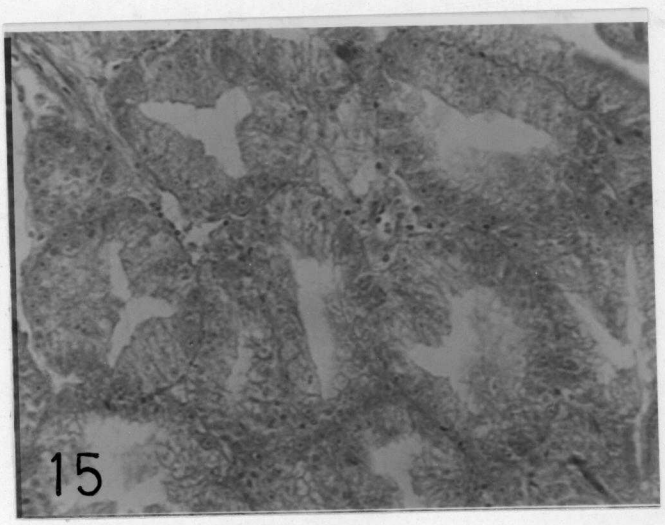




Sunetta scripta : C.S. of gill filaments and digestive tubules of animals maintained under control conditions.

Photomicrograph 13 : Gill filaments x 400

Photomicrograph 14 : Digestive tubules x 100



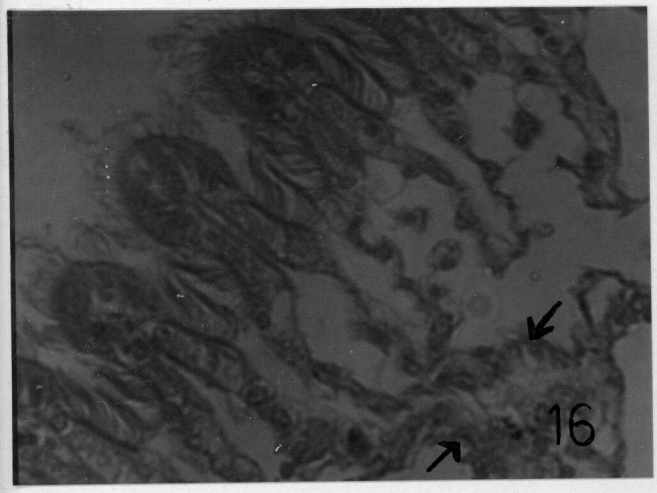
Sunetta scripta : C.S. of digestive tubules of animals exposed to 5 ppm PHC for 5 days.

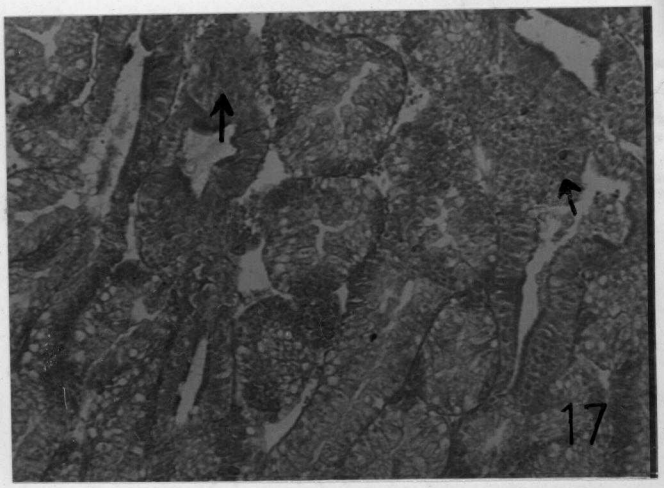
Photomicrograph 15 : Enlargement of digestive tubules x 200

There was an overall shrinkage of the gill filaments after five days of exposure to 5 ppm PHC. Detachment preceded by enlargement and vacuolation resulted in the formation of a space between the epithelial cells and the chitinous rod. The nuclei of the epithelial cells migrated to the apex of the cell. Large number of haemocytes were noticed in the abfrontal area. Enlargement of digestive tubules, and the vacuolation of acidophilic cells were the pathological effects on the tubules (photomicrogrpah 15).

After ten days of exposure, enlargement and partial disintegration of cells in the abfrontal area has resulted in the formation of spaces in the interlamellar regions of the gill (photomicrograph 16). However, cilia were intact in the partially damaged and vacuolated frontal and endothelial cells. Wandering haemocytes were found within the space developed as a result of disintegration of abfrontal cells. In general, the digestive tubules indicated signs of deterioration by the presence of sloughed off cells, haemocytes and at times, granulocytomas (photomicrograph 17). There was relative increase in the number of basophil cells. Migration of basal nuclei to the apex was also visible (photomicrograph 18).

The abfrontal cells of gill filaments were found enlarged due to heavy vacuolation after 15 days exposure.

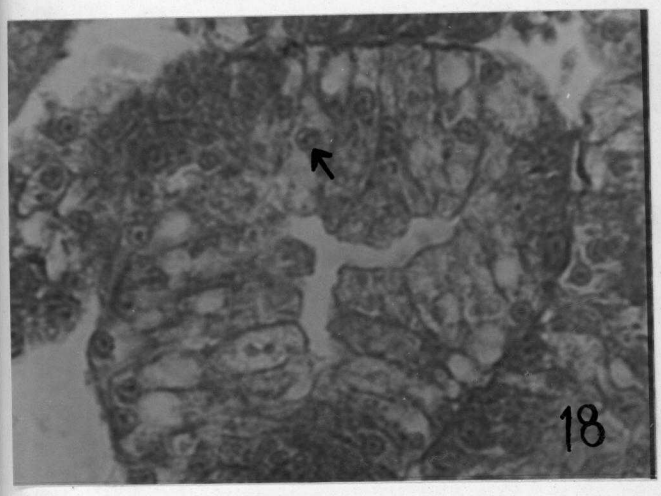


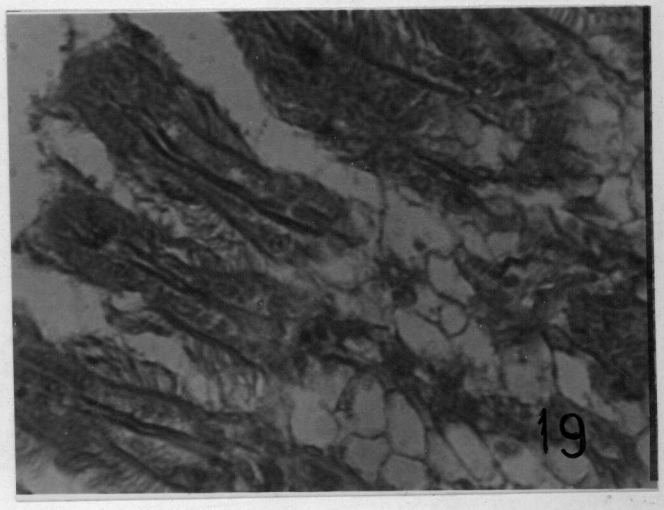


Sunetta scripta : C.S. of gill filaments and digestive tubules of animals exposed to 5 ppm for 10 days

Photomicrograph 16 : Arrows showing formation of interlamellar connections x 400

Photomicrograph 17 : Arrow indicating granulocytoma x 100





Sunetta scripta : C.S. of digestive tubules and gills of animals exposed to 5 ppm PHC for 10 days and 15 days respectively.

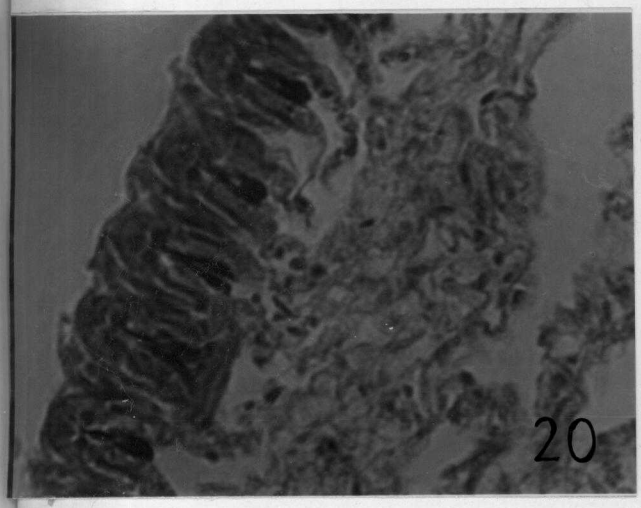
Photomicrograph 18 : Arrow indicating migration of basal nucleus to apex of the cell x 400

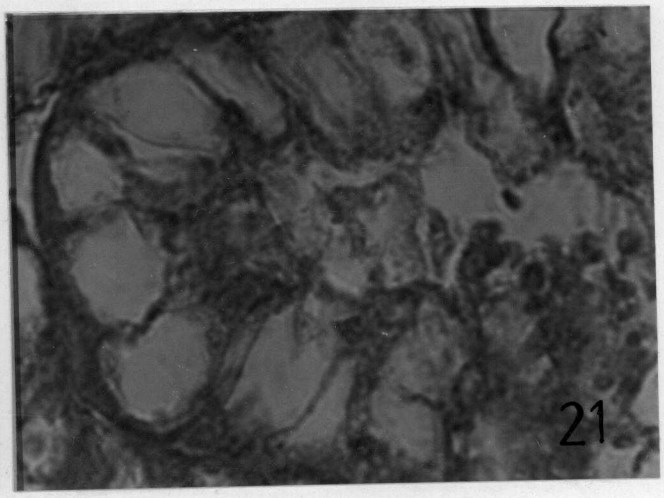
Photomicrograph 19 : Gill filaments showing extensive damage and presence of haemocytes x 400

Large number of haemocytes were found in the gill filaments. The cilia of lateral and laterofrontal portions of the gill filament were clumped (photomicrograph 19). This probably indicates that the cilia bearing cells have been dislodged so as to result in clumping. Presence of large number of haemocytes in the gill indicates internal haemmorhage. There was no further deterioration in the histology of digestive tubules from that of animals exposed to ten days.

Those animals exposed to 15 ppm developed acute pathological conditions. This was reflected in the structure of both the gills and tubules. Mode of degeneration of gills indicated shearing off of cells from the chitinous rod. Wandering haemocytes were present all through the gills. The deterioration of the digestive tubules resulted in breaking up of the collagenous layer.

Prolonged exposure resulted in shrinkage of the gills, enlargement of endothelial and mucus cells (photomicrograph 20). The space created by the disintegration of endothelial cells was found to be filled with haemocytes. Heavy vacuolation of digestive cells resulted in their enlargement (photomicrograph 21). Increase in the number of darkly stained basophilic cells and the lumen getting filled up with sloughed off cells resulting in its obliteration was indicative of the damage of digestive tubules after ten days exposure.

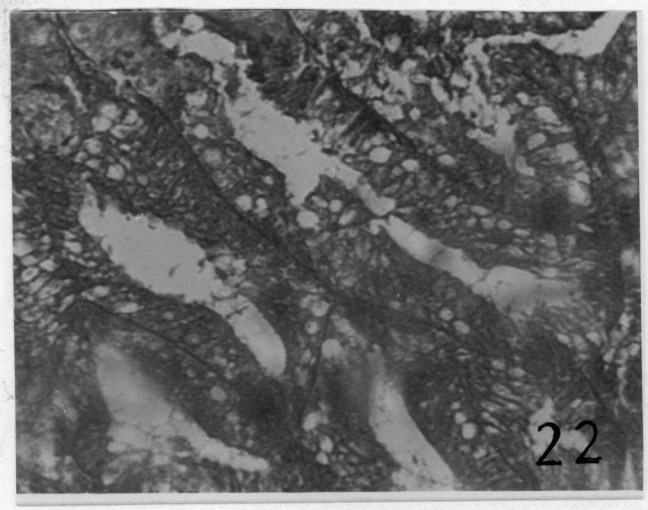




Sunetta scripta : C.S. of gill filaments and digestive tubules of animals exposed to 15 ppm PHC for 10 days

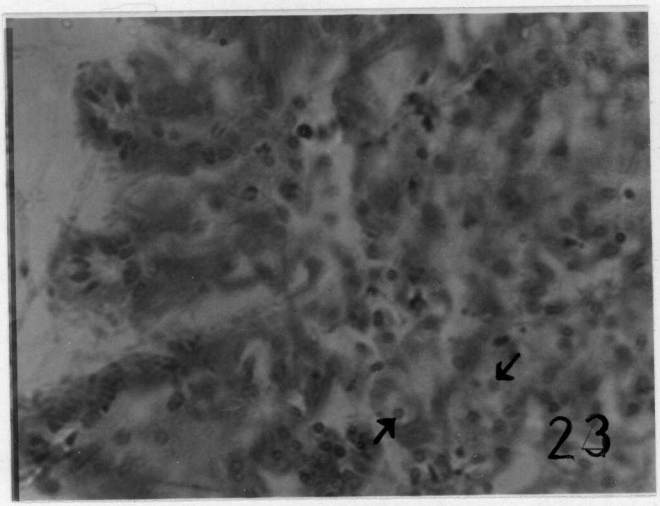
Photomicrograph 20 : Gill filaments shrunken in size x 400

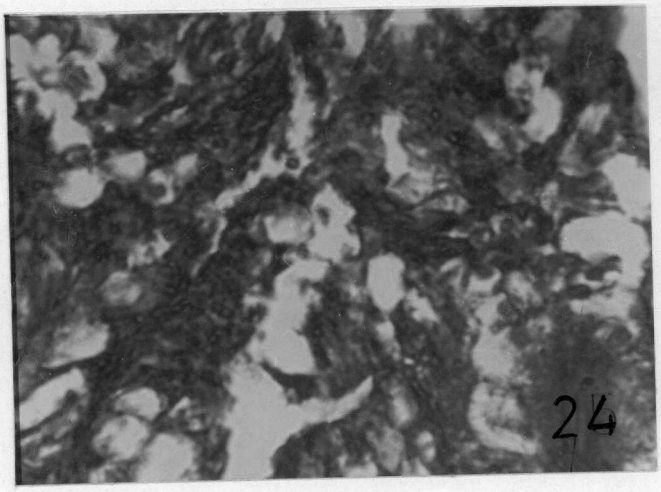
Photomicrograph 21 : Vacuolation of digestive cells x 400



Sunetta scripta : C.S. of digestive tubules of animals exposed to 15 ppm PHC for 15 days

Photomicrograph 22 : Proliferation of darkly stained basophil cells x 400





Sunetta scripta : C.S. of gill filaments and digestive tubules of animals exposed to 50 ppm PHC for 5 days.

Photomicrograph 23 : Presence of haemocytes in gills (arrow) x 400

Photomicrograph 24 : Digestive tubules damaged extensively x 400

This pathological condition of the gills and digestive tubules was further worsened when animals were exposed for fifteen days. But the degeneration of acidophilic cells was compensated by the proliferation of darkly stained basophilic cells so that the digestive tubules had a deeply stained appearance (photomicrograph 22).

S. scripta exposed to 50 ppm of PHC survived only for five days. Examination of histology of gills and digestive tubules showed extensive damages of cells and sporadic disturbance in certain portions of the tissue. As evidenced from the photomicrograph 23, gill filaments were completely damaged and wandering haemocytes were present throughout the gills indicating excessive haemmorhage. The integrity of digestive tubules was thoroughly disrupted indicating excessive lethal damage of this cardinal tissue (photomicrograph 24).

3.4.3 STATISTICAL ASSAY OF TISSUE DAMAGE

3.4.3.1 **Wilcoxon Signed Rank Test**

Wilcoxon signed rank test was conducted to find out whether there was enlargement of digestive tubules and gills as a consequence of exposure to WAF.

3.4.3.1.1 *Perna viridis*

The data in tables XIII and XIV show that there was significant variation in the number of tubules and gill

TABLE XIII

Perna viridis :Number of cells per unit area of digestive tubules and gills in animlas exposed to different concentrations of BHC oil WAF for 15 days.

Treatment		No. of fields examined															
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
		Digestive tubules								Gills							
Control	5th day	9	10	13	7	6	6	10	12	6	5	5	7	6	4	4	6
Control	10th day	12	10	12	7	9	6	8	11	9	4	3	6	5	6	7	4
Control	15th day	12	13	8	9	10	8	11	10	8	5	6	6	7	5	5	4
1 ppm	5th day	11	6	6	13	8	6	8	6	2	3	4	4	3	4	3	3
1 ppm	10th day	7	10	12	8	10	7	10	8	5	7	7	7	8	9	10	5
1 ppm	15th day	10	9	7	8	7	3	4	8	7	4	6	5	5	5	4	5
5 ppm	5th day	8	6	13	8	7	8	9	8	6	4	3	10	6	5	4	8
5 ppm	10th day	11	6	8	6	9	9	9	8	8	9	8	6	7	7	4	6
5 ppm	15th day	10	8	7	8	9	10	10	8	8	4	5	7	5	4	5	6
10ppm	5th day	6	6	5	3	5	8	5	5	3	3	3	4	2	3	3	6

TABLE XIV

Perna viridis : Inference on variation in cell number of animals on exposure to PHC for 15 days with that of control, arrived at employing **WILCOXON SIGNED RANK TEST**

Day of sampling	Treatment	T^- (dig. tubule)	T^- (gill)
5	Control + 1 ppm	12.5	0*
	Control + 5 ppm	13	11
	Control +10 ppm	2*	0*
10	Control + 1 ppm	18	7.5
	Control + 5 ppm	8.5	8.5
15	Control + 1 ppm	0*	5*
	Control + 5 ppm	0*	12

Crititical region at 5% level $T^- \leq 5$

*Significant

filaments per unit area only in the case of animals exposed to lethal concentration (10 ppm) for five days before death set in. However, there was a tendency of enlargement of digestive tubules after 15 days in the case of animals exposed to 1 ppm oil. The tissues of control animals sampled on fifth, tenth and fifteenth day of exposure did not show much difference in structure and also variation in cell number per unit area.

The reduction in number of tubules or gill filaments in unit area can be attributed to the enlargement of these tissues or their degeneration and sloughing off.

In the case of *P. viridis* exposed to 1 ppm PHC, there was significant reduction in the number of gill filaments. This may be because lower concentrations of aromatic compounds are first sensed by the gills which respond by inflammatory reactions or epithelial hyperplasia (Sunila, 1987). When compared with that of control, the variation in number of both digestive tubules and gills were significant for the animals exposed to 10 ppm for five days.

In the case of animals exposed to 1 ppm oil for fifteen days, both digestive tubules and gills recorded enlargement of cells. On the contrary, exposure to 5 ppm for fifteen days resulted in noticeable variation in the size of digestive tubules alone.

TABLE XV

Sunetta scripta : Number of cells/unit area of digestive tubules and gills in animals exposed to different concentrations of BHC oil for 15 days.

Treatment		No. of fields examined															
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
		Digestive tubules								Gills							
Control	5th day	10	13	8	6	6	12	13	9	15	15	14	14	15	14	14	16
Control	10th day	10	12	9	7	9	12	10	9	15	15	12	13	13	10	12	14
Control	15th day	10	9	9	8	10	8	9	9	12	15	13	12	14	10	13	14
5 ppm	5th day	12	11	8	5	8	5	5	6	9	19	14	15	7	8	15	13
5 ppm	10th day	5	2	6	7	9	5	6	10	10	9	10	14	12	9	13	14
5 ppm	15th day	6	5	5	5	6	6	8	8	8	7	5	9	9	9	8	10
15ppm	5th day	7	6	6	9	8	5	7	5	7	10	16	15	9	6	15	15
15ppm	10th day	3	2	2	3	3	4	2	2	14	12	13	13	13	9	8	13
15ppm	15th day	6	10	5	10	6	6	8	8	12	15	13	15	12	8	9	9
50ppm	5th day	6	10	6	6	6	5	6	4	9	9	13	9	9	7	12	13

TABLE XVI

Sunetta scripta : Inference on variation in cell number of treated animals on exposure to PHC for 15 days with that of control arrived at employing **WILCOXON SIGNED RANK TEST**

Day of sampling	Treatments	T^- (dig. tubule)	T^- (gill)
5	Control + 5 ppm	8	10
	Control + 15 ppm	5*	7
	Control + 50 ppm	0*	0*
10	Control + 5 ppm	3*	7
	Control + 15 ppm	0*	4.5
15	Control + 5 ppm	0*	0*
	Control + 15 ppm	6.5	6

Crititical region at 5% level $T^- \leq 5$

*Significant

TABLE XVII

Perna viridis : Histological status by Rank Analysis of digestive tubules and gills of animals exposed to different concentrations of PHC for 15 days.

Parameters selected to assess degree of degeneration	RANKS FOR DIFFERENT CONCENTRATIONS AND DAYS OF SAMPLING									
	Control 5th day	Control 10th day	Control 15th day	1ppm 5th day	1ppm 10th day	1ppm 15th day	5ppm 5th day	5ppm 10th day	5ppm 15th day	10ppm 5th day
Reduction in height of epithelial cells (a)	0	0	0	0	1	3	0	0	1	0
Presence of infiltrated haemocytes (b)	0	0	0	3	0	2	0	3	2	1
Increase in no. of vacuolated cells (c)	0	0	0	3	3	3	1	1	3	1
Presence of sloughed off cells (d)	0	0	0	1	1	3	3	1	3	3
Presence of degenerated epithelial cells (e)	0	0	0	3	2	3	1	1	2	3
Decrease in size of lumen (f)	0	0	0	3	2	2	3	3	1	3
Dilation of branchial vein (g)	0	0	0	3	2	3	3	2	3	3
Infiltrated haemocytes in gills (h)	0	0	0	2	2	1	0	3	3	1
Presence of damaged/distorted cilia (i)	0	0	0	2	1	1	2	1	0	3
Swollen epithelial cells (j)	0	0	0	0	0	1	0	2	0	3
Swollen endothelial cells (k)	0	0	0	1	2	2	3	1	2	3
Ciliary interlocking between gill filaments (l)	3	3	3	1	1	3	1	3	3	1

0 - nil 1 - low 2 - medium 3 - high

Parameters: a - f - digestive tubules g-l - gills

TABLE XVIII

Perna viridis : **SPEARMANN'S CORRELATION COEFFICIENT** for different concentrations based on rank analysis of histological status of cells of digestive tubules and gills of animals exposed to PHC for 15 days.

Day of sampling	Treatments compared	r
5	Control + 1 ppm Control + 5 ppm Control +10 ppm	0.325 0.406 0.437
10	Control + 1 ppm Control + 5 ppm	0.355 0.607*
15	Control + 1 ppm Control + 5 ppm	0.586* 0.576*

Significance at 5% level r $\geq$0.5035

* Significant

3.4.3.1.2 *Sunetta scripta*

The data of Wilcoxon signed rank test (tables XV and XVI indicate that when compared with that of control, the digestive tubules and gills of animals exposed to 5 ppm PHC for 15 days, 15 ppm for ten days and 50 ppm for five days exhibited significant reduction in number of filaments and tubules per unit area. Further, the animals exposed to 5 ppm PHC showed significant enlargement of the epithelial cells of digestive tubules after ten days of exposure to the toxicant.

3.4.3.2 **Rank Analysis**

Characteristics/degenerative changes revealed by digestive tubules and gills, assessed using visual indices, such as variation in vacuolation of cells, sloughing off of cells, swelling of epithelial and endothelial cells, dilation of branchial vein etc., can be used as useful tools to define histological status of the tissue. The method employed was rank analysis. The 'r' value $\geq$ 0.5035 indicates that the extent of damage is significant.

3.4.3.2.1 *Perna viridis*

The results in tables XVII and XVIII have shown that the degree of degeneration, when compared with that of control was significant only for animals exposed to 5 ppm PHC for ten and 15 days and for those exposed to 1 ppm for fifteen days. Even though 10 ppm was considered as lethal concentration and

TABLE XIX

Sunetta scripta : Histological status by Rank Analysis of digestive tubulus and gills of animals exposed to different concentrations of PHC for 15 days

Parameters selected to assess degree of degeneration	RANKS FOR DIFFERENT CONCENTRATIONS AND DAYS OF SAMPLING									
	Control 5th day	Control 10th day	Control 15th day	1ppm 5th day	1ppm 10th day	1ppm 15th day	5ppm 5th day	5ppm 10th day	5ppm 15th day	10ppm 5th day
Reduction in height of epithelial cells (a)	0	0	0	1	0	2	0	0	3	3
Presence of infiltrated haemocytes (b)	0	0	0	0	3	3	3	2	3	2
Increase in no. of vacuolated cells (c)	0	0	0	2	3	1	3	3	1	2
Presence of sloughed off cells (d)	0	0	0	0	2	2	3	2	1	2
Presence of degenerated epithelial cells (e)	0	0	0	0	2	2	1	2	3	3
Decrease in size of lumen (f)	0	0	0	0	1	2	1	2	1	1
Dilation of branchial vein (g)	0	0	0	1	1	0	0	2	1	2
Infiltrated haemocytes in gills (h)	0	0	0	3	2	2	2	3	2	3
Presence of damaged/distorted cilia (i)	0	0	0	2	1	2	1	1	1	3
Swollen epithelial cells (j)	0	0	0	2	1	2	2	1	0	0
Swollen endothelial cells (k)	0	0	0	0	3	3	1	2	3	3
Ciliary interlocking between gill filaments (l)	3	3	3	3	2	2	2	2	2	3

0 - nil 1 - low 2 - medium 3 - high

Parameters: a - f - digestive tubules g-l - gills

TABLE XX

Sunetta scripta : SPEARMANN'S CORRELATION COEFFICIENT for different concentrations based on rank analyses of histological status of cells of digestive tubules and gills of animals exposed to PHC for 15 days.

Day of sampling	Treatments compared	r
5	Control + 5 ppm Control +15 ppm Control +50 ppm	0.640* 0.481 0.589*
10	Control + 5 ppm Control +15 ppm	0.47 0.507*
15	Control + 5 ppm Control +15 ppm	0.533* 0.380

Significance at 5% level $r \geq 0.5035$

* Significant

the animals died on the sixth day of exposure, there was no significant damage to the gills and digestive tubules when assessed by rank analysis. However, Wilcoxon signed rank test shows that there was significant enlargement of both digestive tubules and gills of animals exposed to this lethal concentration.

3.4.3.2.2 *Sunetta scripta*

It was evident from rank analysis that the damages of the tissues of *S. scripta* was much more conspicuous than those of *P. viridis*. The results (tables XIX and XX) also indicated that prolonged exposure need not bring about concomitant degeneration of tissues.

3.5 DISCUSSION

Cellular responses to pollutant-induced sublethal injury provided highly sensitive indicators of environmental impact (Moore, 1985). Damages caused on tissues as evidenced from histological studies could be corroborated with alterations in physiological rate functions. However, delineation of these two could be only through estimation of functional changes and structural damages. Histochemical studies or molecular biology would explain more clearly the variations in rate functions. As pointed out by Cajaraville

et al. (1993), correlation between cellular and organismic responses to experimental oil pollution is generally not significant when both are recorded simultaneously. The histopathological status seem to be normal after long-term exposure to PHC (Ray, 1987).

Microscopical observation of tissues have shown that there were pathological changes resulting mainly in inflammatory and degenerative reactions. Enlargement of cells result in overall increase in volume of cells because of formation of enlarged lysosomes. This subsequently leads to atrophy of digestive cells.

In the case of *P. viridis* exposed to 1 ppm of oil, there was significant enlargement of the gills. The mode of feeding in *P. viridis* is such that the toxicant, mainly in dissolved form comes in contact with the gill filamemts first. On sensing the toxicant, the animal responds by secreting excess quantities of mucus thereby subjecting the concerned cells to over-activity and this probably results in the enlargement of mucus cells rendering them less efficient. Therefore, though the secretion of excess quantities of mucus is a very useful defence mechanism, prolonged exposure to low levels of hydrocarbons would render the gills less efficient with reference to performance. The fact that the enlargement of gill cells is often accompanied by the presence of haemocytes, indicates tissue damage as well, mainly in the

form of inflammation. Sunila (1986) reported similar changes in the case of *M. edulis* exposed to cadmium for 24 h. Prasad (1991) also reported similar changes in *Anabas testudineus* after 48 h exposure to crude oil.

The site and mode of entry of PHC controls the extent of damage on gills and digestive tubules in marine molluscs. Their feeding strategy also is an important criterion controlling the damage. In bivalves, lateral cilia of the gill filaments are responsible for moving the water through the ostia, laterofrontal cilia remove particles from the inflowing water and frontal cilia transport the particles to acceptance or rejection tracts at the margins of the lamella. The lysosome rich digestive cells of the molluscan hepatopancreas are functional in both storage and intracellular digestion of food (Owen, 1972). These cells also accumulate large quantities of PHC and undergo pathological changes due to their toxic action.

Of all the concentrations of PHC employed for the present study, the most marked tissue aberration was observed on short term exposure to relatively high concentrations of WAF. The cytological alterations included hyper-activity of gill mucocyte, increase in vacuolation of the epithelial cells of the digestive tubule, degeneration and sloughing off of epithelial cells. Similar results were reported by Axiak *et al.* (1988) for *Venus verrucosa* exposed to 410 $\mu g\ l^{-1}$ WAF of

Kuwait crude oil. High contaminant concentrations are seen to induce the formation of pathologically enlarged secondary lysosomes in the digestive epithelium of mussels and to cause a disturbance in lipid levels, resulting in alterations in digestive cell architecture (Lowe, 1988). However, in the present study it was noticed that a direct correlation of tissue damages and load of PHC was manifested in the case of *S. scripta*. In *P. viridis*, there was significant enlargement of tubules and gills after five days of exposure to 10 ppm, indicating formation of giant lysosomes and setting in of autophagic changes. Such pathological alterations accelerated by the sudden increase in PHC uptake by the mussel would have resulted in the mortality on sixth day of exposure. Kohler (1990) has reported complete labilization of lysosome and increased content of lipofuscin in *Platichthys flesus* on exposure to contaminants.

A very interesting finding of the present investigation is that *S. scripta* exposed to 15 ppm PHC for 15 days did not record any significant damage to the tissues when assessed by rank analysis. This plus the proliferation of basophilic cells probably indicates extensive tissue repair by the animals. The relatively low damage of tissues of *S. scripta* exposed for fifteen days supports the finding that the tissue load of PHC was also very low during this period. The reason may be the detoxification attempts and the

multixenobiotic resistance mechanism identified in the membrane vesicles from gills, mantle and digestive gland of mussels (Kurelec and Pivcevic, 1991; Kurelec, 1995) which would have helped the effective pumping out of the xenobiotic. The recognition that the mutli-xenobiotic resistance mechanism is operative in mussel may explain the relative resistance of this species to pollution. The proliferation of darkly stained basophil cells which serve mainly in the secretion of detoxifying enzymes mainly the microsomal MFO (Bayne *et al.*, 1985) can be cited as hitological evidence for the induction of the detoxifying mechanism. There is evidence of stimulation of activity of microsomal NADPH-neotetrazolium reductase in *M. edulis* by phenanthrene (100 $\mu g\ l^{-1}$) after 3 days exposure and the stimulation persisted after 12 d recovery period (Moore *et al.*, 1984).

In the present study, the damages caused to non-ciliated cells of the gills were more extensive than that of ciliated cells. This may be because of the fact that the entry of PHC into ciliated cells is made more difficult by synchronous beating of cilia and by mucus on their surface. Sunila (1986) has also reported similar observations in *M. edulis* on exposure to copper and cadmium. She has also opined that what is seen as interlamellar fusions in sections after one month exposure to copper is the formation of a new food groove on the ventral edge of the gill for food transport.

Such interlamellar fusions were found in *S. scripta* in the present study after 15 days exposure to 5 ppm and 15 ppm.

According to Axiak and George (1987), long term exposure to WAF of oil may lead to loss of co-ordination of ciliary activities reducing retention efficiency of particles. In short term exposures, the frontal cilia treat oil droplets as food particles and lead them towards the labial palps. However, on exposure to high concentrations of PHC, many of the oil droplets which may become incorporated in mucus strands are brought to the free edge of the labial palp and are rejected. This confirms the low tissue load of PHC of *P. viridis* when exposed to 10 ppm PHC and relatively less damage to gills due to the protection by mucus layers.

Molluscs exposed to oil reduce their food intake, resulting in reduced growth rates and lower energy reserves. Lysosomal system is involved in proteolysis (Cockle and Dean, 1982) and there is evidence of enhanced autophagy in the digestive cells of mussel exposed to PAH (Lowe and Pipe, 1994) and digestive cells of *L. littorea* in response to phenanthrene as well as nutrient deprivation (Moore *et al.*, 1984).

CHAPTER 4

ULTRASTRUCTURE

4.1 INTRODUCTION

The measurement of the impact of pollutants on marine organisms can be considered at different levels of functional complexity, viz., from the molecular level to the levels of the individuals and population (Bayne *et al.*, 1985). The extent of contamination in most of the polluted sites are moderate to low. More subtle changes might be occurring due to environmental degradation at many of these locations. These changes would go undetected if only the most serious, recognisable bioeffects like mortality, physiological and biochemical alterations, prevalence of tumours etc. are studied. The underlying concept of the "cellular index' approach is that the effects of stress are manifested at lower levels of biological organisation much before perturbations are realized at the organismal, population or community levels.

Cellular assay techniques are employed to study pollutant-induced injuries on the internal organ systems of organisms. Organs are not homogenous arrangements of identical cells and the stressor target often is a specific cell type within a given region of an organ. Further, some cell types may respond quite differently depending on the physiological or structural status of the animal. An understanding of the molecular mechanisms of cell injury and ultrastructural

alterations brought about by the xenobiotics is highly essential for developing effective and comprehensive early warning systems for use in pollution monitoring of the marine environment.

Bivalve molluscs rapidly accumulate organic compounds such as PHC from their environment. The digestive gland is an important storage and metabolising site for accumulated hydrocarbons, whereas the gills and kidney are the main tissues involved in the process of elimination of PHC from the body (Widdows *et al.*, 1983). To monitor, understand and predict the impacts of PHC stress, it is necessary to study the interrelations that affect the availability and toxicity of PHC, including the genetic and cellular mechanisms that influence defence, metabolic compensation and evolutionary adaptation. According to Trump *et al.* (1975), a cell can be in necrotic stage for several hours without showing any discernible change under light microscopy, and this is why electron microscopy with its associated biochemical techniques has found wide applicability in the study of cell reaction to injury.

4.2 REVIEW OF LITERATURE

Xenobiotic induced cellular pathology reflects disturbances of structure and function at the molecular level

of biological organisation. At many instances, the earliest detectable changes or primary events are associated with a particular class of sub-cellular organelle such as the lysosome or endoplasmic reticulum (Slater, 1978). Investigations in mammals have revealed that the primary intracellular disturbances are initiated by the highly reactive derivatives of xenobiotics. These may spread rapidly into a complex network of associated secondary and higher order disturbances which become progressively more difficult for the cell to reverse or modify (Varanasi, 1989).

Studies on the influence of natural and anthropogenic factors such as starvation, quality of food, temperature, toxic compounds, etc. on the liver ultrastructure of teleosts have demonstrated that hepatocytes reflect changes in the environment with high sensitivity (Storch *et al.*, 1984; Kohler, 1989). In livers of flounder *Platichthys flesus* caught from Elbe estuary polluted by mercury and chlorinated hydrocarbons, enlarged Golgi bodies and proliferation of rough endoplasmic reticulum are interpreted as an adaptive sublethal response indicating successful detoxification (Kohler, 1990). Leland (1983) observed only subtle changes in the ultrastructure of hepatocytes of juvenile rainbow trout and mature brown trout exposed to copper or zinc. Leland (*loc. cit.*) has further suggested that apart from the possible sequestering of the metals, which are seen as electron-dense

granules, the ultrastructural changes observed may be largely a non-specific response to stress. In the cat fish *Ictalurus* sp., by the 15th day after administration of phenyl hydrazine, the hepatic parenchyma was manifesting maximum degeneration. An autoregenerative phase of the liver parenchyma was evident on the 21st day (Baruffaldi *et al.*, 1995).

Employing multi-element analysis using electron microscopy with an energy dispersive X-ray analyser, Ishii *et al.* (1985) found granules containing extremely large quantities of manganese and cadmium in the kidneys of the marine bivalve *Cyclosunetta menstrualis*. The spherical fine granules of trace metals appear extra cellularly beside microvilli and develop into larger granules while moving to the centre of the lumen of the kidney tubules (Ishii *et al.*, 1986). Degenerative changes have been reported to occur in the kidney epithelium of mussels treated with high dose of a mixture of diesel oil and copper (Auffret, 1988). In *Littorina littorea* after experimental exposure to 30 $\mu g\ l^{-1}$ of naphthalene, the ultrastructural changes within excretory cells consisted of increased formation or accumulation of lipid droplets, increased occurrence of membrane bound dark bodies which became enlarged after 96 h of exposure to naphthalene, distortion of the Golgi complex and altered mitochondrial structure (Cajaraville *et al.*, 1990).

The gills of aquatic animals are generally the most delicate of the epithelia exposed to the environment. So it is likely that they are most prone to damage by pollutants. Nuwayhid *et al.* (1980) found that 100% WSF of North Sea crude oil caused damage to surface microvilli and to cilia of *Patella vulgata* when viewed under SEM. Transmission electron microscopy revealed severe disruption of normal cellular organisation, increase in the numbers of lysosomes, vacuolation of mitochondria and the extrusion of cytoplasm and damaged organelles through the apical surface. Transmission and SEM studies by Sunila and Lindstrom (1985) have shown that the interfilamentar junction of *M. edulis* gill consists of interlocking pairs of cilia. The tip of each cilium rests at the base of the other and has connections with the microvillus border. During exposure to copper and cadmium, connections between cilia and microvilli break and the ciliary discs slide apart causing uncoupling of the gill filaments. Hyne *et al.* (1992) reported the occurrence of dense membrane bound granules with high concentrations of iron and small amounts of copper and zinc within the cytoplasm of gill of the gastropod *Haliotis rubra* collected from a polluted environment. In the deposit feeding bivalve *Abra alba* collected from a site polluted by industrial effluents, ultrastructural study revealed that endocytosis of fine sediment particles occurs not only in the digestive gland but also in the gills. In both these organs, the lysosomal system

ensured the storage of fine particulate material (Martoja *et al.*, 1988).

Molecular cell biology is identifying many possible biomarkers relating to perturbation of cellular processes or else, defensive reactions involving those systems that protect the cell against hostile environmental agents. These include heat-shock proteins like chaperonins and metallothioneins, cell surface receptors, cellular oncogenes, neurobiological disturbances, the enzymatic machinery for xenobiotic detoxification and activation, as well as the internal membranous components of the cell, such as, the endoplasmic reticulum, Golgi complex, transport vesicles and lysosomes, and the various building blocks of the cytoskeleton (Sanders, 1990; Moore, 1993).

The cell surface or plasma membrane of invertebrate hepatopancreatic cells is a major interface with the environment. Pollutants associated with the diet come into direct contact with the cell surface of digestive glands. Lipophilic xenobiotics may enter the cell by diffusion across the plasma membrane or by endocytosis in association with low density lipoprotein (Mohammed *et al.*, 1990). Perturbation of these and other physiological functions of plasma membrane by toxic chemicals will have profound effects on the cell and consequently on the organ and animal.

Reactions of intracellular membranes to toxic pollutants are perhaps better documented than those of the plasma membrane. The endoplasmic reticulum is a major site of many of the enzymatic reactions involved in xenobiotic detoxification and activation. These processes involve the cytochrome P-450 and epoxide hydrase as well as some of the antioxidation protection enzymes (Livingstone *et al.*, 1990; Stegeman and Lech, 1991). In addition to the functional changes and induction of ER-associated enzymes, the ER itself can undergo proliferation markedly altering the internal organization of the cell, following exposure to many xenobiotics (Hinton and Lauren, 1990). Activated derivatives of certain xenobiotics can be retained within the lipoprotein membranes of the endoplasmic reticulum and converted to potentially damaging oxyradicals.

Damage to lysosomes has been described in diverse species of both fish and invertebrates as a consequence of exposure to a range of environmental contaminants including PHC (Lowe, 1988; Moore, 1990). Lysosomal responses to cell injury can be considered as alterations in : (1) lysosomal contents such as hydrolytic degradative enzymes, (2) rate of membrane fusion events with either the cell membrane or other components of the vacuolar system and (3) lysosomal membrane permeability (Hawkins, 1980). Exposure of marine molluscs to PAH and PHC has been demonstrated to result in the activities

of certain lysosomal enzymes, viz., β-glucuronidase and acid phosphatase (Moore *et al.*, 1986).

Phenanthrene as well as PHC induces abnormal enlargement of secondary lysosomes of digestive cells indicating increased fusion of membrane bound vesicles (Pipe and Moore, 1986). Nott and Moore (1987) are of the opinion that phenanthrene reduces the additional internal lysosomal membranes which are formed by the uptake of material by fusion with vacuoles. This suggests that the digestive processes of the cell are affected. Recent studies using pH sensitive molecular probes have indicated that the enlargement of lysosomes in response to exposure to hydrocarbon contaminants is due to the reduction of internal pH of lysosomes. The rapid decrease in pH on exposure to PAH may be related to enhanced protein break-down in the lysosomes, which is known to accompany cellular autophagy (Anon., 1996).

The third category of lysosomal disturbance involves membrane permeability. Work in the Plymouth Marine Laboratory (1995-96) has shown that one of the first responses of accumulation of organic xenobiotics by lysosomes is an increase in cellular free Ca^{2+} and the resulting Ca^{2+} cytotoxicity probably contributes to the lysosomal membrane changes. Investigations of lysosomal responses to specific PAH have demonstrated that the lysosomal disturbances are complex

and differs markedly for PAH which are structurally dissimilar, such as the isomeric 3-ring forms anthracene and phenanthrene (Moore and Farrar, 1985). Lowe and Pipe (1994) assessed lysosomal damage using the retention of the cationic diazine probe neutral red in the lysosomes of digestive cells of phenanthrene exposed mussels. The results showed that probe retention time was significantly reduced in the lysosomes of cells isolated from exposed mussels indicating the impairment of functional integrity of lysosomal membrane following PAH exposure. Pollutant induced injury of lysosomes in the blood cells of mussels is strongly correlated with depression of phagocytosis of foreign particles. Total and differential blood cell counts, phagocytotic ability of the blood cells and capacity for generation of super oxide radicals by the blood cells are used as measures of immuno-competence. So it can be inferred that PAHs are inducing an immuno-deficiency syndrome in mussels (Lowe *et al.*, 1995; Pipe *et al.*, 1995).

The potential for PAH enhanced oxyradical generation in molluscs is considerable (Lemaire and Livingstone, 1994 (a)). Failure to remove oxyradicals by antioxidant defences can result in oxidative damage to key biological molecules like DNA (Kehrer, 1993), lipid peroxidation etc. There is evidence of enhanced formation of lipofuscin-rich lysosomes and residual bodies in digestive cells following exposure of *L. littorea* to phenanthrene (Varanasi, 1989).

Manisseri and Menon (1995) assessed the pathological disturbances in the hepatopancreas of *Metapenaeus dobsoni* following exposure to copper for 15 days. Damage to nuclei included distortion of nuclei with scalloped edges, damage of the nuclear membrane, overall shrinkage and loss of shape of the nuclei. Endoplasmic reticulum and mitochondria were made dysfunctional due to their partial or total disintegration. They also found damage to the SER, which is concerned with detoxification and lipid synthesis, indicating a possible disruption of the cellular detoxification machinery. They also noted the immobilisation of accumulated copper by the cell by sequestering and eliminating them *via* electron dense granules.

4.3 MATERIALS AND METHODS

This part of the investigation looked into the effects of both sublethal and lethal concentrations of PHC on the fine structure of hepatopancreas/digestive gland of *Perna viridis* and *Sunetta scripta*. The details of the test animals, water used for the experiments, laboratory conditioning of animals and toxicants- concentration and mode of preparation are the same as explained in sections 2.3.1 to 2.3.4.1.

Ten animals each were exposed to 5 l of toxicant solution of the prescribed concentrations for 15 days in a partial media replenishing culture system. Animals were fed on

fortified artificial feed. Controls were also run along with the treatments. Another set of animals were also subjected to starvation for 15 days and the fine structure of such animals was also examined to have a comparative idea of the structural damage.

TEM studies were done on the hepatopancreatic tissues of both *P. viridis* and *S. scripta*. After termination of the exposure period, the animals were cut open and the hepatopancreas was dissected out and fixed immediately in cold ($4^{o}C$) Cacodylate buffered 5% glutaraldehyde solution at pH 7.2 for 12 hours. The following time-chart was adopted for making tissue blocks:

1. Tissues were then trimmed into small pieces of 2 mm^3 size.

2. Tissues were washed thrice in Cacodylate buffer, stored in fresh buffer and refrigerated. Samples can be stored in this manner until further processing.

3. Post fixation of the tissues was done using 1% Osmium tetroxide at $4^{o}C$ for 2 hours.

4. Osmium tetroxide was drained out and tissues were washed in several changes of fresh buffer solution, 15 minutes each time. This is to remove excess osmium tetroxide that could form a black precipitate in the tissue.

5. Tissues washed in double distilled water.

6. Dehydration of the tissues done in ascending series of ethanol by immersing for 15 minutes each in 30%, 50%, 70%, 90% and absolute alcohol at 4^{o}c giving two changes at each step.

7. Infiltration of the tissues was done using different concentrations of ethanol and spurr embedding medium - 75:25, 50:50, 25:75 respectively.

8. Embedding done in spurr embedding media, keeping the moulds at 70^{o}c for 24 hours.

Standard procedure for sectioning and staining for electron microscopy was followed. Silver coloured ultra thin sections were taken using an LKB 2188 "Ultrotome Nova" microtome. Sections were mounted on copper grids and sequentially stained with Uranyl acetate (Watson, 1968) and lead citrate (Reynolds, 1963). The sections were viewed and electron micrographs were taken in a Jeol 100SX transmission electron microscope operating at 80KV.

4.4 RESULTS

In mussels, cells of the digestive tubules are particular targets for the injurious action of PHC and the lysosomes in these cells are sites of PHC accumulation. The

fine structure of the digestive gland of *P. viridis* and *S. scripta* was studied employing electron microscopy. The structural changes akin to disruption or proliferation of organelles like nuclei, lysosomes, endoplasmic reticulum, mitochondria etc. serve as biomarkers of cell injury and enhanced rate of detoxification on account of entry of xenobiotics.

The epithelium of the digestive tubules consist of two cell types, viz., digestive or acidophilic cells and basophilic cells (see Chapter 3). There are two types of basophil cells - mature secretory cells and immature flagellated cells. The digestive cell is characterised by the presence of mitochondria, golgi elements, free ribosomes, elements of both smooth and granular endoplasmic reticulum, numerous membrane bound cytoplasmic vesicles and microvilli which project from the cell apex into the tubule lumen.

The basophil cells are more or less pyramidal in shape (Owen, 1970) with the broad base resting on a basement membrane and the tapering apical region with the microvilli bordering the lumen of the tubules. The dominant feature is the presence of parallel arrays of granular endoplasmic reticulum in the basal, lateral and circumnuclear regions of the basophil cell. Other features include numerous free ribosomes, active Golgi apparatus arranged more or less

concentrically to enclose an extensive cup-shaped Golgi zone, mitochondria, membrane bound microvesicles and secretory vesicles towards the cell apex. Basophil cells are sites of extensive protein synthesis.

The immature flagellated basophil cells differ from the secretory mature basophil cells in the presence of flagellum which projects from the apical region of the cell into the lumen of the tubule. Other major differences are the scarcity of endoplasmic reticulum, presence of numerous free ribosomes distributed throughout the cytoplasm, Golgi apparatus with only one or two stacks of saccules although not as prominent as that of a mature cell, presence of autophagic vacuoles etc.

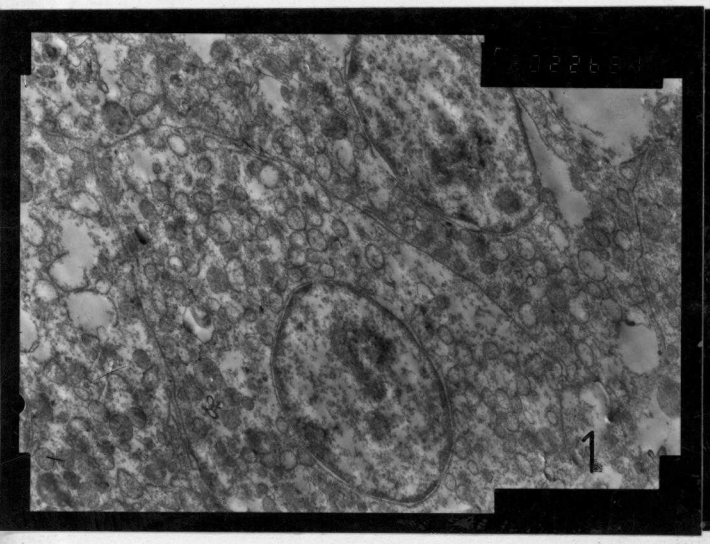
4.4.1 *PERNA VIRIDIS*

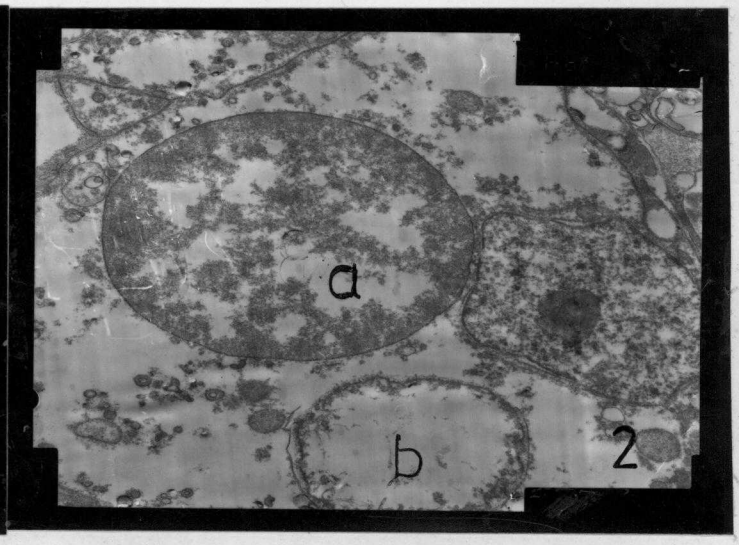
The typical digestive cell of *P. viridis* as shown in photomicrograph 1 has an oval nucleus and different types of macrovesicles and microvesicles. The difference in macrovesicles signifies various stages of digestion (Owen, 1970). Macrovesicles which are essentially vacuoles involved in digestion could be of different constitution. Thus, two macrovesicles, one loaded with electron dense granules and another with dense granules distributed peripherally are seen in photomicrograph 2. This shows that the process of digestion is in progress.

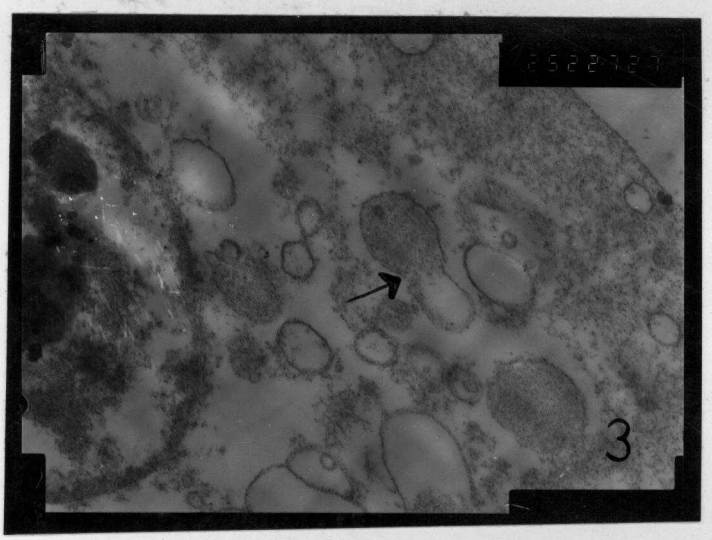
Macrovesicles (phagosomes) are essentially non-secretory. To perform digestion, it is essential to have digestive enzymes to be supplied by primary lysosomes. In photomicrograph 3, the process of fusion of a phagosome with a primary lysosome is seen. The phagosome is loaded with electron dense material.

To examine the influence of starvation in the morphology of digestive cells, sections of digestive tubules of starved *P. viridis* were also examined using electron microscopy. Presence of large number of vesicles of various shapes and size in the digestive cell was the basic indication of starvation. Presence of residual bodies, lysosomes engulfing electron dense vesicles etc. probably indicate basic digestive activity in the cell and the source of food being that which is stored by the animal. Examination of electron microscopic images have shown the presence of damaged nucleus and large number of macrovesicles due to reduction in cytoplasmic volume in the digestive cells. Swollen mitochondria show signs of disintegration (photomicrograph 4).

The structural characteristics of the digestive cells included vacuolated smooth endoplasmic reticulum, shrunken nuclei displaced to the side of the cell. Denuded cells indicating necrosis was another feature. Presence of large number of lipid droplets indicates enhanced fat







Perna viridis : Fine structure of hepatopancreatic cells of animals maintained under control conditions.

Photomicrograph 1 : A typical digestive cell x 8000

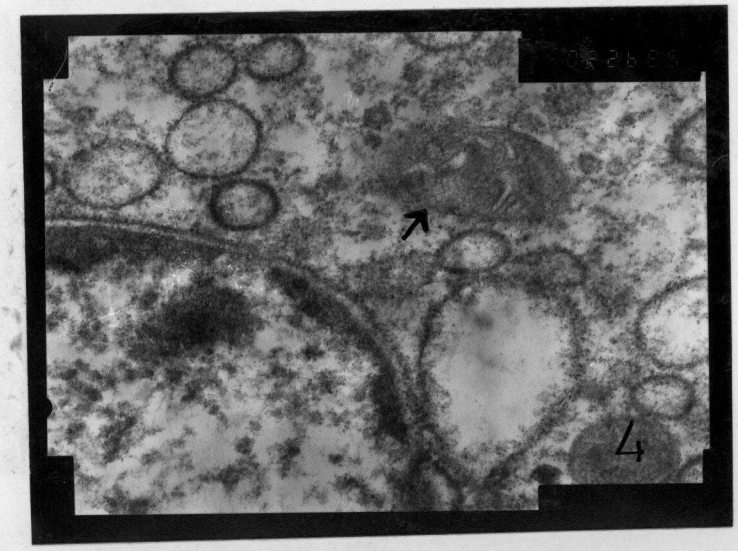
Photomicrograph 2 : Two types of macrovesicles (a) and (b) in the digestive cell x 8000

Photomicrograph 3 : Fusion of a phagosome with a primary lysosome (arrow) x 25,000

metabolism. Large number of cells had stained dark (photomicrograph 5). Lysosomal activity was stepped up resulting in the formation of enlarged secondary lysosomes as seen in photomicrograph 6. The Golgi apparatus of the basophil cells have lost their characteristic shape. Presence of haemocytes in the lumen was also noticed. These were the changes of cells of the digestive tubules of animals exposed to 1ppm of PHC for 15 days. In certain areas, presence of fibroblasts with collagenous fibres were noticed. These indicate the formation of granulocytomas.

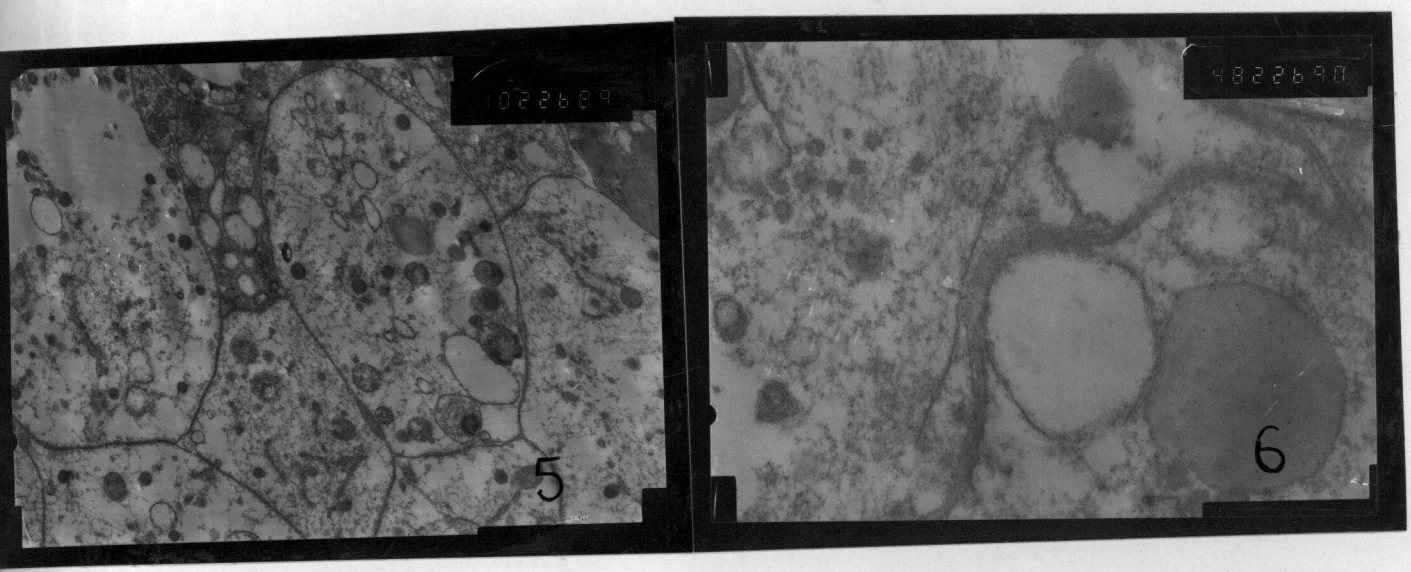
Apart from the damages that were caused to the cell organelles, changes such as presence of lysosomes of varying sizes carrying dense inclusions, disintegration of rough endoplasmic reticulum and mitochondria, disappearance of nucleolus were also noticed in the digestive cells of *P. viridis* exposed to 5ppm PHC for 15 days. Breakages in the basal lamina (photomicrograph 7), concentric arrangement of granular material in certain cells (photomicrograph 8) were also observed.

In the case of *P. viridis* exposed to 10ppm PHC increase in the number of mature basophil cells, proliferation of rough endoplasmic reticulum, shrinking of cells, elongation of nucleus, disappearence of mucleolus and damage of nuclear membrane were indications of cellular pathology (photomicrographs 9 and 10). At many places,



Perna viridis : Fine structure of hepatopancreatic cells of starved animals.

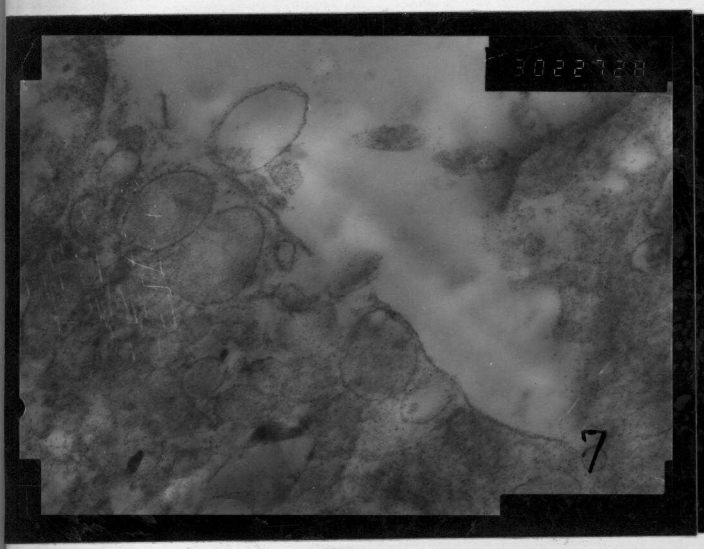
Photomicrograph 4 : Portion of a digestive cell showing disintegrating mitochondrion (arrow) and numerous lysosomes x 40,000.

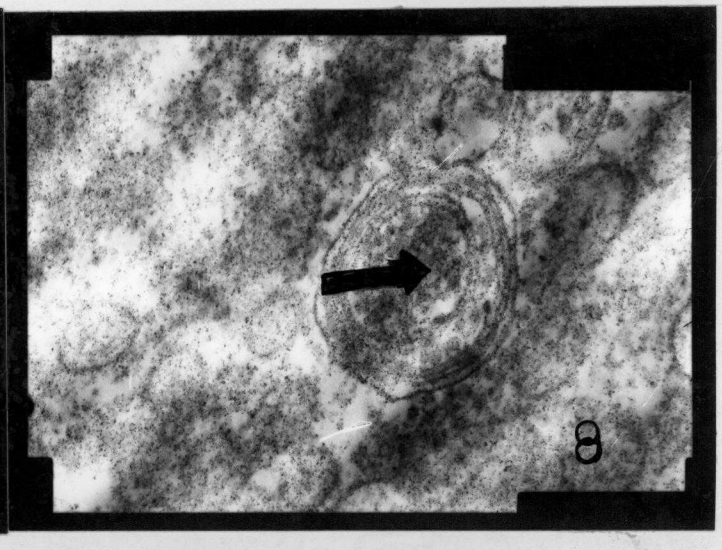


Perna viridis : Fine structure of hepatopancreatic cells of animals exposed to 1 ppm PHC for 15 days.

Photomicrograph 5 : Few cells showing the extent of damage due to toxic insult x 10,000

Photomicrograph 6 : Formation of a secondary lysosome x 40,000

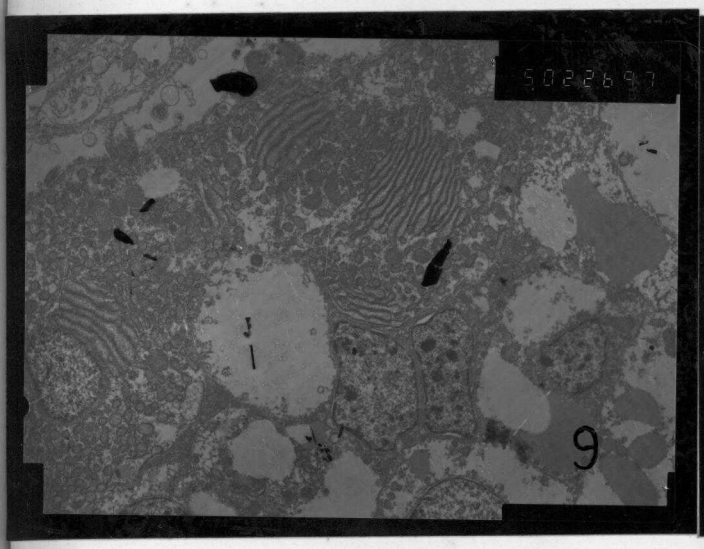


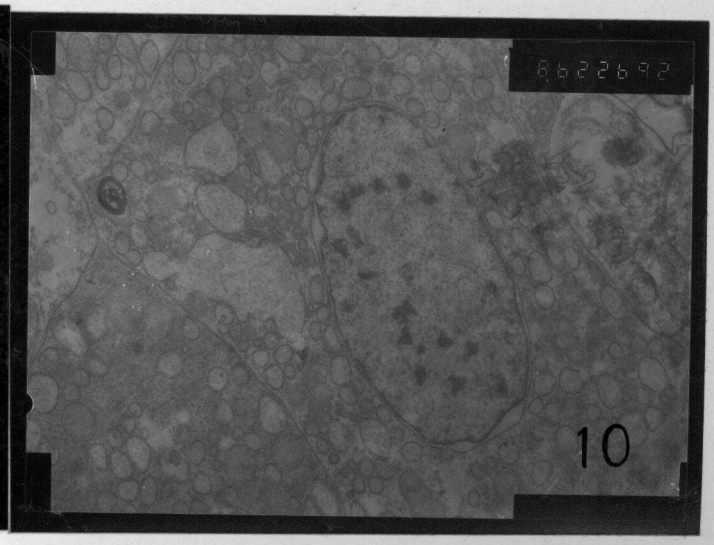


Perna viridis : Fine structure of hepatopancreatic cells of animals exposed to 5 ppm PHC for 15 days.

Photomicrograph 7 : Breakages in basal lamina x 30,000

Photomicrograph 8 : Occurrence of granular material inside a secondary lysosome x 50,000





Perna viridis : Fine structure of hepatopancreatic cells of animals exposed to 10 ppm PHC for 15 days.

Photomicrograph 9 : Proliferation of endoplasmic reticulum x 5,000

Photomicrograph 10 : Nucleus with distorted nuclear membrane x 8,000

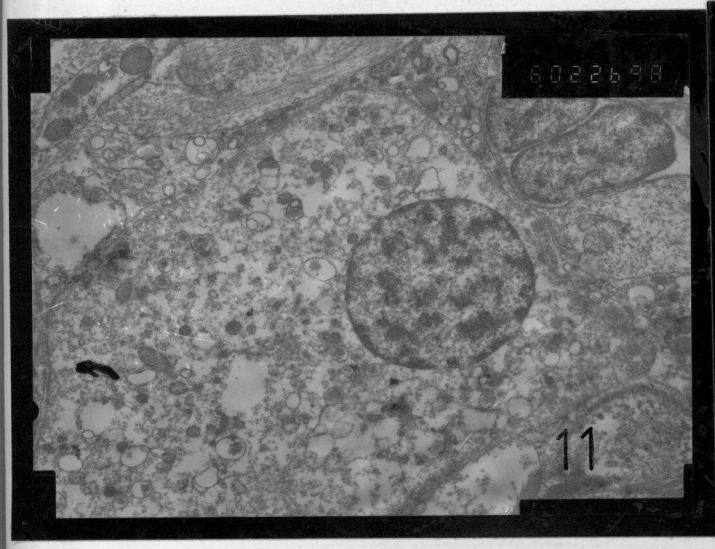
digestive cells were found replaced by flagellated cells indicating nutrient deprivation. Certain digestive cells were devoid of inclusions but for a few enlarged lysosomes. In one cell a lysosome with an engulfed Golgi apparatus was seen. There was reduction in the number of lipid droplets. Mitochondria were found damaged in some cells.

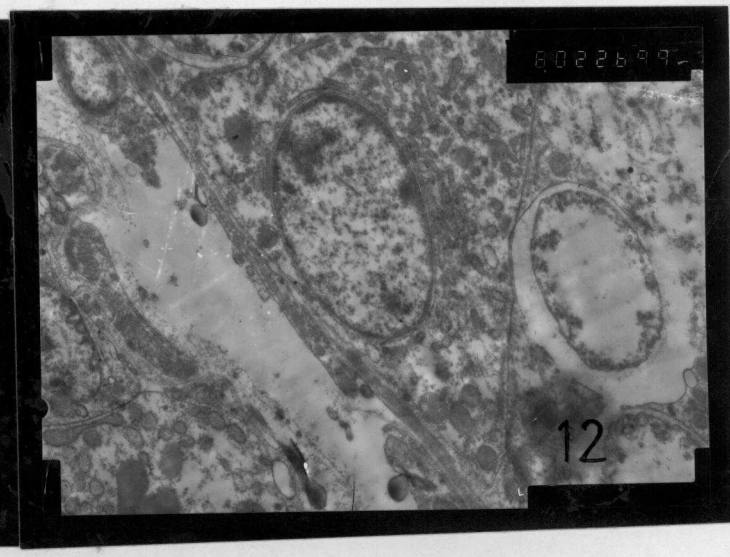
4.4.2 *SUNETTA SCRIPTA*

As in the case of *P. viridis*, representatives of this species were exposed to 5ppm, 15ppm and 50ppm of PHC for fifteen days and the ultrastructure of the digestive tubules examined by TEM.

Two types of cells viz., digesive (phtomicrograph 11) and basophilic (photomicrograph 12) were identified in the digestive tubules. The cells had small rounded nuclei. Cytoplasm contained numerous strands of endoplasmic reticulum, ribosomes, mitochondria, macrovesicles, microvesicles and residual bodies. Microvilli were also found to be intact. A few of the basophil cells were flagellated.

Starved animals had shrunken digestive cells with less dense cytoplasm and grossly degenerated nuclei. There was a total reduction in the number of residual bodies also. The process of degeneration was found to be very extensive in

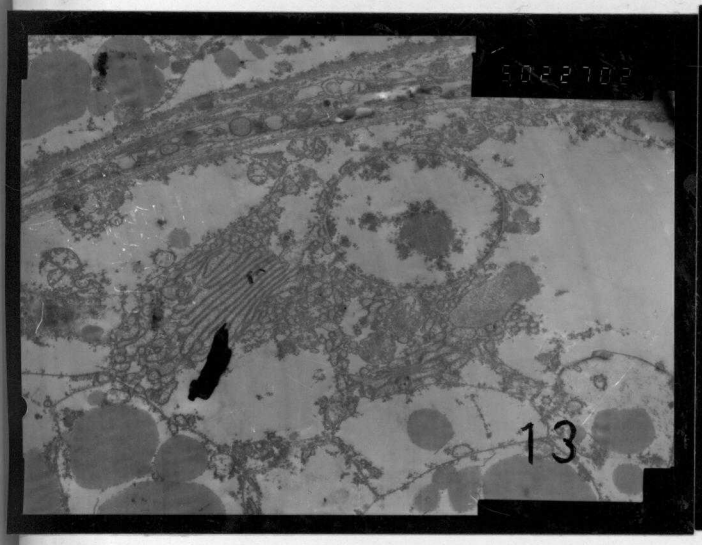


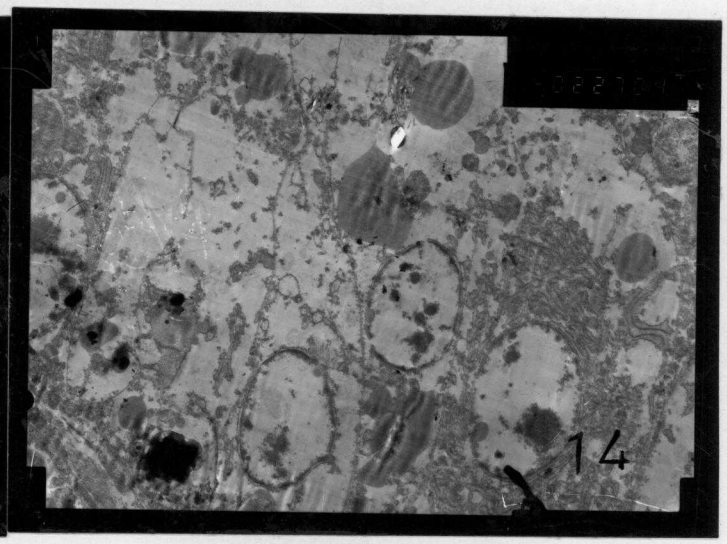


Sunetta scripta : Fine structure of hepatopancreatic cells of animals maintained under control conditions.

Photomicrograph 11 : A typical digestive cell x 8,000

Photomicrograph 12 : A typical basophil cell x 8,000





Sunetta scripta : Fine structure of hepatopancreatic cells of starved animals.

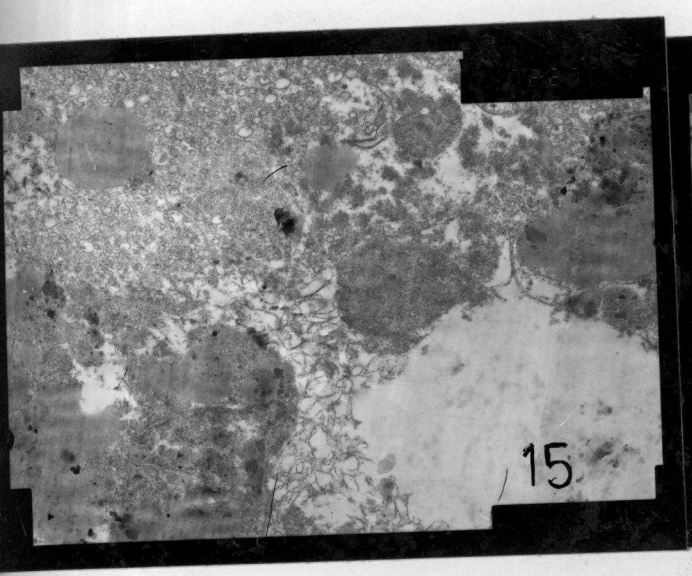
Photomicrograph 13 : Degeneration of a basophil cell x 5,000

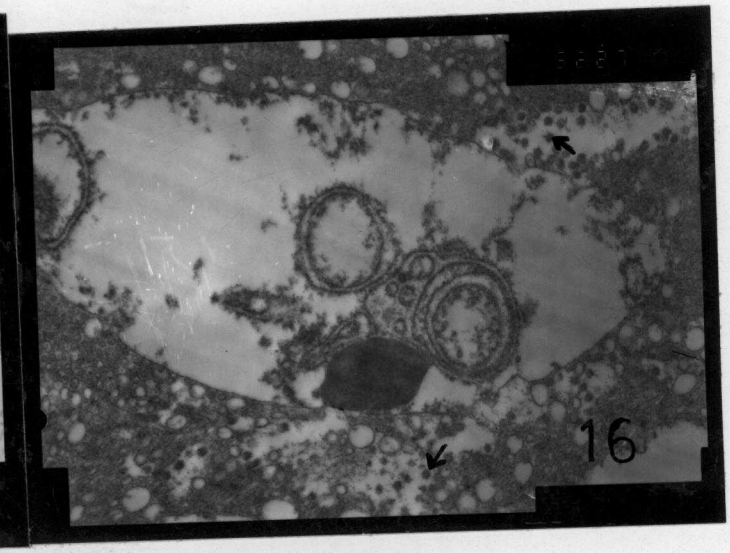
Photomicrograph 14 : Few cells with lesser inclusions and less dense nuclei x 4,000

the basophil cells (photomicrograph 13).This was exemplified by disintegrated rough endoplasmig reticulum, ill-shaped Golgi bodies, nuclei with less dense nucleoplasm (photomicrograph 14) and lysosomes at different stages of fusion with other membrane bound vesicles.

In the case of those animals exposed to 5ppm of PHC for fifteen days, in general, the damages noticed were very similar to those of starved animals. Cytoplasm as seen in photomicrograph 15 was found filled with numerous vacuolar smooth endoplasmic reticulum interspersed with highly electron dense granules and lysosomes. Proliferation of lysosomes accompanied by lack of other inclusions was another feature of the damaged cells. Smaller granules similar to lipid droplets were assumed to be oil droplets. Autophagy is indicated in some secondary lysosomes. Further, microautophagic invagination by lysosomes is also discernible (photomicrograph 16). The endothelial cells contained accumulated lysosomes and residual bodies. Folding and thickening of basal lamina with electron dense granules was also noticed (photomicrograph 17). Damaged microvilli facing the lumen are also seen.

Apart from these damages, those animals exposed to 15 ppm exhibited much more exagerrated cellular damages. Lysosomes were the prominent inclusions in the cell. Even the lysosomal membrane was damaged. In the photomicrograph 18,

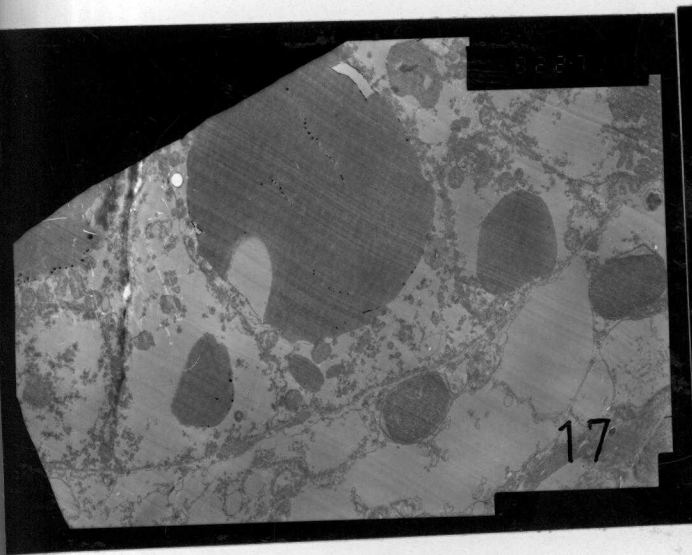


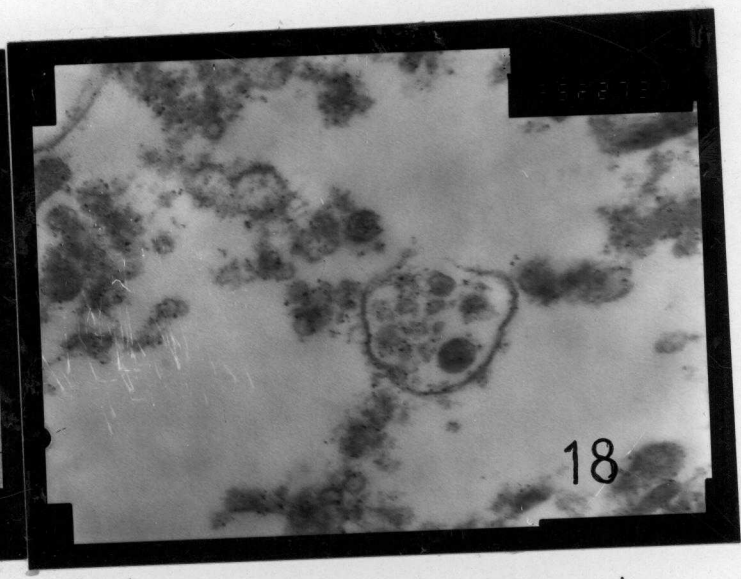


Sunetta scripta : Fine structure of hepatopancreatic cells of animals exposed to 5 ppm PHC for 15 days.

Photomicrograph 15 : Cytoplasm filled with SER, electron dense granules and lysosomes x 10,000

Photomicrograph 16 : Accumulation of lipid or oil droplets x 15,000

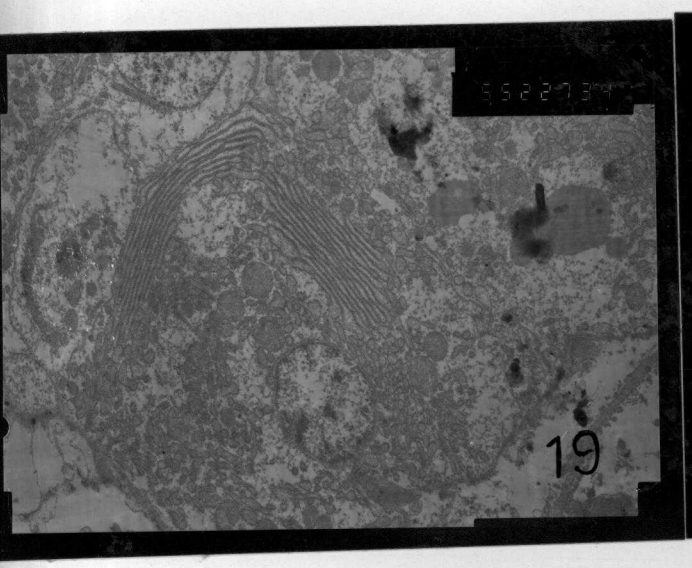


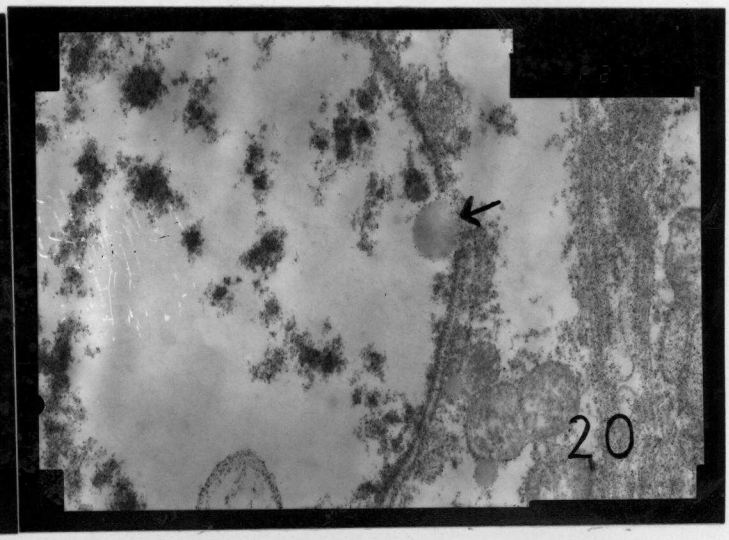


Sunetta scripta : Fine structure of hepatopancreatic cells of animals exposed to 5 ppm and 15 ppm PHC for 15 days.

Photomicrograph 17 : Microautophagic invagination by a lysosome x 4,000

Photomicrograph 18 : Damage and rupture of lysosomal membrane x 50,000

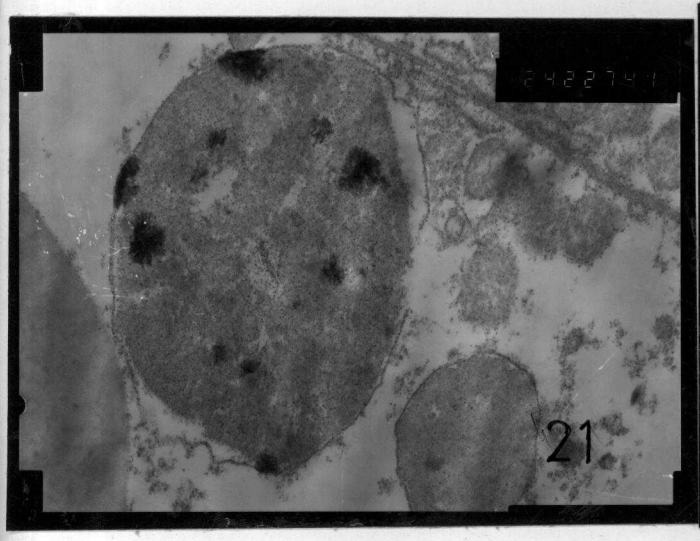


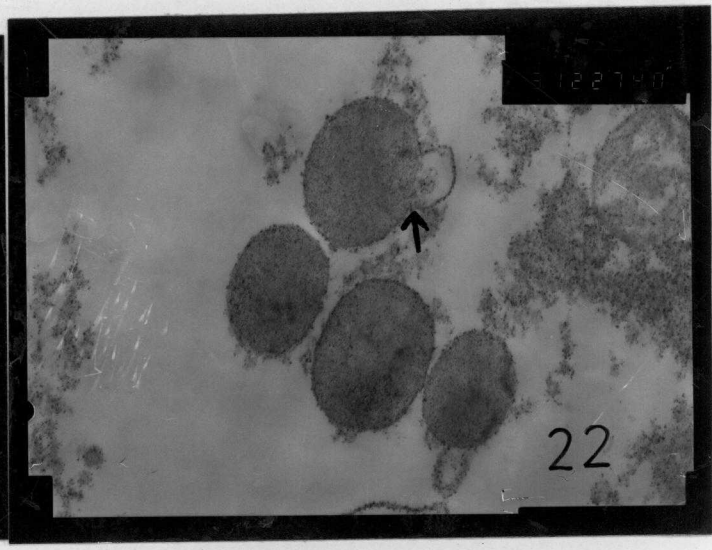


Sunetta scripta : Fine structure of hepatopancreatic cells of animals exposed to 15 ppm and 50 ppm PHC for 15 days.

Photomicrograph 19 : Proliferation of smooth and rough ER x 5,000

Photomicrograph 20 : Entry of a lysosome through the ruptured nuclear membrane (arrow) x 20,000





Sunetta scripta : Fine structure of hepatopancreatic cells of animals exposed to 50 ppm PHC for 5 days.

Photomicrograph 21 : Damage of outer nuclear membrane x 20,000

Photomicrograph 22 : Blebbing of lysosomal membrane (arrow). Portion of the otherwise empty cytoplasm also seen x 30,000

the lysosomal membrane not only shows irregular profiles but also ruptures. Apart from lysosomes, the prominent feature noted in some cells was the proliferation of smooth and rough endoplasmic reticulum (photomicrograph 19). Number of lipid or oil droplet like structures in the cytoplasm was much less.

Animals exposed to 50ppm PHC did not survive beyond six days. Therefore information on fine structure is confined to tissues of animals which were alive upto five days. Extensive damage of digestive tubules was reflected in the fine structure. Nuclei had become electron transparent, devoid of nucleoli or dense chromation as evidenced in photomicrograph 20. Entry of a secondary lysosome through the ruptured nuclear membrane also seen. In photomicrograph 21, damage of outer nuclear membrane which has become detached could also be seen. Cytoplasmic inclusions were minimal. Mitochondria were found to lose their characteristic shape. Damaged lysosomal membranes with ruptures and distentions and blebbing of the outer membrane were also noticed (photomicrographs 22). Inclusions resembling oil droplets were also noticed in the cells.

4.5 DISCUSSION

Xenobiotics first induce changes at the molecular and cellular levels of biological organization. These

biomarkers serve as distress signals and provide early warning of pollutant exposure and damage to the health of affected individuals.

Moore (1985) has opined that the earlier detectable histopathological or ultrastructural changes induced by xenobiotics are associated generally with subcellular organelles such as mitochondria, lysosomes, endoplasmic reticulum and biological membranes. Further, lysosomes, most particularly those in the digestive cells of the hepatopancreas of the mussels, have the ability to accumulate organic contaminants like PHC, which results in enhanced toxicity and cell injury through lysosomal damage (Moore, 1990). The present findings on the cellular damages of *P. viridis* and *S. scripta* by PHC support the above findings.

A general picture of the structure and function of the digestive tubule of bivalve mussel can be drawn from the reviews by Owen (1970, 1972), Thompson *et al.* (1974) and Bayne *et al.* (1976). Extracellular digestion in bivalves takes place in the stomach and intracellular digestion occurs in the digestive diverticuli, in alternating phases (Owen, 1974). The epithelium lining the tubule consists of two types of mature cells, such as, secretory basophil cells and digesitve cells. There are also immature flagellated basophil cells which may replace one or both types of mature cells under

suitable conditions. Digestive cells are specialized for the uptake and further processing of exogenous material directed to the tubules from the stomach by way of lysosomal action. The exogenous material ingested by means of pinocytosis in the cell is initially accumulated in the macrovesicle named phagosome. These fuse with the primary lysosomes which release its hydrolytic enzymes to digest the food material. The resultant macrovesicles which range in appearance from being empty to completely full with homogenous, moderately or highly electron dense granules are qualified as phagolysosomes. These are the main sites of digestion. Residual bodies represent the end point of intracellular digestion and are excreted out into the lumen. The contents are invariably distributed in electron-dense clumps in the periphery of the vesicle. Most probably all these macrovesicles represent stages in the heterophagic cycle rather than discrete bodies.

The role of basophil cells is secretion of proteinaceous enzymes. Proliferation of basophilic cells in the digestive epithelium of PHC treated mussels have been reported by many workers (Lowe and Clarke, 1989; Cajaraville *et al.*, 1993). This proliferation is possibly associated with the simultaneous loss of digestive cells after injury and with disintegration or regeneration processes of digestive tubules (Cajaraville *et al.*, 1990). It is not clear whether there was

an absolute increase in basophil cells or simply in their proportion relative to numbers of digestive cells. Alternatively, many of the cells that appear to be pyramidal may in fact be epithelial precursor cells in the process of differentiating into digestive cells. Thompson *et al.* (1974) in their study of the effects of starvation on the digestive epithelium of *M. edulis* found that the degenerating digestive cells are replaced by flagellated basophil cells. These flagellated cells may be regarded as immature digestive cells because of their high mitotic rate (Purchon, 1971). Cajaraville *et al.* (1993) have found that the volume density of basophilic cells in mussels exposed to crude oil WAF strongly correlated with catalase levels in the digestive epithelium. Catalase is involved in the detoxification of excess free oxygen radicals generated during hydrocarbon metabolisom. This leads us to infer that the basophilic cells are involved in the detoxification of PHC. In the present study, increase in number of basophilic cells was noticed both under light and electron microscopy in *P. viridis* exposed to 5 and 10ppm of PHC and in *S. scripta* to 5 and 15ppm of PHC.

RER, with the RNA containing ribosomes, is especially well developed in cells actively engaged in protein synthesis, such as enzyme-producing cells. Detoxification and lipid synthesis are among the special functions of SER (De Robertis and De Robertis, 1980). Xenobiotics like PAH are

metabolised by many marine invertebrates (Varanasi, 1989). Activated derivatives of these xenobiotics can be 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 and Di Giulio, 1991). The resultant bulging and disintegration of the endoplasmic reticulum may have deleterious effects on the vital functions of this organelle. Hinton and Lauren (1990) have reported that on exposure to xenobiotics, endoplasmic reticulum in tissue cells can undergo proliferation, thus, markedly altering the internal organization. Such proliferation was noted in the cells of *P. viridis* and *S. scripta* exposed to higher concentrations of PHC. Metals can also exert negative effects on the membranes of both the rough and smooth endoplasmic reticulum, one of which is due to lipid peroxidation (Buss and Gibson, 1979).

Studies with invertebrates have shown that contaminant induced increases in oxyradicals can induce antioxidant enzymes, viz., superoxide dismutase, catalase, Glutathione peroxidase (Winston *et al.*, 1990) and low moleculer weight scavengers like Vitamins A, E, C, β-Carotene and Glutathione (Livingstone *et al.*, 1990; Steadman *et al.*, 1991). Cajaraville *et al.* (loc.cit.) have identified SER as

site of synthesis of antioxidant enzymes like catalase. Further, detoxifying enzymes of PAH- mixed function oxidases - are localised in the microsomes in the SER of the hepatopancreas of bivalves (Varanasi,1989). NADPH - neotetrazolium reductase, a component of MFO has been used as a marker enzyme for SER and stimulation of MFO system in *M. edulis* exposed to aromatic hydrocarbons and phenobarbital (Nott and Moore, 1987; Kohler, 1990). Proliferation of SER is an indication of induction and increased activity of the MFO system. This indicates the relevance of study of ultrastructure in pollution monitoring activities. In the present study ample proof is provided by the photomicrographs regarding proliferation of SER in both *P. viridis* and *S. scripta*. Studies show that inductions of MFO in fishes is very rapid, occurring within hours but the induction is slow in invertebrates and dependent on the concentration of toxicant. As the entry of PHC into the tissues of bivalves is a passive process, the rate of accumulation in the tissues may surpass the detoxification and elimination process of PHC. This assumption is supported by the finding that damage of tissues of exposed animals is rampant notwithstanding the proliferation of SER and the resultant detoxification.

Bulging or disintegration of mitochondrial membrane was noticed in *P. viridis* exposed to all test concentrations and in *S. scripta* exposed to 15ppm of PHC. At

lethal concentrations of PHC, mitochondria lost their characteristic shape. Structural damages will render the mitochondria non-functional. Swelling of membrane-bound organelles like Golgi complex, mitochondrial cristae occurs on exposure to naphthalene (Cajaraville *el al.*, 1990) and other PHC (Nott and Moore, 1987). Viarengo (1985) is of the opinion that PAH act on the mitochondria by inhibiting or uncoupling oxidative phosphorylation. Once damaged, the mitochondria are degraded by autophagic process by the lysosomes.

Lysosomal-vacuolar system is considered to be the major degradative system within the cell. Lysosomes can also accumulate and sequester heavy metals and organic pollutants (Moore, 1985). Multiple membrane of lysosomes reflect the addition of membrane bound material to the lysosome. Surplus membranes are returned to endoplasmic reticulum and blebbing phenomenon may be part of this process (Nott *et al.*, 1985).

Nott and Moore (1987) observed that the net effect of phenanthrene on molluscan lysosome is a reduction in the additional internal membranes which are associated with the uptake of material by fusion with vacuoles. This suggests that the digestive processes of the cell are affected. Apart from these changes, breaks in the lysosomal membrane was noted in the present study. Nott and Moore (1987) opined that the breaks are not ruptures, but discontinuities made by the

overlapping membranes. Fusion of vacuoles or membrane bound bodies with the large secondary lysosomes represent macroautophagy and the accumulation of material by invagination of the limiting membrane represent microautophagy (Mortimore *et al.*, 1983). Similar observations were made in the present study also. Enhanced catabolism of cytosolic proteins in the digestive gland of mussels exposed to 150 and 200 ug l^{-1} of phenanthracene indicate that PAH may increase the rate of some autophagic processes (Moore and Viarengo, 1987).

Mechanism of lysosomal membrane injury involve lipid peroxidation process resulting in the formation of lipofuscin. Kohler (1990) found a close relationship between enlarged lipid droplets and accumulation of lipophilic substances in flounder *P. flesus* caught from a highly contaminated site. These droplets resembled large vacuoles filled with finely granular material. These vacuoles might play a role in the transport of lipoproteins. Increase of lipid droplets in the digestive cells seems to be a feature of PHC exposed molluscs. (Pipe and Moore, 1986; Cajaraville *et al.*, 1990). Carles *et al.* (1986) explain this phenomenon as the induction of lipid synthesis by PHC, while Raber and Carter (1986) suggest that the accumulation of lipid could be due to the blockade in the intracellular transport of lipid at the Golgi complex or between the endoplasmic reticulum and the Golgi complex. The latter hypothesis was supported in part by the

occurrence of ultrastructural alterations in the Golgi complex of the basophil cells of the digestive gland of *M. galloprovincialis* after 6 days exposure to crude oil (Carles *et al.*, loc. cit.). In the present study also, lipid droplets of varying sizes resembling oil globules were noticed in PHC treated *P. viridis* and *S. scripta* with increased occurrence in the latter.

The results obtained on the ultrastructural changes of hepatopancreatic tissues on starvation, agree with the results obtained by Thompson *et al.* (1974) on *M. edulis*. They noticed that the protein secretory cells are especially susceptible during starvation. As in the present study, Manisseri (1993) also noticed a large number of vacuoles or vesicles and general reduction in the cellular inclusions in starved *Metapenaeus dobsoni* which, according to her, could indicate utilization of reserve food, burning out of mitochondria or spent lysosomes. Hepatopancreas of juvenile *Penaeus monodon* starved for 7 days showed a decrease in the size of the R cell, depletion of stored lipid inclusions and an increase in the thickness of the basal lamina (Storch, *et al.*, 1984).

Thompson *et al.* (1974) have identified flagellated immature cells as prominent structures in the digestive gland following starvation. In the present study, this feature was

noted not only in the starved animals but also in *P. viridis* exposed to the lethal concentration of 10 ppm. Further, the damages noticed in *S. scripta* were very similar to those of starved animals. The inference is that the animals would have been starved or deprived of nutrients either due to termination of feeding by shell closure or due to inefficient digestion and assimilation by the disintegrating digestive cells. Lysosomal structure and activity indicates that at times of stress, autophagy increases mainly due to the fragility of the autolysosomal membrane and reduced latency of their hydrolytic enzymes (Bayne *et al.*, 1985).

The damage caused by PAH to nuclei and its membrane is such that some of the active metabolites can covalently bind to DNA and damage them (Varanasi *et al.*, 1986). According to Viarengo (1985), in organisms exposed to Cd, Hg and Cu, the rate of protein synthesis is reduced not only by reducing the rate of RNA synthesis but also by influencing the attachment of polyribosomes to the RER. In the present investigation also, RER in the exposed cells were found damaged. This along with the proliferation of SER would have led to insufficient protein synthesis resulting in a decline in the body condition of the treated animals.

Apart from the proliferation of SER, other subcellular changes noticed on exposure to PHC appear to be

the general pathological responses brought about by toxic insult. Similar damages were recorded in the lysosomes by Pipe and Moore (1985) in *M. edulis* as a response to increased salinity and by Manisseri and Menon (1995) in the hepatopancreatic cells of *M. dobsoni* on exposure to copper. The present findings on fine structure and general histology indicate that these changes could be manifested in a much more pronounced manner accompanying subtle behavioural changes.

SUMMARY

The thesis deals with different aspects connected with the distribution and toxicity of PHC in crude oils in the aquatic environment and marine molluscs. Information has been gathered and presented on the present status of research in the field based on a thorough review of recent literature. It is seen from the literature that the resident time of PHC becomes lesser and lesser when crude oil is discharged in large quantities into the coastal waters where excess oxygenation light penetration, temperature availability and strong tidal currents persist. This results in the reduction of the onslaught of oil pollution. This probably shows that toxic insult of crude oil in tropical shores will be relatively lesser than that occurring in temperate and boreal waters. However, there is no record of major episodal oil pollution comparable in quantum and extent to that of Torrey Canyon or Amoco Cadiz which prevents a more reliable analysis on the issue. Considerable controversy exists on the method of estimation of PHC in seawater. However, ultra-violet fluorescence spectroscopy has been widely recommended as an analytical tool for the determination of total hydrocarbons. Similarly representation of values as chrysene equivalents has been recognised as the most reliable method of quantum representation.

The first chapter is devoted towards an understanding of spatial and temporal distribution of PHC in water and sediments in the Cochin estuary and the adjacent sea. A clear cut gradient was discernible on the distribution of PHC in the sediments of Cochin estuary. The dry months were characterised by higher PHC concentration in the sediment and waters. Probably monsoon flushing and dilution due to freshwater run off resulted in reduced distribution of PHC in this area during the monsoon.

The chapter on experimental studies represent the result obtained utilising transplants and native species of bivalves *viz., Perna viridis* and *Sunetta scripta*. Existence in polluted localities was found to result in decreased rate of oxygen consumption and filtration in the molluscs tested. This was found to be coupled with increased body burden of PHC in *Perna viridis*. Enchanced rate of oxygen consumption is a common response of molluscs to low or moderate concentrations of PHC in the surrounding waters. It was also noticed that relationship exists between the trend in increase of body burden and rate of oxygen uptake. Further, respiratory behaviour or filtration rate need not be tissue load dependent if the load is not severe enough to hamper behaviour. In the case of *Sunetta scripta* also, rate of oxygen consumption and filtration was dose dependent. *S. scripta* seems to be better adapted to overcome PHC insult. It is known that some bivalves

have the capacity to partially neutralize the toxic effects of hydrocarbons.

The present study proved that suspension feeding bivalves which dwell in the bottom of oil polluted estuaries are inclined to take more PHC through suspended particles than through water.

The third chapter deals with histopathology of *P. viridis* and *S. scripta*. Histopathology is a field which has received limited attention from toxicologists. Knowledge of the basic structure of organs and organelles is an important pre-requisite for a correct understanding of histopathological effects brought about by toxic reactions caused by xenobiotics. At many instances literature remains silent on cardinal damages brought about by chemical toxicity. Pathological changes resulting from exposure to PHC were mainly inflammatory and degenerative with respect to gills and digestive tubules. In the case of *P. viridis*, cytological alterations included hyperactivity of gill mucocytes, increase in vacuolation of epithelial cells of digestive tubules, etc. Similarly in the case of *S. scripta*, damages caused to the gills and gastric tubules resulted in various types of damages. The damage caused to ciliated cells of gills were less than those caused to non-ciliated cells. Assessment of damages using statistical methods indicated that this is a

useful tool to define histological status of the pathology of the tissue.

Chapter on ultrastructure deals with evidences brought about by a study of fine structure of cells of hepatopancreas of the bivalves exposed to PHC. This section also gives information on the nature of damage caused to this cardinal tissue on starvation. Variations in structure brought about by lack of food and similar natural changes resulting in stress have been documented profusely in the case of fishes. Information of similar nature on bivalves is rather lacking. The findings have shown that basophilic cells are involved in the detoxification of PHC. This was mainly evidenced by proliferation of basophilic cells which was clear in the light microscopic study also. Increase in the number of lipid droplets in the digestive cells is an indication of toxic effect of PHC on molluscs. In literature this is referred to as induction of lipid synthesis by PHC or accumulation of lipid due to the blockade of intracellular transport of lipids. The latter is normally supported by ultrastructural damages to the Golgi complex of the basophil cells. Proliferation of smooth endoplasmic reticulum was another reaction to PHC toxicity.

A list of references which have been consulted during the course of the study has been presented at the end of the thesis.

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