STUDIES ON THE EFFECT OF POLYCYCLIC AROMATIC HYDROCARBONS IN THE LIFE AND ACTIVITY OF *Perna spp.*

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Ph.D Thesis

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Dedicated to

My beloved Parents
who always kept praying for me to achieve my goal

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Certificate

This is to certify that the thesis entitled **"*Studies on the effect of Polycyclic Aromatic Hydrocarbons in the life and activity of Perna spp.*"** is an authentic record of research work carried by Smt. Nikitha Divakaran 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 for the award of any degree, diploma, associateship, in any university.

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May, 2012

Declaration

I hereby do declare that the thesis entitled **"Studies on the effect of Polycyclic Aromatic Hydrocarbons in the life and activity of *Perna spp.'*** is a genuine record of research work done by me under the scientific supervision and guidance of Prof.Dr.N.Ravindranatha Menon, Emeritus Professor, School of Marine Sciences, Cochin University of Science and Technology, Kochi and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or other similar title of any university or Institution.

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Preface

The marine ecosystem is under immense pressure due to anthropogenic activities. Exploration and transportation of petroleum products is directly connected to marine ecosystem. This leads to accidental and episodial contamination of the seas with crude and its derivatives. Oil is a complex mixture of aliphatic, alicyclic and aromatic hydrocarbons. Among these, hydrocarbons the aromatic fraction is responsible for toxicity of the oil to marine organisms. The problem of oil pollution is worldwide and it is acute at least in certain regions of the Indian seas. Although the influence of pollutants on marine ecosystems is known to some extent, only limited information regarding their nature of action, physiological and biochemical effects on marine organisms is known.

Ecotoxicology mainly involves understanding the biological fate of foreign substances in various individual species and the resultant stress induced physiological, biochemical and pathological changes. Ecotoxicological studies are usually conducted on some basic principles; Primarily it is directed towards the impact assessment on the ecological systems. The fate and effect of toxicants when they come into contact with marine organism is another major concern of ecotoxicology. In the field of ecotoxicology, the use of biomarkers has emerged as a new tool for detecting rate function anomalies due to exposure to environmental contaminants. Biomarkers provide rapid toxicity assessment and throw light on community stress. They are also potential tools that could be used to assess the effect of specific chemical on the life and activity of target organisms. Unlike most chemical monitoring procedures, biomarker indices have the potential to reflect and assess the bioavailability of complex mixtures present in the environment. Further, they are capable of throwing light on the biological significance of toxicants.

Toxicity tests have become important tools in determining the permissible levels of pollutants in the environment. Detailed experimental studies are essential to understand the differences noticed in the vulnerability of toxicants on target species and in species dependent rate of variation in tolerance. *In situ* studies to analyse functional and structural responses to toxicant are also recommended to understand toxic effects. Field exposure studies, apart are useful approach for ultimately assessing environmental impacts, and also giving information on *in situ* effects also would help to understand and eventually predict the possible reactions of test organisms under laboratory conditions. Mussels are frequently used to study on the effects of oil on marine organisms. The source of test organisms is an important biological consideration. Choice of species is governed by availability, sensitivity to contaminants and tolerance to test conditions.The utility of bivalve molluscs, and mussels, in particular has been proved as effective. Since they are proved to accumulate high levels of PAHs in their tissues, thereby providing a time-integrated indication of environmental contamination.

In the present study an endeavour has been made to analyse the acute toxicity of WAFs of Bombay High crude and Light Diesel oil on commercially important bivalve species *Perna viridis* and *Perna indica* by static bioassay methods. The toxic effects of chemicals in the WAF on the organisms ; their tissues and eventually on rate functions have been elucidated.

Marine oil pollution not only affects productivity and quality of marine organisms but also eventually affects the health of human population due to a possible health risk by way of consumption of oil contaminated seafood.

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Abbreviations

ANOVA	:	Analysis of Variance
B(a)P	:	Benzo(a)pyrene
BHC	:	Bombay High Crude
DMSO	:	Dimethylsulfoxide
FSW	:	Filtered Seawater
LC50	:	Lethal Concentration to kill 50% of test animals
LDO	:	Light Diesel Oil
PAHs	:	Polycyclic Aromatic Hydrocarbon
PHC	:	Petroleum Hydrocarbons
ROS	:	Reactive Oxygen Species
WAF	:	Water Accommodated Fractions

Part I

GENERAL INTRODUCTION

Natural environment is vulnerable to various anthropogenic sources. This is particularly so in heavily industrialized areas and urban settlement. The marine environment is the ultimate sink for many of the pollutants as the end stage collector of domestic and industrial wastes, urban and agricultural runoff.

Human activity on land, water and air contributes to contamination of sea, marine sediments and organisms with harmful substances. Oil exploration activities is an environmental threat since oil and other toxic substances are released into the sea intentionally or accidently. Oil is mainly composed of saturated hydrocarbons, but also contains variable amounts of polycyclic aromatic hydrocarbons (PAHs). Even though PAH constitutes only a minor fraction of the crude oil, they are very resistant against degradational and weathering processes. This renders them suitable for analysis as indicators of oil pollution. Their chemical stability and persistency, leads to accumulation into various components of the marine environment, resulting in their exposure to marine organisms.

Marine environment supports many different forms of life, from viruses to mammals. Alarming number of toxic chemicals is a major threat for the health of that organism inhabiting the seas.

Marine organisms are capable of absorbing and accumulating contaminants from water or sediments. Marine organisms are incapable to fully excrete accumulated contaminants in the tissues. This can result in biomagnifications of contaminants in the higher trophic levels of the food web. There are members of different marine taxa

capable of accumulating large quantities of toxicants in their body with out depicting any deleterious effects, although very sensitive species reacting violently to toxicants slowly disappears from the seas.

Heavy metals and less water-soluble organic compounds have a tendency to get accumulated in cultivated marine organisms. This would be harmful to the organisms and to humans, as many of the farmed organisms form food for man. Some of the contaminants in very small quantities can cause serious problems associated with taste. Limited capability of bivalves to detoxify and excrete toxic substances, make them dangerous food for human consumption.

Petroleum products include kerosene, gasoline, diesel fuel, lubricating oil, and asphalt. They reach marine environment through oil spills and routine tanker operations (e.g. discharge of ballast water. Hydrophobic nature and low biodegradability, of petroleum products could result in relatively higher levels of these in the tissues of marine organisms when compared to ambient concentrations. Some of the PAHs are known as mutagens and/or carcinogens requiring metabolic activation to exhibit genotoxicity. The quantity of the carcinogen produced depends on the competing activation and detoxification pathways.

PAHs are primarily products of incomplete combustion processes and comprise of two to six fused aromatic rings. The low molecular weight (two and three rings) PAHs have a significant acute toxicity, whereas some of the higher molecular weight PAHs are carcinogenic.

Mussels have a number of characteristics, which make them useful bioindicators of chemical pollution: They have a wide geographical distribution, are easy to collect and are abundant in areas of the sea which are subjected to chronic pollution. They are filter-feeding organisms, and are capable of tolerating baseline levels of pollution; consequently, and hence could accumulatye large quantities of chemical pollutants

although contaminants are present only in low concentrations. Mussels are also capable of bioconcentrating xenobiotics to higher levels than that of the biotope. Being sessile in habit, mussels are particularly desirable for bioindicators since they are likely to reflect changes in pollution levels of the sampling area (Manduzio *et al.*, 2004). Mussels are known to accumulate high levels of metals and organic contaminants including PAHs and PCBs in their tissues with observable cellular and physiological responses (Livingstone *et al.*, 2000, Menon and Menon, 2000). Biological markers or biomarkers indicate that the organism has been exposed to pollutants (exposure biomarker) and / or the magnitude of the organism's response to the pollutant (effect biomarker or biomarker of stress) (Cajaraville *et al.*, 2000). Alterations in cellular metabolism are used as biomarkers for the detection of pollutant induced cellular responses and they serve as early warning signals of exposure to contaminants in environmental monitoring.

Important factors for tissue distribution of PAHs are lipid levels of tissues and the route of uptake. Hydrocarbons can enter the marine food web by several means. One is through adsorption to particles, both living and dead, followed by ingestion of these particles (Menon and Menon, 2000). Another is through active uptake of dissolved or dispersed petroleum, mainly via the gills. A third means would be passage into the gut of animals that gulp or drink water (Menon and Menon, 2000).

In polluted environments organisms exhibit several strategies at the cellular level to protect themselves from the toxic effects of organic and metallic compounds. The major ones are the antioxidant defence systems, sequestration capacity, binding to specific proteins, and detoxification processes including metabolism and exclusion by multixenobiotic resistance transporters. Steps in contaminant processing within tissues and cells are uptake, sequestration and excretion.

In general, the cell possesses various biotransformation enzymes for the metabolism of contaminants. Biotransformation can be divided into functionalization (Phase I) and conjugative processes (Phase II). During phase I metabolism the foreign compound is chemically modified (e.g. oxidation, reduction, hydrolysis etc.) to obtain a reactive group for the biotransformation in phase II.

Impairment of physiological functions and cell structures are the most important detectable parameters of pollutional effects. Analysis of such impairments based on laboratory investigations is the most important aspect of pollution research. Cytological and histopathological alterations provide a direct record of stress response. Assessment of histopathological manifestations provides insight into the degree of stress, susceptibility and adaptative capability of the stressed organism. The sensitivity of ultrastructure studies is superior to and more accurate than light microscopic studies.

Histopathology as an integrative tool to monitor health effects of chemicals has been widely used in monitoring programmes using mussels. In the present thesis, pathological changes were investigated at the subcellular level. In parallel, the distribution of the contaminants was analysed with the electron microscopic technique to elucidate direct toxicant-induced effects.

At the cellular and molecular levels, numerous pathological changes and biochemical markers have been identified that signal exposure to pollutants (McCarthy and Shugart, 1990). Relationships between particular pollutants at known concentrations and pathological or biochemical responses to which they give rise have been established readily. Notwithstanding of this physiological effects, reflected as individuals responses leading to changes in populations and communities have generally been proved difficult.

The science of ecotoxicology has revealed that pollution of natural water bodies will have diverse, complex, and often unpredictable effects on the ecosystem. The lack of

ecological relevance of many standard ecotoxicological tests are often noticed by investigation of natural ecosystems. Extrapolation of laboratory findings to real-world situations is often impractical (Giddings, 1986). This situation has spurred efforts to find more ecologically relevant methods to assess the effects of pollutants in marine, estuarine and brackish waters (Hopkin. 1993).

The presence of a xenobiotic compound in a segment of an aquatic ecosystem does not, by itself, indicate injurious effects. Connections must be established between external levels of exposure, internal levels of tissue contamination and adverse effects (Van der Oost *et al.*, 2003). Over the years, researchers have used living organisms to monitor the concentration and effect of pollutants in the environment. PAHs are lipophilic and therefore are preferentially accumulated in the lipids of organisms where they cause a variety of sublethal effects. The interest in using organisms to monitor marine pollution relies on the fact that analyses of the tissues give an indication of the bioavailable fraction of the environmental contaminant.

Tissue burden of an organism to a pollutant is dependent on the biological availability of the compound, the duration of exposure, and the organism's capacity of the organisms for metabolic transformation (Van der Oost *et al.*, 2003). Apart from bioavailability, another key factor that affects organisms' tissue burden is the uptake route (feeding mode). Elaborating on the feeding mode, Van der Oost *et al.* (2003) reported that persistent hydrophobic chemicals may accumulate in aquatic organisms through different mechanisms: via the direct uptake from water by the gills or skin by uptake of suspended particles or consumption of contaminated food. Organisms exposed to only the dissolved phase, tend to accumulate more of the soluble fraction of the contaminant available in the water. While, organisms exposed to high turbid water or living close to the sediment tend to accumulate high molecular weight PAHs and less soluble compounds from the sediment and particulate matters. Menon and

Menon (2000) have proved that marine sediment is the most important component that control the quantity of PAH accumulated in *Meretrix casta.*

Invertebrates have less ability to metabolise xenobiotics than vertebrates and hence can high tissue concentrations even when the contaminant has virtually disappeared from the environment.

In the marine environment, organisms are often exposed to complex mixtures of pollutants, including PAHs, PCBs, and other persistent organic chemicals. The accumulation of PAHs in mussel tissues has been suggested as an early warning signal and most sensitive biochemical response for assessing environmental contamination.

Among the available environmental monitoring techniques, the integrated use of chemical analysis and biochemical response to pollutants is an effective procedure for detecting the impact of contaminants in the aquatic systems. In this context we aimed to quantify total PAH concentrations in mussel tissues, water and sediment that were collected from different areas of the Cochin backwaters.

The biomarker concept has been extended from purely biochemical measurements to include cellular pathology, physiological processes, and even behaviour of organisms (Depledge *et al.*, 1993). This enhancement creates the possibility of using a hierarchy of biomarker measurements (Depledge, 1994). Initially, effects of pollutants might be detected by relatively non-specific biomarkers, usually high in the hierarchy (e.g., behavioural and physiological biomarkers). Detection of abnormalities with these non-specific biomarkers at a site at risk from pollution might then justify the measurements of more costly, lower hierarchy, specific biochemical and cellular biomarkers to seek to identify the class of pollutant responsible.

Scope of the Present Study

Oil causes disfunction in marine animal communities, death of some marine organisms and sometimes the whole community. Though accidental spill of oil at sea represent a relatively small proportion of total oil inputs to the environment, they are likely to be of most concern from the conservation point of view. Since this result in slicks which are likely to affect marine birds, mammals, shore life and emergent vegetation and tourism.

Shell fish can be used as an ideal and reference candidate for monitoring the intensity of pollution. The present study considers responses to oil-derived hydrocarbons at the molecular, subcellular, cellular and whole animal levels of organization, with particular emphasis on the use of marine molluscs as sentinel organisms for assessing pollutant effects. Biological effects measurements are described which have been used in the development of early-warning systems. These include functional and structural responses of lysosomes to hydrocarbons, quantitative structural alterations in the cells of the digestive and respiratory systems and effects on physiological scope for growth. Recovery processes were also documented.

The present investigation will open up a very interesting aspect of toxicology, the understanding of which would help gain a better knowledge on the effect of WAF of PAHs on the physiological, biological, biochemical, histopathological and fine structure aspects of marine mussels.

The main objectives of the present study are:

- Quantify the degree of pollution by measuring the behavioural and physiological stress responses
- Analyze the histopathological changes caused by WAF of PAHs.

- Find out the alteration in cell and tissue architecture in mussels exposed to different concentrations of WAF.
- Elucidate the role of antioxidant enzymes in mussels exposed to WAF of PAH.
- Measurement of lysosomal membrane stability, and Acetylcholinesterase as integrative biomarker of cellular stress
- Assess marine environmental quality by using transplanted mussels as an in-situ bioassay

Outline of the Thesis and Rationale

The thesis is structured into three parts:
Part I- General Introduction

In part I, a general overview on the research assumptions, as well as scope of the work, the objectives and structure of the thesis is presented.

Part II - Review of relevant literature.

Part III
Chapter 1- Bioaccumulation and Certain physiological responses of *Perna spp* exposed to PAHs.

In this chapter the pattern of accumulation of Bombay High Crude (BHC) and Light Diesel oil (LDO) WAF by *Perna viridis* and *Perna indica* is evaluated. Additionally, short-term toxic effects of WAF of PAHs on these species have also been elucidated. Lethality as well as behavioural and physiological responses of *Perna spp.* to WAF of PAHs have also been found out.

Chapter 2 and 3 explains histopathological and ultrastructure analyses in *Perna spp.* Pathological changes were investigated at the subcellular level. The distribution of

the contaminants was analysed by means of electron microscopic technique to elucidate direct toxicant-induced structural damages.

In chapter 4, the responses of a battery of biomarkers were investigated to understand the toxicity mechanisms induced by PAHs in marine mussels.

Chapter 5 examines the lysosomal membrane stability, and acetylcholinesterase activity in digestive gland (hepatopancreas).

In Chapter 6, an attempt was made to study PAHs monitoring in Cochin estuary. Areas of the Cochin estuary are chronically exposed to PAHs. Considering the deleterious effects of these contaminants in aquatic organisms, a monitoring program was developed to assess the status of PAHs contamination in the Cochin estuary.

Part II

REVIEW OF LITERATURE

Polycyclic aromatic hydrocarbons is one of the several classes of pollutants that reach the marine environment largely due to human activities (Neff, 2002).The carcinogenic properties and anthropogenic origins (particularly via combustion processes) of PAH have led to considerable concern to understand their distribution and fate in the marine environment. Polycyclic aromatic hydrocarbons are a class of chemical carcinogens and mutagenic pollutants that are toxic to aquatic organisms (Malins *et al* 1988).

PAHs are primarly products of incomplete combustion processes and comprise of two to six fused aromatic rings. The low molecular weight (two and three rings) PAHs have a significant acute toxicity, whereas some of the higher molecular weight PAHs are carcinogenic (Neff, 1979, 2002).

The low boiling fractions of oil are toxic and readily soluble in seawater causing biological damage at the very moment where as oil spill occurs. The solubility of oil is increased in the in shore waters by agitation and thus toxic fractions dissolve more rapidly and reach higher concentrations.

Although, there are marked temporal and spatial variations in the environmental levels of PAHs, in heavily-developed coastal areas and harbours, concentrations may be as high as 505 pg/litre in waters. The stable chemical structure and low solubilities of PAHs may result in their persisting in the environment for many years (McElroy *et al.,* 1989).The aromatic components of crude oil, such as polycyclic aromatic

hydrocarbons (PAHs) are known to be toxic to many marine organisms (Varanasi, 1989).

Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic pollutants occurring in the bottom sediments, overlying waters, and biota in aquatic environment. PAHs, is a priority pollutant, a compound selected on the basis of its known or suspected carcinogenicity, teratogenicity or acute toxicity to aquatic organisms (Livingstone *et al.*, 1990; Venier and Canova 1996; Akcha *et al.*, 2000; Law *et al.,* 2002; White, 2002).

The mode of toxic action is likely to be interference with membrane function and fluidity, a phenomenon called non polar narcosis .Several papers have shown that marine organisms are prone to bioaccumulate these substances, particularly in lipid rich tissues (Neff, 2002; Francioni *et al.*, 2005; Dugan *et al.*, 2005).

Organisms that accumulate contaminants but are resistant to their toxicity can be used in monitoring contamination in the marine environment (Cossa, 1989). Among various aquatic organisms, invertebrates have been preferred for environmental assessment. They are the major components in all ecosystems and, because of the usually numerous population, they can be sampled for analyses with little damage to population dynamics (Depledge and Fossi, 1994).

Mussels are most frequently used as biomonitors of water contamination because of the abundance, resilience, and high bioaccumulation of several contaminants of concern.One way to monitor the fate of the bioavailable fraction of PAHs in the environment is through the use of a sentinel species such as the mussel. It possesses the capability to bioaccumulate persistent hydrophobic organic contaminants to very high concentrations in their tissues (Farrington *et al.,* 1983). This high bioaccumulation potential is due to numerous factors including a minimal ability to metabolize hydrophobic organic contaminants, such as PAHs (James 1989; Neff 1979), as well as

the large quantity of water that mussels are capable of filtering through their gills (Naimo 1995). While different mussel species may vary in their ability to metabolize hydrophobic organic contaminants, the fast rate of uptake relative to the slow elimination and substantial bioconcentration of these compounds indicate that metabolism is outweighed by high accumulation rates. Mussels have been used as sentinel species for many years to monitor the overall health of various ecosystems,including coastal and marine environments (Farrington *et al.,* 1983) . However, to adequately acquire and interpret bioconcentration and bioaccumulation data, it is necessary to understand the toxicokinetic parameters that influence PAH bioaccumulation; such as uptake rates elimination rates (and times required to reach a certain percentage of steady state. Elimination is relatively easy to measure because of the simplicity of placing contaminated mussels in clean water and measuring tissue burdens over a designated time period.

Marine molluscs are known to accumulate and tolerate high concentrations of metallic and organic pollutants (Goldberg *et al.*, 1978). Moreover, bivalves, such as *Mytilus*, are widely employed in laboratory studies where either the uptake, loss, or the biological effects of heavy metals, petroleum compounds, and other chemicals is investigated (Bayne *et al.,* 1993, 1985 Widdows *et al.* 1990; Moore 1985).

A description of the major physiological responses of marine organisms to chemicals is presented by Vernberg and Vernberg (1974); Vernberg *et al.* (1977), Dorigan and Harrison (1987), and Holwerda and Opperhuizen (1991). The rationale underlying extensive physiological monitoring, revealing disturbances of almost all physiological systems, has been fully discussed by Depledge and Bjerregaard *(*1989), providing encouragement that a strategy for changes in physiology to be related to alterations in the Darwinian fitness of individuals.

Acquisition of physiological data, whether in the laboratory or *in situ,* has been severely hampered by the lack of suitable transducers capable of recording data over long periods, from several test organisms, simultaneously, without imposing undue stress. However, the introduction of computer-aided monitoring systems and non-invasive transducer techniques is beginning to alleviate this situation (Depledge and Andersen, 1990; Aagaard *et al.,* 1990).

Mayer *et al.* (1992) reviewed methods to assess chemical-induced changes in whole animal physiology.

Mussels are well known for their ability to survive in hydrodynamically stressful environments (Bell and Gosline, 1996; Carrington and Gosline, 2004). In the intertidal zone, waves breaking on the shore create small-scale turbulence superimposed on a directional current, and intense wave action creates extreme hydrodynamic forces, which in turn increase the risk of dislodgment and mortality (Bell and Gosline, 1997; Denny, 1987; Hunt and Scheibling, 2001).

Marine mussels are experts at rapid, versatile, and permanent adhesion to solid surfaces in wave-swept seashores. This is a noteworthy achievement. Usually, the last few layers of water molecules adsorbed on hydrophilic surfaces are extremely difficult to remove, causing the failure of most man-made adhesives. Mussel adhesion is mediated by a holdfast structure known as the byssus, essentially a leathery bundle of threads tipped by flattened adhesive plaques that attach the mussel to a variety of hard surfaces (Lee *et al.,* 2006).

The key to survival on rocky wave and wind-swept seashores for many mussel species is a beard-like array of tethering attachment threads known collectively as the byssus, which is used to secure the mussel to the hard substratum against the lift and drag forces of waves (Yonge, 1962). In addition, the byssus is emerging as an important paradigm for the bioinspired engineering of water-resistant adhesives (Lee *et*

al., 2006), functionally graded polymers (Waite *et al.*, 2004), and injection molding of liquid crystals (Hassenkam *et al.*, 2004).

The blue mussel, *Mytilus edulis* Linnaeus, withstands these large hydrodynamic forces by tethering itself to the substrate with a byssal complex composed of multiple extracellular collagenous byssal threads that radiate from a central stem. Individual threads are secreted by a gland in the foot and comprise three regions: the corrugated proximal region, the smooth distal region and the terminal adhesive plaque, which attaches each thread to the substrate (Brown, 1952; Waite, 1992). Individual threads are both strong and extensible, and these material properties confer a mechanical toughness that exceeds that of mammalian tendon and other collagenous fibers (Gosline *et al.*, 2002). The strength of byssal attachment, or tenacity, has been shown to vary spatially and temporally; solitary mussels maintain a stronger attachment in comparison with bed mussels (Bell and Gosline, 1997), which presumably experience lower wave exposure.

It has often been suggested that the dynamics of attachment strength reflect the mussels' adaptive response to increased flow; producing a greater number of byssal threads enables mussels to remain attached during wavier conditions (Dolmer and Svane, 1994; Hunt and Scheibling, 2001; Lee *et al.*, 1990).

The role of thread production in enhancing mussel attachment, however, has recently been questioned. Moeser *et al.*, 2006) examined multiple flow levels and found a curvilinear relationship between flow and thread production, with thread production peaking around 10–14 cms^{-1} and decreasing dramatically at higher levels of flow (Moeser *et al.*, 2006). In addition, both laboratory and field experiments indicate that thread production is highest when mussels are most weakly attached (Moeser *et al.*, 2006). These findings indicate that increased thread production due to heightened

wave action is not the mechanism controlling the dynamics of mussel attachment strength, and alternative explanations warrant investigation.

One mechanism that could lead to the observed differences in attachment strength throughout the year is seasonal variation in thread mechanical properties. Byssal threads are composed of collagenous proteins with both silk and elastin domains in the distal and proximal regions, respectively (Qin and Waite, 1995). The presence of both metal chelates and DOPA crosslinks combined with specific oxidizing conditions is necessary to maximize the assembly of the individual protein fibers found in threads (Waite, 1992; Waite *et al.*, 2002). Just as the composition of human hair, a proteinaceous extracellular structure, is altered when humans are malnourished ,the structure and adhesion of byssal threads may also vary with fluctuations in food supply and water chemistry .Threads are known to reflect the geochemical nature of their environment .However, these geochemical signatures are metabolically transported to the threads rather than adsorbed onto the surface of the threads (Waite *et al.,* 2004). Several studies have quantified the tensile mechanical properties of individual byssal threads (Bell and Gosline, 1996; Carrington and Gosline, 2004; Lucas *et al.*, 2002; Smeathers and Vincent, 1979). Such tensile testing places an individual byssal thread in tension and extends it until failure, providing both a detailed description of thread behavior and an estimate of breaking force and breaking strain. Breaking force is the maximum force supported by a thread, whereas breaking strain is defined as the total distance a thread extends before failure, divided by the initial thread length. Overall byssus strength increases as the breaking force (strength) and breaking strain (extensibility) of individual threads increase. This latter effect is less intuitive; extensibility allows individual threads to stretch further within the byssal complex to realign and recruit more threads with which to withstand hydrodynamic forces (Bell and Gosline, 1996).

Byssal threads are subdivided into four morphologically and mechanically distinct regions: the stem, the plaque, and the proximal and the distal portions of the thread. The stem attaches the thread to the mussel tissue, and the plaque contains the adhesive that connects the thread to the hard substratum. The thread connecting the stem to the plaque is further divided into two mechanically distinct regions. The proximal end (closest to the organism) is extensible up to 200% of its original length, has a low initial stiffness, and a corrugated appearance. The distal portion of the thread, in contrast, is characterized by a high initial stiffness followed by a yield point at about 15% strain and a noticeable stress softening (Bell and Gosline, 1996). For most engineered polymeric materials, yield is not reversible and leads to permanent deformation rendering the material functionally useless. However, in distal threads, damage due to yield is reversible in a time-dependent, self-healing manner with threads recovering 25% of the lost modulus and strain energy in 10·min following a cycle to 35% strain (Carrington and Gosline, 2004).

Thread formation begins when the mussel foot touches down on a surface it finds suitable for attachment (Waite, 1992). Once committed, soluble thread precursors are secreted into the ventral groove of the foot where muscular contractions mold them into functional threads. The whole process takes a total of 2–5·min. The main protein component preCol, also known as byssal collagen, makes up 96% of distal thread and 66% of the proximal (Waite *et al.,* 2002).

Different sessile species of mussels develop byssus threads in order be safely attached to a suitable substratum. It has been shown that this formation could be a sensitive indicator to study impact of pollution (Eknath and Menon, 1979).

Mussels are filter feeders, and for this reason they play an important role in marine ecosystems. They reduce plankton populations, increase water clarity, and benefit plants, invertebrates, fish and bird populations .Bivalves, uses their foot, to

burrow and to move, and anchor themselves in one location even when water currents are rapid They pump the water through their gills, which are suspended within the dorsal side of their bodies, to filter out nutrients from the water. Each species can filter out different sized particles, and it has been reported that some species can retain particles with a diameter less than one 1 µm and others up to a diameter of 4 µm .

In the case of the clearance rate of bivalve molluscs, filtration has been considered to be subjected to physiological regulation (Hawkins *et al.,* 1996, Bayne and Thurberg 1998) with the purpose of maximizing energy uptake (Bayne *et al.,* 1993).

The ability to maintain a relatively constant uptake of oxygen under conditions of low oxygen availability is important for organisms that may encounter low dissolved oxygen (hypoxia) frequently in their environment (reviewed by Herreid, 1980). If the rate of oxygen consumption (OC) is not maintained at low levels of dissolved oxygen, anaerobic metabolism, which is inefficient with respect to energy production, may be activated in the tissues .This ability to maintain OC (generally called metabolic regulation) in water of reduced DO has been widely investigated for marine species of bivalves from a range of habitats (Bayne *et al.*, 1987).

Polycyclic aromatic hydrocarbons (PAHs) affect organisms through toxic actions. A major concern in animals is the ability of the reactive metabolites of some PAHs to bind cellular proteins and DNA, leading to mutations, developmental malformations, tumours and cancer (Hoffman, 2003). Relationships between the occurrence of neoplasia and hepatopacreas tumours in mussel and PAH concentrations in sediment where they were collected have shown that there is a strong causal link between these factors (Malins *et al.*,1988; Varanasi, 1989; Hoffman, 2003; Ariese *et al.*, 2005). Polycyclic aromatic hydrocarbons affect humans primarily through food consumption. Once ingested, PAHs can be absorbed by the human body and may cause cancers

and decreased fecundity, among other health problems (Fleming *et al.*, 2006). Besides ecological consequences, seafood safety is an issue of concern in every oil spill incident. Commercial and recreational fisheries and subsistence seafood use could potentially be affected as a consequence of the fauna and flora exposure to oil. In order to guarantee public health, restrictions or closure of seafood harvesting might be necessary (Yender *et al.*, 2002).

Mode of uptake and subcellular localisation of chemicals determine their toxicity as well as their pharmaceutical effects. Polyaromatic hydrocarbons (PAHs) are ubiquitous lipophilic contaminants in marine and terrestrial ecosystems with a high persistence and ability to accumulate in organisms and in the food web. Most of these pollutants are waste products from industrial processes and transportation activities that enter the marine environment through the atmosphere or rivers. These contaminants are of major concern because they are known to cause a variety of adverse effects to animal and human health.

Many contaminants are bound to particulate material and enter the digestive tract with food material. Particulate-associated pollutants are accumulated and further redistributed in the digestive gland, which is a major site for the detoxification of contaminants. Intracellular digestion of food particles, taken up by endocytosis, takes place in a well-developed lysosomal vascular system of digestive cells (Pipe and Moore 1986, Dimitriadis *et al.,* 2004).

The digestive gland was selected because it is considered the target organ in environmental pollution assessment. It accumulates pollutants of diverse nature and actively participates in detoxification processes (Marig´omez *et al.,* 1996; Moore and Allen, 2002). The digestive epithelium consists of two cell types, namely, the digestive and basophilic cells (Morton, 1983). Digestive cells are specialized in the intracellular

digestion and posses a well-developed endolysosomal system whereas basophilic cells are less abundant secretory cells (Dimitriadis *et al.*, 2004).

Water solubility and vapor pressure are the key physical/chemical characteristics of PAHs which influence their distribution amongst the soluble and particle components of the atmosphere, hydrosphere and biosphere. lipophilic and hydrophobic properties which make them preferentially accumulate in lipid rich tissues of organisms.

The digestive gland epithelium is mainly composed of two cell types: digestive and basophilic cells. Digestive cells, characterized by a well-developed endo-lysosomal system, are mainly involved in intracellular digestion and the detoxification of xenobiotics (Viarengo 1989, Cajaraville *et al.* 1995). The role of the basophilic cells is not clear, but they appear to be involved in extracellular digestion by producing and secreting hydrolytic enzymes (Cajaraville *et al.* 1990a, Marig´omez *et al.* 1998). Under control conditions, digestive cells are much more abundant than basophilic cells but, after exposure to pollutants, basophilic cells may outnumber digestive cells (Cajaraville *et al.*, 1990a,b, Marig´omez *et al.*, 1990 a,b, 1996, 1998).

The toxicity of organic xenobiotics such as polycyclic aromatic hydrocarbons (PAH) to aquatic organisms is likely partially mediated by the production of reactive oxygen species (ROS). These ROS are produced normally as by-products of the cellular metabolism but ROS production can be enhanced by xenobiotic metabolism (Di Giulio *et al.*,1995, Livingstone *et al.* 1994, Lackner 1998). It is well established that the metabolism of the model PAH benzo(*a*)pyrene (B(*a*)P) and in general aromatic hydrocarbon quinones by mussel digestive gland microsomes involves the formation of ROS (Livingstone 1998, Sjolin and Livingstone 1997, Mitchelmore *et al.*, 1998) with the concomitant risk of oxidative stress processes that can damage DNA and other cellular macromolecules (Mitchelmore *et al.*,1998). The activity of antioxidant enzymes, among

other mechanisms, plays a key role in preventing cellular damage to vital cellular components.

PAH treatment of molluscan digestive cells results in oxidative stress, probably by direct oxidative attack on intra-lysosomal PAH by oxyradicals normally generated in the lysosomal compartment. Such oxidative attack will generate AH-quinones which will result in redox cycling contributing to oxidative stress (Moore, 1990; Moore *et al.*, 1985; Sjolin and Livingstone, 1997; Winston *et al.,* 1991).

Glutathione (GSH) is a ubiquitous tripeptide that is regarded as one of the most important non-protein thiols in biological systems (Kosower and Kosower, 1978; Mason and Jenkins, 1996). GSH functions as an important overall modulator of cellular homeostasis, and serves numerous essential functions including detoxification of metals and oxy-radicals (Meister and Anderson, 1983; Christie and Costa, 1984). While exposure to pollutants or stressful conditions can result in elevated GSH levels, there is evidence that adverse effects are associated with GSH depletion in marine bivalves (Viarengo *et al.*, 1990; Ringwood *et al.*, 1999), as well as mammalian systems (Dudley and Klaasen, 1984). Organisms may also be more susceptible to additional stressors when GSH is depleted (Conners and Ringwood, 2000; Ringwood and Conners, 2000), and GSH status has been proposed as a potential risk factor in human-based risk assessments (Jones *et al.,* 1995).

Past research on various bivalve species subjected to a number of stressors has shown that the site of earliest detectable stress response is in the lysosome, an organelle of the hemocytes (Moore *et al.*, 1978, Moore 1980, Bayne *et al.*, 1980, 1981; Lowe *et al.,* 1995a, b). The rate of lysosomal changes following the addition of the neutral red dye is indicative of the stress response and can be directly related to the degree of stress being imposed on the mussels. Lysosomal membrane stability was

measured in freely circulating haemocytes by the neutral red retention time (NRRT) which quantifies the capability of these organelles to retain the vital dye (Regoli 2000).

Lysosomes are intracellular organelles that are involved in many essential functions, including membrane turnover, nutrition, and cellular defense (Adema *et al.*, 1991; Auffret and Oubella, 1994). The internal acidic environment of the lysosome, integral for the optimal activity of acid hydrolases, is maintained by a membrane-bound, ATPase-dependent proton pump (Ohkuma *et al.,* 1982). Lysosomes also act to sequester metals and other contaminants, which may lead to membrane destabilization (Lowe *et al.*, 1981, 1995a; Moore 1982, 1985). Disruption of the proton pump by chemical contaminants can lead to the impairment of vital functions and cell death (Moore, 1994; Lowe, 1996). Lysosomal destabilization has been used as a valuable indicator of cellular damage in a variety of fish and shellfish (Lowe *et al.*, 1992; Moore, 1994; Ringwood *et al.*, 1998b) and has been regarded as a valuable indicator of compromised biotic integrity (Moore, 1994).

A relatively simple assay using neutral red retention is used to assess lysosomal stability. This assay has been conducted successfully with hemocytes and hepatic cells.Hepatic tissues are frequently a major site of accumulation of toxins and a likely target for adverse effects. Cells incubated in neutral red accumulate the lipophilic dye in the lysosomes, where it is trapped by protonization. In healthy cells, neutral red is taken up and retained in stable lysosomes, whereas in damaged cells it leaks out of lysosomes and into the cytoplasm. The leaking of neutral red reflects the efflux of lysosomal contents into the cytosol, which ultimately causes cell death (Lowe *et al.,* 1995b).

Several studies have reported pollutants effects on the basis of the cellular biomarkers used in this investigation. Destabilization of lysosomal membranes in mussel cells has been observed in areas near urban and industrial discharges, with

presence of either organic chemicals (aromatic hydrocarbons, polychlorinated biphenyls, DDTs, HCH, and Aroclor 1254; Moore *et al.* 1978; Widdows *et al.* 1982; Krishnakumar *et al.*1994; Lowe *et al.* 1995) or metals (mercury, cobalt, zinc, and copper; Ward 1990; Krishnakumar *et al.* 1994; Lowe *et al.*1995). There is evidence of lysosomal enlargement in digestive cells of mussels obtained from native populations living in zones polluted by heavy metals such as manganese, iron, and lead (Regoli 1992) and from areas near sewage discharges of urban and industrial origin with high presence of organic and inorganic pollutants (Etxeberria *et al.* 1995). There is also evidence of increasing levels of lipofuscin granules in digestive cells of mussels exposed to organic and inorganic pollutants (Regoli 1992; Krishnakumar *et al.* 1994).

Several physiological responses of the mussel, *M. edulis,* have been tested under laboratory conditions for their suitability to detect environmental stressors. Those that offer potential as physiological biomarkers include respiration and filtration rates, pumping rates, valve activity and heart beat (Balogh and Salanki, 1987).

Histopathological examination of bivalve digestive gland has shown it to be a sensitive target for the injurious action of many pollutants (Lowe *et al.*, 1981; Moore and Viarengo,1987; Lowe, 1988). Pathological alterations are a reflection of disturbances at the molecular level (Moore and Simpson, 1992) and identification of these disturbances can aid in the understanding of the whole animal consequences of early cell response to pollutant exposure.

Lysosomal perturbations have been widely used as early indicators of adverse effect to various factors, including pollutant exposure (Galloway *et al.,* 2004; Moore, 2002; Moore *et al.,* 2004). Consequently, lysosomal function can be used across a range of animals, including annelids, molluscs, crustaceans and fish to detect responses to environmental stress (Cajaraville *et al.*, 2000; Galloway *et al.,* 2004; Hankard *et al.*, 2004;

Hwang *et al.*,2002; Kohler, *et al.*, 1992; Kohler, *et al.*,, 2002; Lekube, *et al* ., 2000; Lowe, *et al*,1992,1995; Svendsen and Weeks, 1995; Wedderburn, *et al.*,1998).

Lysosomal stability constitutes a very useful index of cellular damage. Lysosomes in the digestive gland of mussels are organelles dealing primarily with nutrition, tissue repair and cellular components' turnover. In addition, they constitute main sites of toxic metal and organic pollutant's sequestration and detoxification (Moore, 1985; Viarengo *et al.,* 1987), which may lead to lysosomal membrane damage, and to the leaking of the lysosomal contents into the cytoplasm. The stability of the lysosomal membranes is evaluated using the lysosomal membrane stability test (LMS) of the digestive gland (e.g. Domouhtsidou and Dimitriadis, 2001; Krishnakumar et al., 1994; Petrovic *et al* 2001; Regoli, 1992; UNEP, 1997), as well as in vitro using the neutral red retention assay (NRR) of the haemocyte lysosomes. NRR measures the lysosomal content efflux into the cytosol which, in stressed mussels, reflects a physiological process after membrane damage and comparatively measures the capacity of cellular processes to adapt to stress conditions (Lowe and Pipe, 1994). The efflux of lysosomal contents into the cytosol has been shown to be a rapid and sensitive test for determining the lysosomal stability of indigenous invertebrate populations (e.g. Fernley *et al.,* 2000; Lowe, *et al* .,1995).

Chapter 1

BIOACCUMULATION AND CERTAIN PHYSIOLOGICAL RESPONSES OF *Perna spp.* EXPOSED TO PETROLEUM HYDROCARBONS

Contents

1.1 Introduction
1.2 Materials and methods
1.3 Result
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1.1 Introduction

Environmental degradation by oil spill is one of the most serious problems facing the coastal ecosystem. The distribution of hydrocarbons in the environment can vary from one area to another. Considerable quantities of petroleum products are discharged into the marine environment through run off, atmospheric input, industrial effluents, sewage storm water drains, shipping activities, spillage, etc. Besides natural oil seeps can also be considerable in some areas. Significant changes in hydrocarbon composition can occur due to selective dissolution, evaporation, chemical and photo-oxidation and biodegradation.

Crude oil is a mixture of compounds ranging from low-molecular-weight hydrocarbons to very complex polynuclear aromatic hydrocarbons containing numerous isomers. When crude oil is mixed with water, a much more physically and chemically complicated mixture is formed. The bulk of the oil is mostly nonpolar and forms into spheres of various sizes. Compounds then partition between the oil droplets and the water (Shaw, 1997). It has been shown that crude oil droplets in water are coated by a layer of water molecules (Malcolm and Commaert, 1981) which prevents

the droplets from coalescing on contact with each other. Apart from tending to keep oil in an emulsion, these water jackets on the oil droplets could be important in increasing contact between oil biological components such as mucus.

Crude oil is a naturally occurring complex mixture of hydrocarbons and non hydrocarbons which, at appropriate concentrations, possesses a measurable toxicity towards living systems (Halstead, 1971). There is a considerable evidence of oil degradation process in natural environments through laboratory experiments, extrapolation of such data to environmental conditions – especially when sub-lethal concentrations are involved – is a formidable exercise. Therefore, while the toxicity of crude oil cannot be denied it is clear that there is a vast lack of knowledge concerning the process.

Each contaminant has its own contamination scenario, which differs not only according to the system being considered, but also according to the state of the receiving environment. The initial impact of oil in coastal and marine systems can vary from minimal to the death of everything in a particular biological community.

The significant environmental PAH range is between naphthalene and coronene ($C_{24}H_{12}$). Physical and chemical properties of PAH vary approximately in a regular trend with molecular weight. Susceptibility to redox reactions increases with increasing molecular weight, whereas aqueous solubility and vapour pressure decreases almost logarithmically with increasing molecular weight (Neff, 1979). Thus PAH differ in their environmental behaviour and interactions with biological systems. Some of PAH's are well known carcinogens and mutagens. The low molecular weight (two and three rings) PAHs have a significant acute toxicity, whereas some of the higher molecular weight PAHs are carcinogenic (Witt 1995). The aqueous solubility of PAHs is low (Chu and Chan, 2000) and their hydrophobic nature to particulate associations. In addition, there is increasing evidence that PAH may be occluded in soft particles (eg. Readman *et al.*, 1984 ; American Chemical Society, 1997) hindering exchange and isomer specific alterations through microbial degradation, photo-degradation and chemical oxidation.

The light aromatic compounds (benzene naphthalene) are found to be one of the most immediate toxic component of petroleum other than the carcinogenic polycyclic aromatic hydrocarbons and according to Neff, (1979) the low molecular weight PAH i.e. 2-3 ring aromatics (Naphthalene, fluorine, phenanthrenes and anthracenes) have significant acute toxicity to aquatic organisms, than PAH like 4-7 ring aromatic (chrysene, coronene). The PAHs of interest (chrysene, benzo (b) fluoranthene, benzo (a) pyrene, indeno (1,2,3 – c,d) pyrene and dibenzo (1,2,5,6) anthracene) were potential carcinogens (Kennish, 1996). Naphthalene, fluorenes, phenanthrenes, and anthracenes are volatile to some extent and are relatively toxic to aquatic lives.

The primary repository of the larger PAHs, four, five, and six-ring species, in water bodies is the sediment (Latimer and Zheng, 2003). This has consequences in sedimentation areas such as estuaries with the risk of high exposure of benthic organisms to PAH (Den Besten *et al.*, 2003). PAHs can be bioconcentrated and /or bioaccumulated by certain aquatic invertebrates low in the food chain that they either lack or have a poorly developed capacity for effective biotransformation.

Bivalves are the most commonly used sentinel organisms for the health assessment of the marine environment. The special properties of a sentinel species are that it is able to survive in a polluted habitat, and accumulates chemicals in its tissues. Due to their ability to accumulate and reflect a wide range of contaminants, mussels have been widely used in marine pollution monitoring (Goldberg 1975, Cajaraville *et al.,* 1990 ; Livingstone *et al.,* 1990 ; Smolders *et al.*, 2003 ; Marigómez *et al.,* 2006 ; ICES, 2006). Mussels, particularly *Perna* spp., have several advantages as a bioindicator, such as a wide distribution, abundance, sedentary behaviour, and a pronounced ability to accumulate organic compounds.

Toxicity tests are one experimental approach that measures the response of living organisms to contaminants. These responses may be lethal effects, e.g. death of

the organism over 96 h, or sub-lethal effects such as inhibition of growth, reproduction or enzyme activity. Toxicity is a generic measure of the particular biological response or end-point. Responses can be assessed at any level of biological organisation, and testing usually includes a range of endpoints and test species from different levels of the food chain.

The idea of quantifying the accumulation of potentially toxic chemicals in environmental compartments in order to evaluate a safe ecotoxicity threshold in aquatic environments has been formally applied by Miller (1992). The concept assumes that the ability of an aquatic ecosystem to restore itself to pre-pollution state is related to the retention/transport rates in and outside environmental compartments (i.e. sediment).

The rate at which accumulation occurs in an organism depends not only on the availability of the pollutant, but also a whole range of biological, chemical and environmental factors. The ultimate levels reached are governed by the ability of the organisms to excrete the pollutant or alternatively store it. Organisms, which happen to accumulate the PAHs, do also have the capacity to depurate. One fundamental but probably not startling insight is that continuous attention to sub-lethal physiological/ biochemical effects of pollutants is an essential ingredient in any broad examination of environment pollution. Sub-lethal effects – many of them physiological – can provide understanding of causes of toxicity – an understanding that is rarely gained from acute tests. Of course, physiological studies are but part of a matrix of interlocking research efforts, having especially critical synapses and anastamoses with animal behaviour, population genetics, pathology, and ecosystem analyses.

Numerous accumulation studies on the common mussel, *Perna viridis* and *Perna indica*, have been carried out under true marine conditions. The present study was carried out under controlled conditions to determine the effects of different concentrations of PAHs on the filtration activity, byssogenesis, oxygen consumption

rate and the subsequent level of toxicity acquired by *Perna spp.* In order to study the biological significance of these concentrations, physiological and histological measurements were made. The lethal concentration (LC_{50}) were determined. The byssogenesis test, opening response and production of mucus were used to detect the effects of sublethal concentrations on the mussels.

1.2 Materials and Methods

Experimental set-up

Small sized mussels having an average body length of 12 mm and weighing 0.35 g were selected for the test. Duplicates and controls were maintained. The bioassay tests were repeated 4 times till consistent results were obtained. The mussels, *Perna viridis* and *Perna indica* were collected and transported from Thangassery ($9^{0\prime}$ N, 76^{0} 35′ E). They were cleaned off the epibiotic growths. Prior to the experiments, the mussels were acclimatized to laboratory conditions for at least 7 days in well aerated natural sea water (salinity 32 ppt, temp 28^{0}C, pH 7.8, dissolved oxygen >4ml/l). The animals were fed daily with artificial feeds.

Bioassay studies

The effect of PAHs on the survival of *Perna viridis* and *Perna indica* were assessed following the static renewal type of bioassay method. Acute toxicity studies were conducted to determine the 96 hr LC_{50} value of PAHs.

Preparation of petroleum solutions for toxicity testing

The preparation of water accommodated fraction (WAFs) for use in ecotoxicity testing of crude oils involves a 20 h mixing period and at least 1 h standing for phase separation. This approach is based on the work performed by Anderson *et al.,* (1974).

The concentration of the accommodated oil was estimated in ppm basis after extraction of oil from the WAF using n-hexane.The fluorescence intensity of the hexane

extract was determined against a standard of chrysene, using fluorescence spectrophotometer at wave lengths 310 nm (EX) and 360 nm (EM).

The respective oil concentration in the 100% WAF was computed from the standard graph, Calculated volumes of the WAF of the respective oil was then added to the test media to get the required PHC concentration.

Spectrofluorometric Measurement of Total Polyaromatic Hydrocarbons in Seawater

500 ml of seawater was taken in a separatory funnel and the oil was extracted with 25 ml 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 50 ml by addition of hexane (IOC, 1984). The concentration of PHC was determined against a standard of chrysene, using fluorescence spectrophotometer at wave lengths 310 nm (EX) and 360 nm (EM).

Spectrofluorometric Measurement of Total Polyaromatic Hydrocarbons in Sediment

Wet sediment samples of 50 grams each were digested for 2 hours in soxhlet apparatus with 50 ml of 0.5 N methanolic KOH (HPLC grade). The resultant organic phase was extracted twice with 25 ml 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, 10 cm). The hydrocarbons were diluted with n-hexane and made up to a final volume of 25 ml each (IOC, 1982). The concentration of PHC is expressed in chrysene equivalents using the fluorescence spectrophotometer 310 nm (EX) and 360 nm (EM).

Spectrofluorometric Measurement of Total Polyaromatic Hydrocarbons in Mussel tissue

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

Byssogenesis

Byssus thread production was determined following procedures outlined by VanWinkle (1970) and Rajagopal *et al.,* (1997).

Byssal apparatuses were obtained from *Perna viridis* and *Perna indica* after exposure of WAF of PAHs. For SEM, samples were fixed by immersion in 3% gluteraldehyde in phosphate buffer, pH 7.4, either *in toto* or after partial dissection. The specimens were then dehydrated, cut with the aid of a stereomicroscope, and fixed on metal supports. After shadowing with gold-palladium they were examined with a Leo 435 VP SEM Cambridge.

The oxygen consumption was determined following the method of Bricelj and Malouf. (1984). Filtration rate was measured following the method described by Coughlan (1969).

Statistical Analysis

Data were presented as the mean ± standard deviation. Data were first tested for normality distributions using the Kolmogorov-Smirnov test and checked for homogeneity of variance by the Levene test. A 3-way ANOVA for hydrocarbons, using an additive model for three factors (tissue type, treatment and time), was performed. Statistical analyses were carried out by using Sigmastat 3.5 statistical software package.

1.3 Results

The effect of PAHs on the survival of ***Perna viridis*** and ***Perna indica*** were assessed following the static renewal type of bioassay method. Acute toxicity studies were conducted to determine the 96 hr LC_{50} value of PAHs.

Results obtained from acute toxicity tests are expressed as LC_{50}-96h values. The 95% confidence intervals associated with these endpoints were also observed, as an estimate of the precision (uncertainty) around the LC_{50} value. In tests without partial

kills, the 95% confidence intervals were estimated as follows: the lower limit was the highest concentration with 0% mortality, and the upper limit was the lowest concentration with 100% mortality. Pooled estimates of 96h LC_{50} were reported for repeated tests with WAF of BHC and LDO because the 96 h LC_{50} values calculated for each test were not significantly different. The LC_{50} values were estimated by probit analysis (PASW 18) and the results are summarized in Table 1.1.

For the 96-h WAF of BHC experiment, LC_{50} values of 6.49 ppm was for *P. indica* and, 10.30 ppm for *P. viridis* .

For the experiment with WAF of LDO, *P. indica* was more sensitive with an LC_{50} of 4.60 ppm, while it was 5.20 ppm for *P. viridis* .

The exposed mussels detached their byssus thread and secreted mucous, resulting in foaming of the culture media. The frothing increased with increasing PAHs concentration. Another response of the mussels to WAF of PAHs exposure was sustained valve adduction, particularly at higher concentrations. Reaction to irritation (closing of the valves, withdrawing of the foot) was slow or absent and mussels were immobile at lower concentrations of PAHs.

Table 1.1: Acute toxicity values (96-h LC_{50} ppm; 95% confidence interval in parentheses) of *Perna viridis* and *Perna indica* tested with WAF of LDO and BHC.

Exposure media	Species	96-h LC_{50} value (ppm)
LDO WAF	*Perna indica*	**4.60 (2.41-6.79)**
BHC WAF	*Perna indica*	**6.49 (3.63-9.36)**
LDO WAF	*Perna viridis*	**5.20 (2.59-7.81)**
BHC WAF	*Perna viridis*	**10.30 (5.32-15.29)**

Additionally, short-term toxic effects of PAHs on *Perna viridis* and *Perna indica* have also been evaluated.The rate of PAHs accumulation in both the individuals of

Perna viridis and *Perna indica* , exposed to various concentrations of BH crude WAFs for periods ranging from 1 to 20 days (Phase of accumulation) and subsequently exposed to raw sea water for 15 days (Phase of depuration) are presented in tables 1.2-1.5. The mussels survived 35 days in confined experimental set up and part of the PAHs accumulated in the mussel tissue.

Summary table of 3-way ANOVA revealed that there was a significant increase ($P<0.001$) between control and different concentrations of WAF treated groups. Accumulation of total PAHs were significant ($P <0.001$) in the tissues. The maximum accumulation was found to occur in hepatopancreas followed by gill and mantle tissue. Adductor muscle accumulated the least quantities of WAF.

Among the tissues petroleum hydrocarbons accumulation was maximum in hepatopancreas, followed by gill, mantle and adductor muscle, irrespective of the concentration of WAF of PAHs. Hence it was inferred that a small quantity of WAF of crude oil would lead to considerable accumulation of petroleum hydrocarbons in the tissues. The responses measured are all dose and time dependant though the rates are variable.

BAF was found for WAF of BHC and WAF of LDO in *Perna viridis* were 0.046 to 2.734 and 0.039 – 2.739 respectively. *Perna indica* the BAF for WAF of BHC and WAF of LDO were 0.061-1.908 and 0.048-2.131.

Oxygen consumption rates of the mussels under the WAF of BHC treatments are presented in (Table 1.6-1.9). The mean oxygen consumption rate ranged from 0.74 to l.38 µg O_2 $h^{-1}mg^{-1}$ dry wt for *P.viridis* BHC WAF exposed mussels and 0.69 to 1.57 µg O_2 $h^{-1}mg^{-1}$ dry wt for LDO WAF exposed ones. The oxygen consumption rate ranged from 0.61 to l.30 µg O_2 $h^{-1}mg^{-1}$ dry wt and 0.49 to l.54 µg O_2 $h^{-1}mg^{-1}$ dry wt for *P. indica* exposed to WAF of BHC and LDO respectively. However, no significant statistical changes ($p > 0.05$) in oxygen consumption was recorded between treatments and exposure time.

Table 1.2: Accumulation of PHC in different tissues of *Perna viridis* when exposed to sublethal concentrations of WAF of BH Crude oil (μg/g wet wt)

Exposure Concentrations	Days	Tissues			
		Hepatopancreas	Gill	Adductor Muscle	Mantle
Control					
		0	0	0	0
0.1 ppm					
	1	14.02 ± 0.01	12.13 ± 0.02	0.92 ± 0.10	8.52 ± 0.30
	5	16.00 ± 0.01	14.50 ± 0.35	1.50 ± 0.01	9.16 ± 0.01
	10	19.32 ± 0.06	18.31 ± 0.02	2.38 ± 0.09	11.10 ± 0.18
	15	24.30 ± 0.08	20.00 ± 0.35	6.52 ± 0.07	13.51 ± 0.15
	20	33.40 ± 0.05	24.12 ± 0.17	7.30 ± 0.23	14.22 ± 0.26
1 ppm					
	1	16.25 ± 0.05	13.02 ± 0.32	1.22 ± 0.45	10.52 ± 0.13
	5	21.02 ± 0.01	14.77 ± 0.17	5.61 ± 0.23	11.48 ± 0.34
	10	23.51 ± 0.16	18.58 ± 0.21	8.22 ± 0.75	12.01 ± 0.16
	15	32.79 ± 0.01	22.05 ± 0.16	10.18 ± 0.12	14.11 ± 0.13
	20	39.70 ± 0.08	25.01 ± 0.19	13.30 ± 0.24	15.06 ± 0.21
5 ppm					
	1	21.04 ± 0.24	13.79 ± 0.18	1.58 ± 0.17	12.66 ± 0.13
	5	24.12 ± 0.15	15.33 ± 0.18	6.30 ± 0.14	12.18 ± 0.12
	10	28.92 ± 0.19	21.52 ± 0.13	9.20 ± 0.32	14.54 ± 0.18
	15	36.16 ± 0.15	23.10 ± 0.18	11.01 ± 0.14	17.00 ± 0.15
	20	51.05 ± 0.24	26.44 ± 0.18	16.44 ± 0.32	18.15 ± 0.13
8 ppm					
	1	23.52 ± 0.21	15.05 ± 0.18	2.01 ± 0.01	13.02 ± 0.18
	5	26.38 ± 0.16	15.48 ± 0.15	6.55 ± 0.22	12.99 ± 0.13
	10	37.04 ± 0.32	22.18 ± 0.15	9.50 ± 0.18	16.95 ± 0.13
	15	38.17 ± 0.16	25.01 ± 0.12	13.62 ± 0.15	19.01 ± 0.18
	20	54.69 ± 0.25	28.95 ± 0.12	20.99 ± 0.13	23.11 ± 0.12

Three Way Analysis of Variance

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	4098.591	1366.197	617.961	<0.001
PAH CONC	**3**	546.113	182.038	82.340	<0.001
TIME	**5**	6246.412	1249.282	565.078	<0.001
Residual	**45**	99.487	2.211		
Total	**95**	12705.715	133.744		

Table 1.3: Accumulation of PHC in different tissues of *Perna viridis* when exposed to sublethal concentrations of WAF of LDO (µg/g wet wt).

Exposure concentrations	Days	Tissues			
		Hepatopancreas	**Gill**	**Adductor Muscle**	**Mantle**
Control					
	1	0	0	0	0
0.05 ppm					
	1	23.68 ± 0.24	13.72 ± 0.18	0.79 ± 0.02	11.21 ± 0.08
	5	26.92 ± 0.12	16.21 ± 0.17	0.96 ± 0.01	18.44 ± 0.02
	10	32.46 ± 0.12	18.67 ± 0.13	1.52 ± 0.04	21.69 ± 0.12
	15	38.54 ± 0.12	22.56 ± 0.15	2.78 ± 0.13	29.76 ± 0.18
	20	40.28 ± 0.10	26.71 ± 0.13	5.31 ± 0.04	32.51 ± 0.18
0.5 ppm					
	1	27.32 ± 0.21	15.36 ± 0.15	2.18 ± 0.04	19.39 ± 0.17
	5	31.59 ± 0.16	17.63 ± 0.12	10.52 ± 0.09	22.65 ± 0.31
	10	36.17 ± 0.04	19.79 ± 0.01	18.79 ± 0.02	30.70 ± 0.07
	15	42.78 ± 0.02	22.76 ± 0.05	22.36 ± 0.02	46.54 ± 0.01
	20	48.60 ± 0.01	38.79 ± 0.12	30.19 ± 0.11	48.20 ± 0.15
2 ppm					
	1	30.71 ± 0.12	18.79 ± 0.16	15.79 ± 0.07	24.23 ± 0.05
	5	32.56 ± 0.18	21.36 ± 0.04	20.56 ± 0.06	31.90 ± 0.14
	10	31.79 ± 0.02	26.54 ± 0.01	22.67 ± 0.18	37.67 ± 0.20
	15	42.86 ± 0.03	29.31 ± 0.12	29.79 ± 0.05	40.39 ± 0.15
	20	49.70 ± 0.12	35.46 ± 0.06	33.76 ± 0.22	46.78 ± 0.27
4.8 ppm					
	1	32.78 ± 0.06	22.56 ± 0.02	20.64 ± 0.23	30.71 ± 0.16
	5	35.62 ± 0.07	26.79 ± 0.35	25.29 ± 0.27	35.26 ± 0.16
	10	38.76 ± 0.22	28.21 ± 0.18	32 .74 ± 0.19	39.30 ± 0.15
	15	42.63 ± 0.18	34.67 ± 0.30	39.20 ± 0.07	43.79 ± 0.22
	20	54.79 ± 0.02	42.34 ± 0.16	46.76 ± 0.18	48.65 ± 0.18

Three Way Analysis of Variance

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	3225.496	1075.165	112.289	<0.001
PAH CONC	**3**	2514.974	838.325	87.553	<0.001
TIME	**5**	14830.502	2966.100	309.775	<0.001
Residual	**45**	430.875	9.575		
Total	**95**	23155.147	243.738		

Table 1.4: Accumulation of PHC in different tissues of *Perna indica* when exposed to sublethal concentrations of WAF of BH Crude oil (μg/g wet wt)

Exposure concentrations	Days	Tissues			
		Hepatopancreas	Gill	Adductor Muscle	Mantle
Control					
	1	0	0	0	0
0.1 ppm					
	1	18.54 ± 0.02	16.71 ± 0.01	0.69 ± 0.01	8.76 ± 0.02
	5	20.21 ± 0.08	19.87 ± 0.01	1.25 ± 0.02	10.95 ± 0.04
	10	25.44 ± 0.06	20.32 ± 0.02	2.78 ± 0.01	11.21 ± 0.04
	15	29.61 ± 0.04	25.16 ± 0.03	4.86 ± 0.05	15.78 ± 0.03
	20	32.78 ± 0.02	30.80 ± 0.01	10.78 ± 0.05	19.22 ± 0.06
0.6 ppm					
	1	22.66 ± 0.04	18.93 ± 0.22	12.20 ± 0.17	10.39 ± 0.15
	5	25.31 ± 0.02	24.67 ± 0.11	17.54 ± 0.14	15.41 ± 0.09
	10	28.49 ± 0.13	28.66 ± 0.10	19.22 ± 0.13	18.63 ± 0.07
	15	36.90 ± 0.12	32 .79 ± 0.11	26.30 ± 0.03	20.77 ± 0.01
	20	42.03 ± 0.04	36.99 ± 0.01	29.81 ± 0.03	25.65 ± 0.05
2 ppm					
	1	23.29 ± 0.20	21.16 ± 0.08	18.64 ± 0.05	16.31 ± 0.02
	5	26.77 ± 0.07	24.33 ± 0.01	22.11 ± 0.08	22.73 ± 0.01
	10	27.69 ± 0.05	27.66 ± 0.12	26.64 ± 0.05	26.64 ± 0.02
	15	32.59 ± 0.06	28.00 ± 0.02	29.31 ± 0.02	30.12 ± 0.04
	20	39.55 ± 0.05	30.10 ± 0.01	33.64 ± 0.14	35.49 ± 0.12
5 ppm					
	1	25.66 ± 0.02	26.33 ± 0.01	24.01 ± 0.02	29.38 ± 0.04
	5	28.32 ± 0.02	29.10 ± 0.11	26.84 ± 0.15	31.44 ± 0.07
	10	31.24 ± 0.12	32.19 ± 0.14	32.70 ± 0.22	35.09 ± 0.15
	15	36.77 ± 0.14	39.22 ± 0.12	36.11 ± 0.09	38.46 ± 0.10
	20	49.20 ± 0.15	42.16 ± 0.12	39.06 ± 0.07	47.21 ± 0.05

Three Way Analysis of Variance

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	893.607	297.869	50.642	<0.001
PAH CONC	**3**	2682.321	894.107	152.012	<0.001
TIME	**5**	11103.132	2220.626	377.541	<0.001
Residual	**45**	264.682	5.882		
Total	**95**	16604.633	174.786		

Table 1.5: Accumulation of PHC in different tissues of *Perna indica* when exposed to sublethal concentrations of WAF of LDO (μg/g wet wt).

Exposure concentrations	Days	Tissues			
		Hepatopancreas	Gill	Adductor Muscle	Mantle
Control					
	1	0	0	0	0
0.04 ppm					
	1	20.72 ± 0.12	16.52 ± 0.10	6.54 ± 0.03	10.59 ± 0.10
	5	25.79 ± 0.12	18.71 ± 0.06	11.79 ± 0.05	12.63 ± 0.02
	10	30.64 ± 0.15	20.55 ± 0.12	16.33 ± 0.09	14.59 ± 0.15
	15	32.70 ± 0.15	25.74 ± 0.08	19.19 ± 0.14	19.36 ± 0.09
	20	40.50 ± 0.14	33.69 ± 0.22	22.56 ± 0.14	21.54 ± 0.03
0.4 ppm					
	1	26.59 ± 0.12	20.64 ± 0.07	15.79 ± 0.04	18.96 ± 0.06
	5	28.43 ± 0.15	26.94 ± 0.11	21.33 ± 0.12	25.41 ± 0.05
	10	32.76 ± 0.13	33.70 ± 0.05	25.69 ± 0.17	27.99 ± 0.13
	15	39.41 ± 0.04	35.67 ± 1.20	32.16 ± 0.07	32.55 ± 0.12
	20	45.29 ± 0.07	49.31 ± 0.16	37.05 ± 0.18	38.21 ± 0.30
1 ppm					
	1	29.77 ± 0.27	20.79 ± 0.23	18.76 ± 0.16	20.66 ± 0.30
	5	35.66 ± 0.22	25.33 ± 0.18	23.68 ± 0.15	25.44 ± 0.16
	10	38.99 ± 0.07	28.01 ± 0.02	25.16 ± 0.15	28.74 ± 0.04
	15	40.20 ± 0.02	30.72 ± 0.18	28.04 ± 0.12	32.69 ± 0.19
	20	41.35 ± 0.15	35.60 ± 0.18	32.60 ± 0.07	37.21 ± 0.15
3.5 ppm					
	1	35.27 ± 0.12	26.77 ± 0.08	22.79 ± 0.07	30.20 ± 0.11
	5	40.81 ± 0.22	35.64 ± 0.16	30.19 ± 0.14	35.19 ± 0.14
	10	42.76 ± 0.11	44.27 ± 0.10	36.68 ± 0.12	40.20 ± 0.16
	15	45.33 ± 0.11	49.21 ± 0.158	42.89 ± 0.09	40.79 ± 0.05
	20	58.70 ± 0.07	52.77 ± 0.12	55.70 ± 0.23	49.21 ± 0.19

Three Way Analysis of Variance

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	1030.526	343.509	69.474	<0.001
PAH CONC	**3**	3255.190	1085.063	219.451	<0.001
TIME	**5**	15987.064	3197.413	646.667	<0.001
Residual	**45**	222.500	4.944		
Total	**95**	21789.071	229.359		

Mean filtration rates of experimental animals were less than those of control bivalves for all recorded intervals over the 20 days of exposure (Table 1.6-1.9).

Table.1.6: Physiological responses of *Perna viridis* exposed to WAF of BH Crude oil during the experimental periods

Experimental condition	Days	Filtration rate (ml $h^{-1}mg^{-1}$dry wt)	Respiration rate ($\mu gO_2h^{-1}mg^{-1}$dry wt)
Control			
		1.72 ± 0.07	1.52 ± 0.11
0.1 ppm			
	1	1.50 ± 0.06	1.38 ± 0.09
	5	1.29 ± 0.09	1.24 ± 0.12
	10	1.25 ± 0.10	1.05 ± 0.11
	15	0.54 ± 0.08	1.08 ± 0.14
	20	0.52 ± 0.14	0.98 ± 0.08
1 ppm			
	1	1.35 ± 0.06	1.25 ± 0.08
	5	1.32 ± 0.11	1.11 ± 0.05
	10	0.89 ± 0.08	1.03 ± 0.04
	15	0.76 ± 0.12	0.98 ± 0.09
	20	0.72 ± 0.14	0.95± 0.07
5 ppm			
	1	1.20 ± 0.11	1.03 ± 0.15
	5	0.82 ± 0.09	1.10 ± 0.11
	10	0.54 ± 0.06	1.05 ± 0.12
	15	0.31 ± 0.07	0.93 ± 0.11
	20	0.25 ± 0.12	0.74 ± 0.16
8 ppm			
	1	1.22 ± 0.07	1.20 ± 0.08
	5	0.88 ± 0.06	0.99 ± 0.07
	10	0.67 ± 0.05	0.95 ± 0.06
	15	0.48 ± 0.06	1.12 ± 0.10
	20	0.36 ± 0.10	1.20± 0.14

Table 1.7: Physiological responses of *Perna viridis* exposed to WAF of LDO during the experimental periods

Experimental condition	Days	Filtration rate (ml $h^{-1}mg^{-1}$dry wt)	Respiration rate ($\mu gO_2h^{-1}mg^{-1}$dry wt)
Control			
		1.65 ± 0.09	1.41 ± 0.11
0.05 ppm			
	1	1.58 ± 0.06	1.36 ± 0.11
	5	1.05 ± 0.04	1.56 ± 0.15
	10	0.97 ± 0.08	1.09 ± 0.14
	15	0.70 ± 0.10	1.02 ± 0.11
	20	0.54 ± 0.07	0.95 ± 0.12
0.5 ppm			
	1	1.69 ± 0.09	1.48 ± 0.10
	5	1.48 ± 0.12	0.94 ± 0.08
	10	1.23 ± 0.11	0.91 ± 0.06
	15	0.68 ± 0.08	0.81 ± 0.05
	20	0.61 ± 0.05	0.68 ± 0.08
2 ppm			
	1	1.49 ± 0.02	1.57 ± 0.03
	5	0.98 ± 0.15	1.01 ± 0.06
	10	0.72 ± 0.11	0.84 ± 0.08
	15	0.69 ± 0.08	0.82 ± 0.11
	20	0.62 ± 0.06	0.91 ± 0.07
4.8 ppm			
	1	1.30 ± 0.05	1.02 ± 0.08
	5	0.75 ± 0.04	0.94 ± 0.07
	10	0.63 ± 0.01	0.77 ± 0.09
	15	0.55 ± 0.01	0.69 ± 0.11
	20	0.52 ± 0.08	1.20± 0.14

Table 1.8: Physiological responses of *Perna indica* exposed to WAF of BH Crude oil during the experimental periods

Experimental condition	Days	Filtration rate (ml $h^{-1}mg^{-1}$dry wt)	Respiration rate ($\mu gO_2h^{-1}mg^{-1}$dry wt)
Control			
		1.92 ± 0.05	1.56 ± 0.12
0.1 ppm			
	1	1.09 ± 0.11	1.30 ± 0.12
	5	0.79 ± 0.12	1.29 ± 0.11
	10	0.78 ± 0.09	1.10 ± 0.14
	15	0.65 ± 0.05	0.95 ± 0.10
	20	0.55 ± 0.02	0.68 ± 0.09
0.6 ppm			
	1	1.37 ± 0.09	1.24 ± 0.05
	5	1.13 ± 0.05	1.02 ± 0.11
	10	1.09 ± 0.02	0.94 ± 0.13
	15	0.59 ± 0.12	0.77 ± 0.15
	20	0.51 ± 0.05	0.61 ± 0.10
2 ppm			
	1	2.04 ± 0.05	1.14 ± 0.07
	5	1.67 ± 0.06	1.01 ± 0.05
	10	0.90 ± 0.12	0.87 ± 0.11
	15	0.51 ± 0.07	0.80 ± 0.03
	20	0.62 ± 0.06	0.78 ± 0.09
5 ppm			
	1	0.86 ± 0.12	1.05 ± 0.11
	5	0.83 ± 0.07	0.99 ± 0.13
	10	0.73 ± 0.05	0.87 ± 0.10
	15	0.40 ± 0.03	0.77 ± 0.07
	20	0.38 ± 0.04	0.80 ± 0.09

Table 1.9: Physiological responses of *Perna indica* exposed to WAF of LDO during the experimental periods

Experimental condition	Days	Filtration rate (ml $h^{-1}mg^{-1}$dry wt)	Respiration rate ($\mu gO_2h^{-1}mg^{-1}$dry wt)
Control			
		1.86 ± 0.06	1.39 ± 0.08
0.04 ppm			
	1	1.70 ± 0.09	1.43 ± 0.02
	5	1.22 ± 0.05	1.54± 0.05
	10	1.13 ± 0.08	1.14 ± 0.06
	15	0.83 ± 0.06	1.11 ± 0.09
	20	0.78 ± 0.11	0.89 ± 0.03
0.4ppm			
	1	1.39 ± 0.05	1.47 ± 0.09
	5	1.22 ± 0.07	1.49 ± 0.07
	10	1.09 ± 0.04	0.95 ± 0.01
	15	0.66 ± 0.05	0.87 ± 0.06
	20	0.61 ± 0.03	0.72 ± 0.05
1 ppm			
	1	1.09 ± 0.02	1.44 ± 0.02
	5	0.77 ± 0.05	1.05 ± 0.09
	10	0.79 ± 0.02	0.82 ± 0.10
	15	0.52 ± 0.05	0.77± 0.01
	20	0.50± 0.04	0.56 ± 0.07
3.5 ppm			
	1	0.83 ± 0.05	1.05 ± 0.02
	5	0.78 ± 0.07	0.97 ± 0.07
	10	0.54 ± 0.11	0.82± 0.03
	15	0.49 ± 0.12	0.71 ± 0.01
	20	0.42 ± 0.09	0.49 ± 0.06

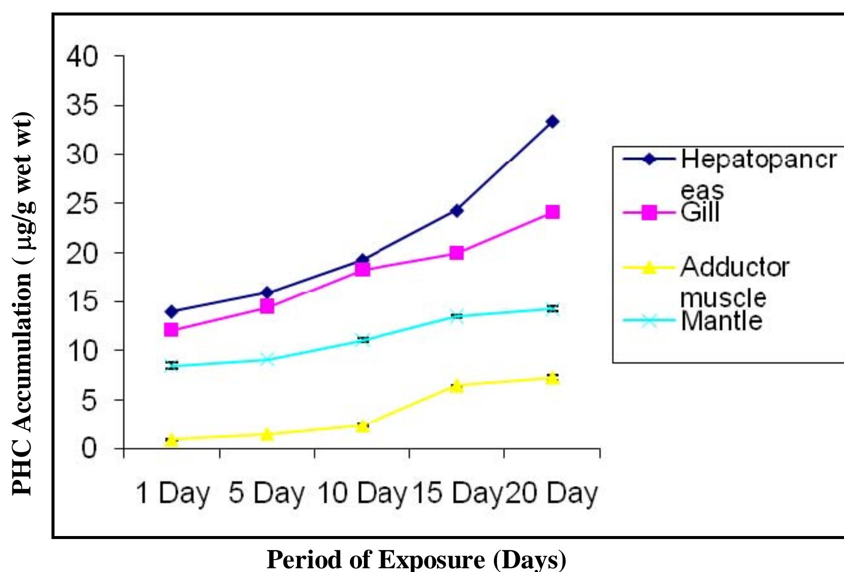


Figure 1.1: Accumulation of PHC in different tissues of *Perna viridis* exposed to 0.1 ppm WAF of BH Crude oil (µg/g wet wt)

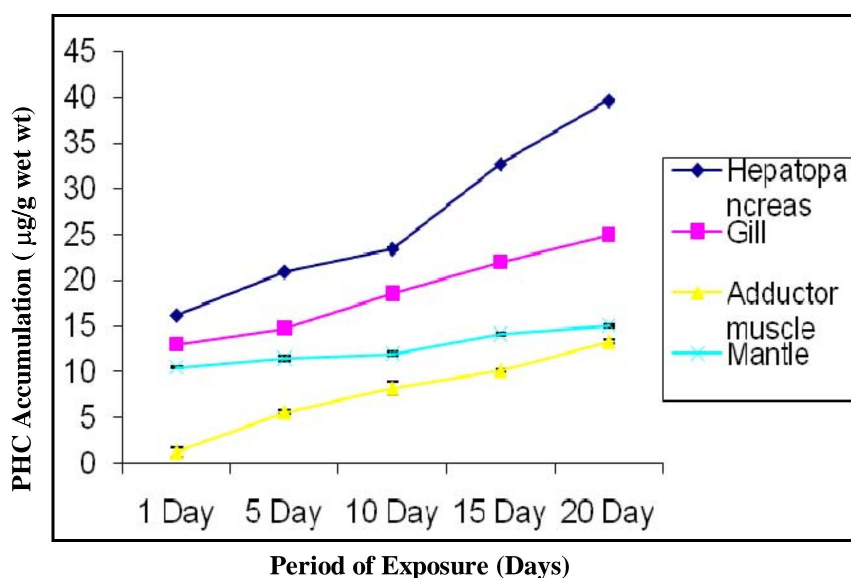


Figure 1.2: Accumulation of PHC in different tissues of *Perna viridis* exposed to 1 ppm WAF of BH Crude oil (µg/g wet wt)

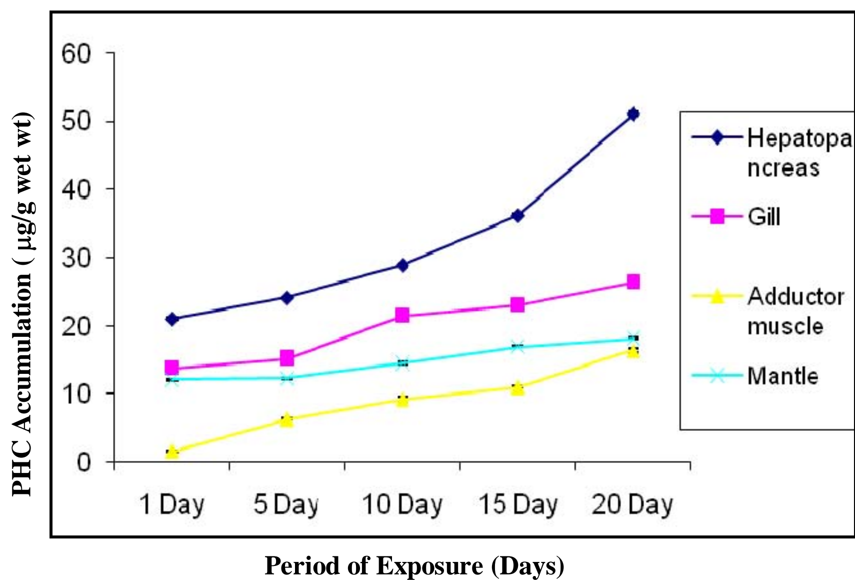


Figure 1.3: Accumulation of PHC in different tissues of *Perna viridis* exposed to 5 ppm WAF of BH Crude oil (µg/g wet wt)

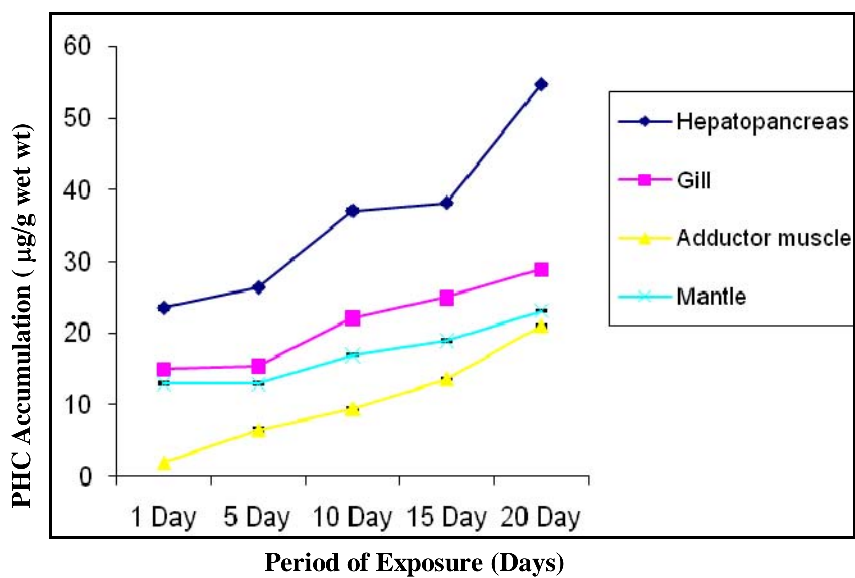
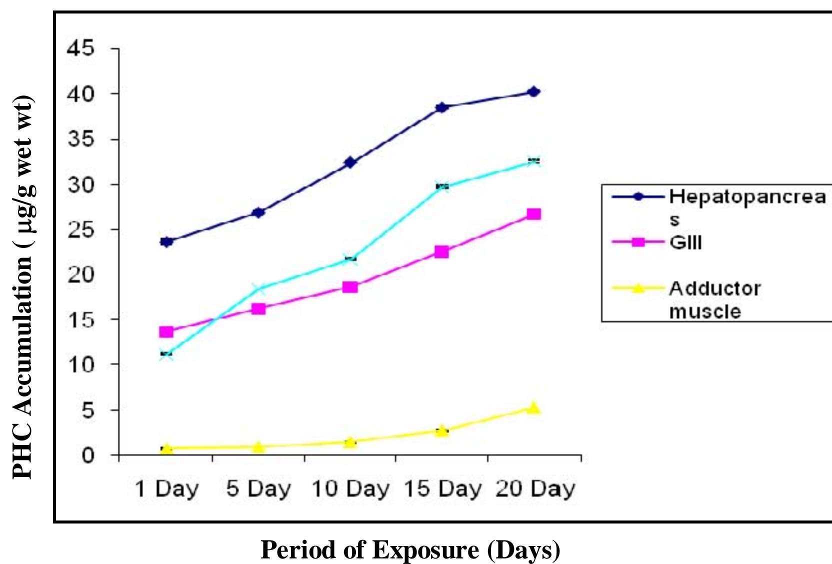
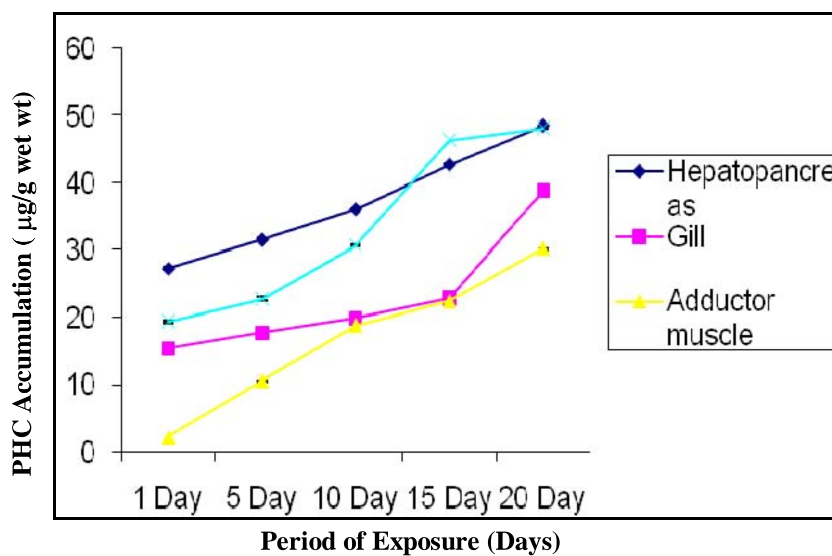


Figure 1.4: Accumulation of PHC in different tissues of *Perna viridis* exposed to 8 ppm WAF of BH Crude oil (µg/g wet wt)



Period of Exposure (Days)

Figure 1.5: Accumulation of PHC in different tissues of *Perna viridis* under 0.05 ppm WAF of Light Diesel oil (µg/g wet wt)



Period of Exposure (Days)

Figure 1.6: Accumulation of PHC in different tissues of *Perna viridis* exposed to 0.5 ppm WAF of Light Diesel oil (µg/g wet wt)

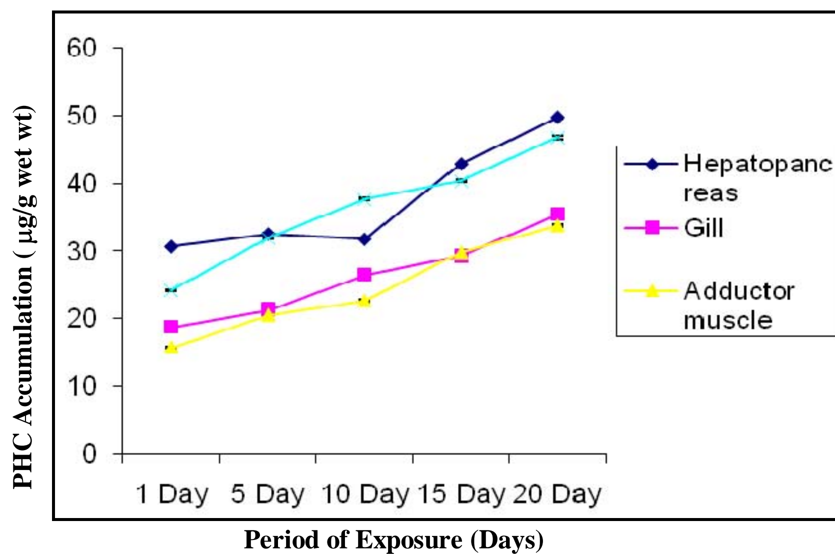


Figure 1.7: Accumulation of PHC in different tissues of *Perna viridis* exposed to 2 ppm WAF of Light Diesel oil (µg/g wet wt)

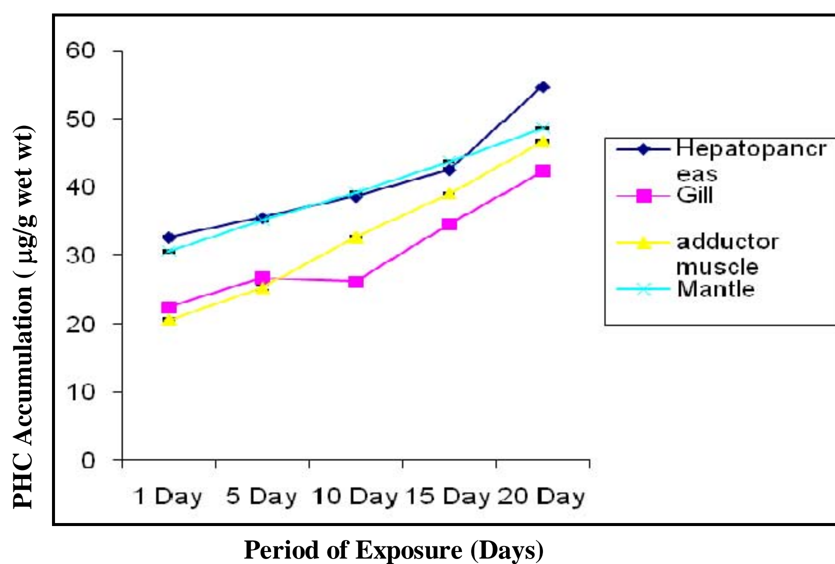


Figure 1.8: Accumulation of PHC in different tissues of *Perna viridis* exposed to 4.8 ppm WAF of Light Diesel oil (µg/g wet wt)

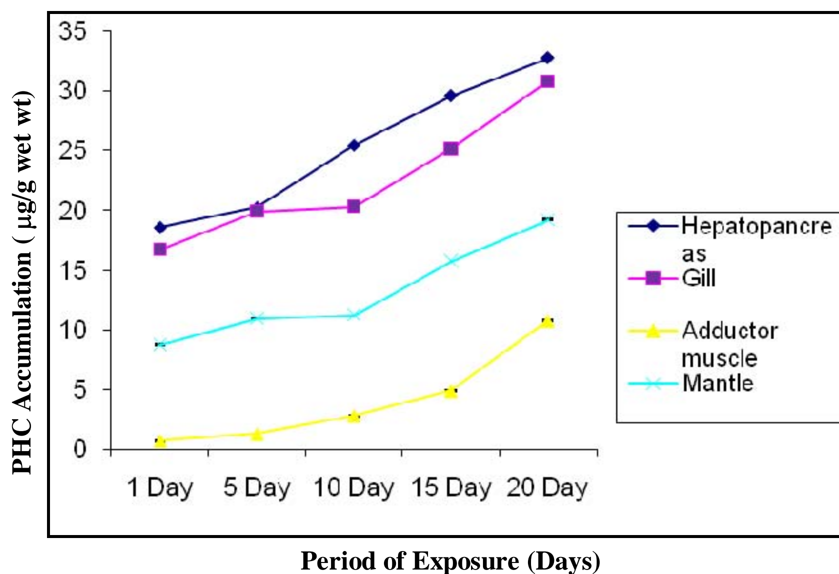


Figure 1.9: Accumulation of PHC in different tissues of *Perna indica* under 0.1 ppm WAF of BH Crude oil (µg/g wet wt)

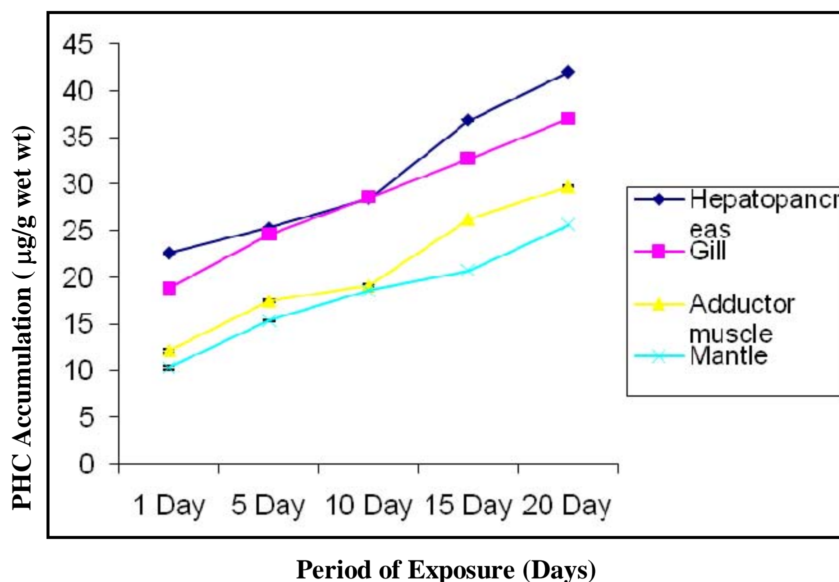


Figure 1.10: Accumulation of PHC in different tissues of *Perna indica* exposed to 0.6 ppm WAF of BH Crude oil (µg/g wet wt)

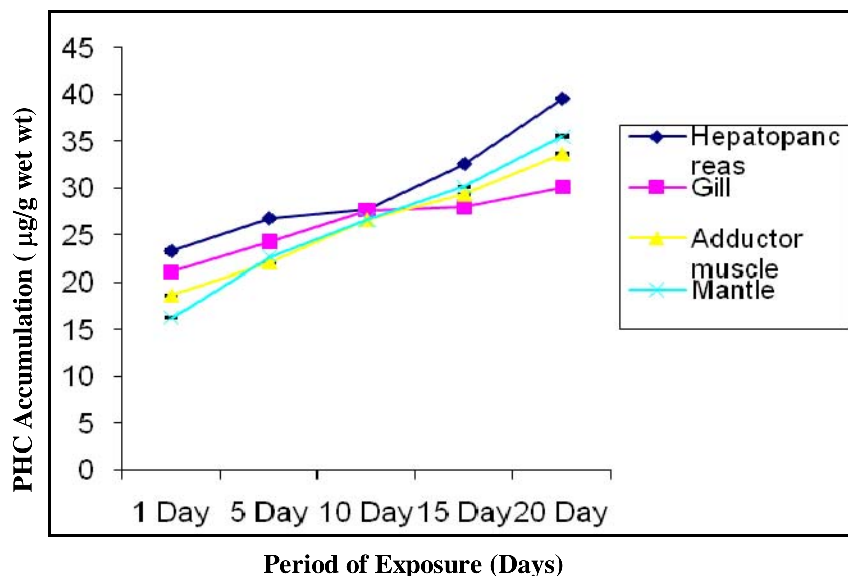


Figure 1.11: Accumulation of PHC in different tissues of *Perna indica* exposed to 2 ppm WAF of BH Crude oil (µg/g wet wt)

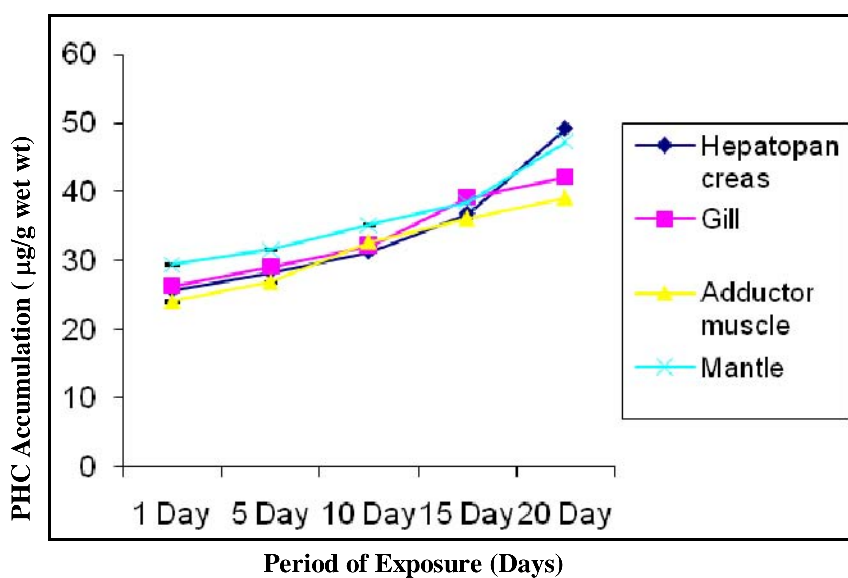


Figure 1.12: Accumulation of PHC in different tissues of *Perna indica* exposed to 5 ppm WAF of BH Crude oil (µg/g wet wt)

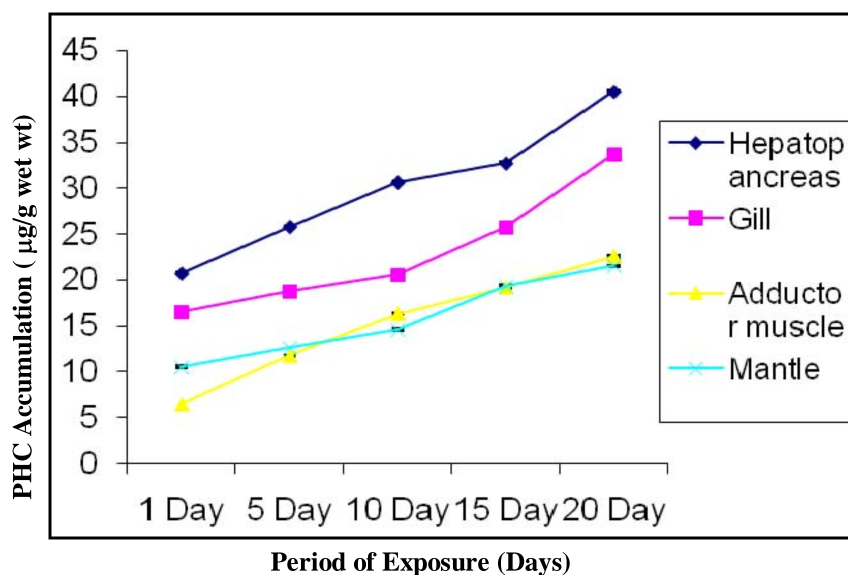


Figure 1.13: Accumulation of PHC in different tissues of *Perna indica* exposed to 0.04 ppm WAF of Light Diesel oil (µg/g wet wt)

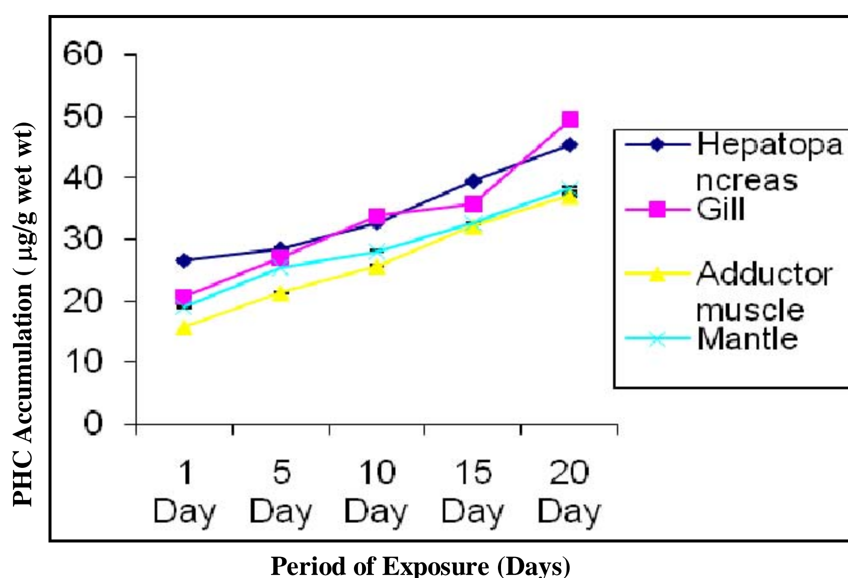


Figure 1.14: Accumulation of PHC in different tissues of *Perna indica* exposed to 0.4 ppm WAF of Light Diesel oil (µg/g wet wt)

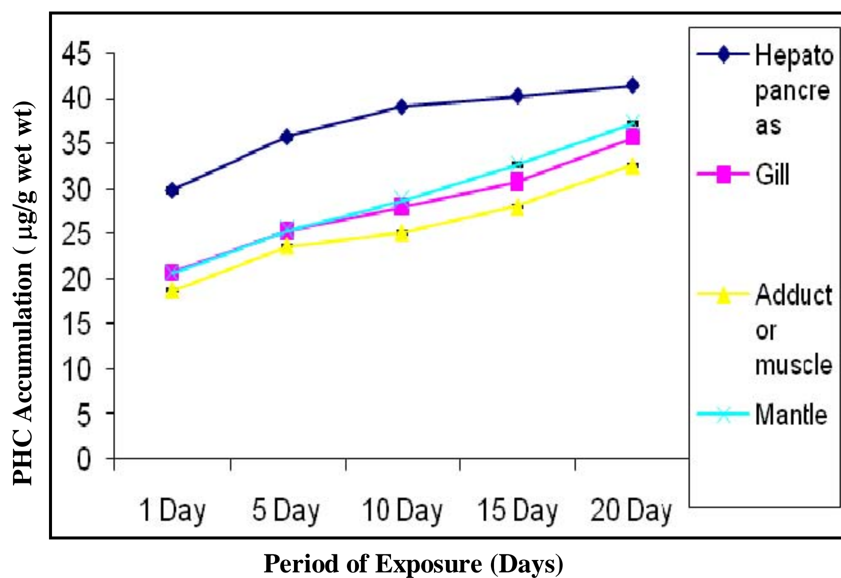


Figure 1.15: Accumulation of PHC in different tissues of *Perna indica* exposed to 1 ppm WAF of Light Diesel oil (µg/g wet wt)

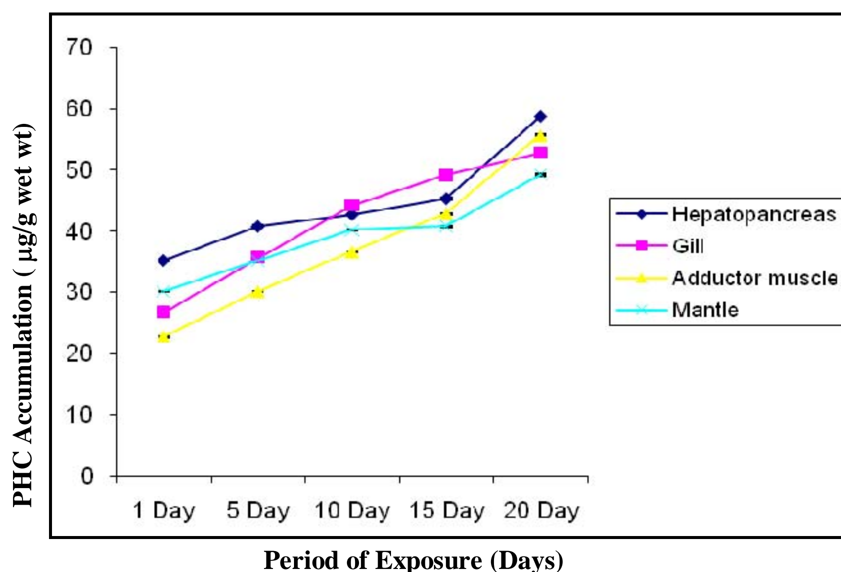


Figure 1.16: Accumulation of PHC in different tissues of *Perna indica* exposed to 3.5 ppm WAF of Light Diesel oil (µg/g wet wt)

It is clear from table 1.6 that the filtration rate decreased as a function of increase in duration of exposure. In terms of measurements of clearance rate, an apparent decrease was noted on 1st day onwards. The mean clearance rate ranged from 0.25 to 1.50 ml h^{-1} mg^{-1} dry weight.

Table 1.7 show the average filtration rate of *Perna viridis* exposed to various concentrations of LDO WAFs. Here also, animals reduced their filtration rate and the reduction was directly proportional to the increase in the duration of exposure. The mean clearance rate ranged from 0.52 to 1.69 ml h^{-1} mg^{-1} dry weight.

Table 1.8 and 1.9 explain the data obtained on the rate of filtration of *Perna indica* exposed to WAF of BH crude and LDO respectively. Increase in the concentration of both the WAF of BH crude and LDO resulted in drastic reduction in filtration rate.

In terms of clearance rate, an apparent decrease was noted at the end of the exposure(20 day). Results of ANOVA indicated that only exposure time was significant ($p < 0.001$) in affecting the clearance rate of the mussels.

The mean number of byssus thread produced ranged from 0.12 to 6.35 $individual^{-1}$ $12h^{-1}$ and 0.42 to 8.57 $individual^{-1}$ $12h^{-1}$ for *P. viridis* BHC WAF and LDO WAF exposed mussels respectively. Significant reduction ($p < 0.001$) in byssus production was found to be time dependant (Table 1.10-1.13).

Common feature of the byssus in Mytilidae is a root attached to the byssal retractor muscle, a stem extending from the root, and individual byssal threads attached to overlapping cuffs of the root.

Adhesive proteins are produced by the foot and are stockpiled in the foot and then secreted or released into the byssal groove, which creates a template for thread and a plaque for the strong attachment to the substrate.

Table 1.10: Mean number of byssus threads produced by *Perna viridis* exposed to sublethal concentrations of WAF of BHC

WAF concentration (ppm)	**Days**	**Byssogenesis (Individual^{-1} 12h^{-1})**
		Mean(± SD)
Control		
		10.55 ± 0.23
0.1 ppm		
	1	6.35 ± 0.62
	5	6.10 ± 0.71
	10	5.40 ± 0.80
	15	4.57 ± 0.59
	20	2.60 ± 0.73
1 ppm		
	1	5.40 ± 0.93
	5	4.92 ± 0.78
	10	3.87 ± 0.60
	15	3.50 ± 0.75
	20	1.47 ± 0.84
5 ppm		
	1	4.97 ± 0.52
	5	3.45 ± 0.93
	10	3.00 ± 0.66
	15	1.75 ± 0.85
	0.57	0.57 ± 0.25
8 ppm		
	1	4.52± 0.62
	5	3.45 ± 0.53
	10	2.55 ± 0.57
	15	1.65 ± 0.46
	20	0.12 ± 0.35

Table 1.11: Mean number of byssus threads produced by *Perna viridis* exposed to sublethal concentrations of WAF of LDO

WAF concentration (ppm)	**Days**	**Byssogenesis (Individual^{-1} 12h^{-1})**
		Mean(± SD)
Control		
		10.55 ± 0.23
0.05 ppm		
	1	8.57 ± 0.26
	5	8.25 ± 0.21
	10	7.43 ± 0.41
	15	5.12 ± 0.33
	20	2.80 ± 0.56
0.5 ppm		
	1	6.25 ± 0.23
	5	4.00 ± 0.68
	10	2.17 ± 0.65
	15	1.95 ± 0.63
	20	0.60 ± 0.42
2 ppm		
	1	2.87 ± 0.08
	5	2.15 ± 0.15
	10	2.10 ± 0.22
	15	0.80 ± 0.29
	20	0.42 ± 0.34
4.8 ppm		
	1	2.32± 0.83
	5	1.55 ± 0.57
	10	0.77 ± 0.24
	15	0.55 ± 0.65
	20	0.00

Table 1.12: Mean number of byssus threads produced by *Perna indica* exposed to sublethal concentrations of WAF of BHC

WAF concentration (ppm)	Days	Byssogenesis (Individual^{-1} 12h^{-1}) Mean(± SD)
Control		
		8.98 ± 0.25
0.1 ppm		
	1	7.52 ± 0.62
	5	6.49 ± 0.58
	10	5.31 ± 0.35
	15	2.45 ± 0.25
	20	1.10 ± 0.29
0.6 ppm		
	1	8.01 ± 0.62
	5	6.09 ± 0.81
	10	3.42 ± 0.73
	15	2.01 ± 0.25
	20	0.54 ± 0.37
2 ppm		
	1	6.05 ± 0.29
	5	4.57 ± 0.31
	10	2.25 ± 0.35
	15	0.42 ± 0.49
	20	0.22 ± 0.23
5 ppm		
	1	1.40 ± 0.61
	5	0.50 ± 0.52
	10	0.18 ± 0.24
	15	0.05 ± 0.38
	20	0.00

Table 1.13. Mean number of byssus threads produced by *Perna indica* exposed to sublethal concentrations of WAF of LDO

WAF concentration (ppm)	**Days**	**Byssogenesis (Individual^{-1} 12h^{-1})**
		Mean(± SD)
Control		
		8.98 ± 0.25
0.04 ppm		
	1	8.09 ± 0.22
	5	8.00 ± 0.29
	10	6.02 ± 0.53
	15	4.57 ± 0.61
	20	3.00 ± 0.35
0.4ppm		
	1	5.20 ± 0.40
	5	3.59 ± 0.10
	10	2.45 ± 0.62
	15	1.10 ± 0.48
	20	0.42 ± 0.72
1 ppm		
	1	3.00 ± 0.39
	5	2.42 ± 0.48
	10	1.95 ± 0.62
	15	0.35± 0.65
	20	0.05 ± 0.47
3.5 ppm		
	1	1.60 ± 0.74
	5	1.65 ± 0.36
	10	0.87 ± 0.52
	15	0.22 ± 0.39
	20	0.00

Scanning electron micrographs provided more detailed structure of byssus. The byssus thread of *Perna viridis* and *Perna indica* had four distinct regions (Plate.1.1a). The stem of both *Perna viridis and Perna indica ,* emerged from a raised area at the base of the foot (Plate.1.3a).

Several thread clusters branched off with variable shapes of attachment discs (Plate. 1.2b). Additional observations showed that the ventral groove extended to the distal tip of the foot.

Cuffs or sheaths, formed by the outer laminae, were observed at the bases of the threads in some specimens. The thread connecting-rings appear more like cuffs of variable extension (Plate. 1.4d). Tile transition area between the cuff and the thread is keel-shaped (Zone Z, Plate.1.3c). The two most markedly protruding portions originate from the side faces of the filaments and diverge into the connecting cuff so that a tiny, triangular- shaped slot is formed (Plate. 1.3d). This opens towards the stem end, and is closed by the extension of the cuff itself, which is wedged under cuffs of the preceding threads. Examination of intact specimens of *Perna spp.* showed that threads attached to the stem by overlapping rings, and branched progressively at distal locations (Plate. 1.3a).

The proximal portion of the thread appears flattened; its typical shape is shown in Plate 1.3b and 1.5 with a edge and had corrugations or folds around their circumferences After exposure to WAF of PAHs it became extended state and lacked surface corrugations (Plate. 1.2a).The byssus thread has broken off (Plate. 1.2c) in some cases.

Plate 1.3b and 1.5a show a large number of corrugations in the proximal portion of the thread. The top edge has protruding folds of fairly constant length, which appear larger than the folds of the thread's sides. In the bottom edge. The longer axis of the folds lies across the length of a thread, often at an angle. Proximal-portion vary from

thread to thread; and may be straight, but are more often curved or spiral-shaped (Plate. 1.5b), with the folds more or less closely packed and protruding as convex or concave surfaces. The proximal portion of the thread, therefore, is formed by a corrugated superficial layer which envelops a smooth cylindrical inner portion, (Plate. 1.5c,d).

Threads of *Perna viridis* and *Perna indica formed* oblique angles with the substratum and ended distally in thin, oval plaques (bottom portions of Plate. 1.4a). The byssus thread has broken off and the thin centre of the triangle has collapsed (Plate.1.4b).Threads of *Perna spp.* however, trifurcated and formed thin, root-like extensions that continued into the plaque (top portion of Plate.1.3c). The narrow axis of the plaque was oriented perpendicular to the longitudinal axis of the thread (Plate. 1.3d).

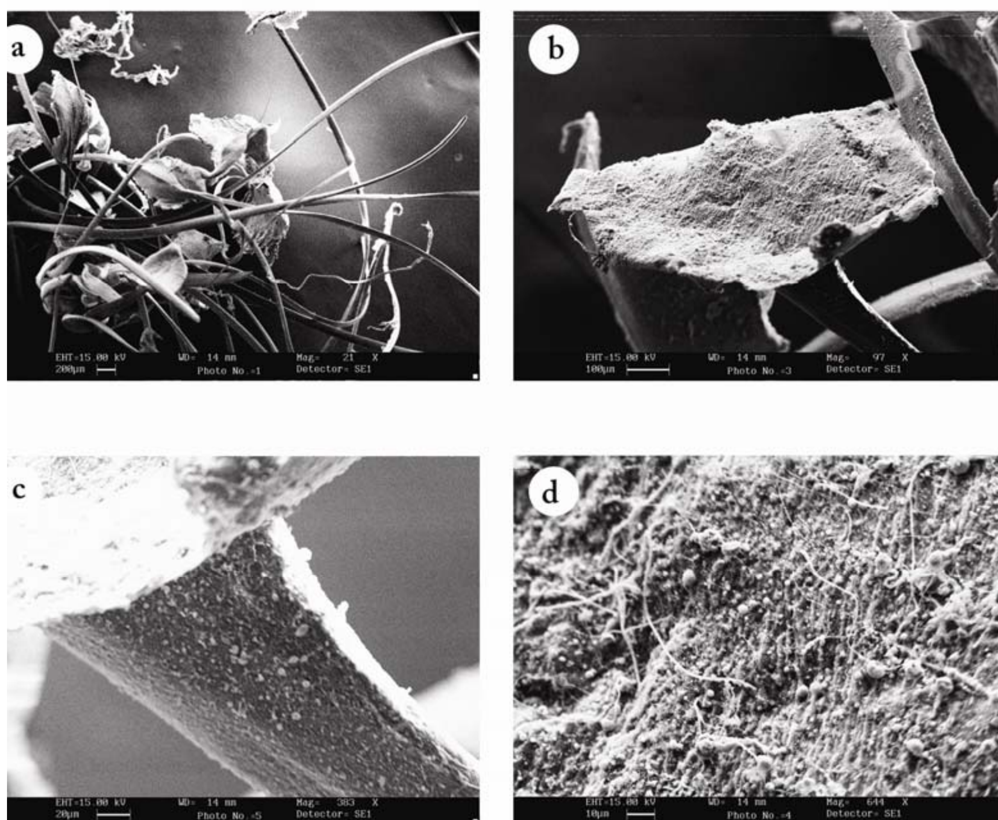


Plate 1.1: P*erna viridis.* SEM micrograph of a) Byssus fragment, thread morphology ; Tpp : proximal portion of thread; Tdp: distal portion of thread.

b) Structure of attachment disc (D). c) Attachment disc with distal thread portion.

d) Details of ventral surface showing heterogenous material embedded in disc matrix (Dm).

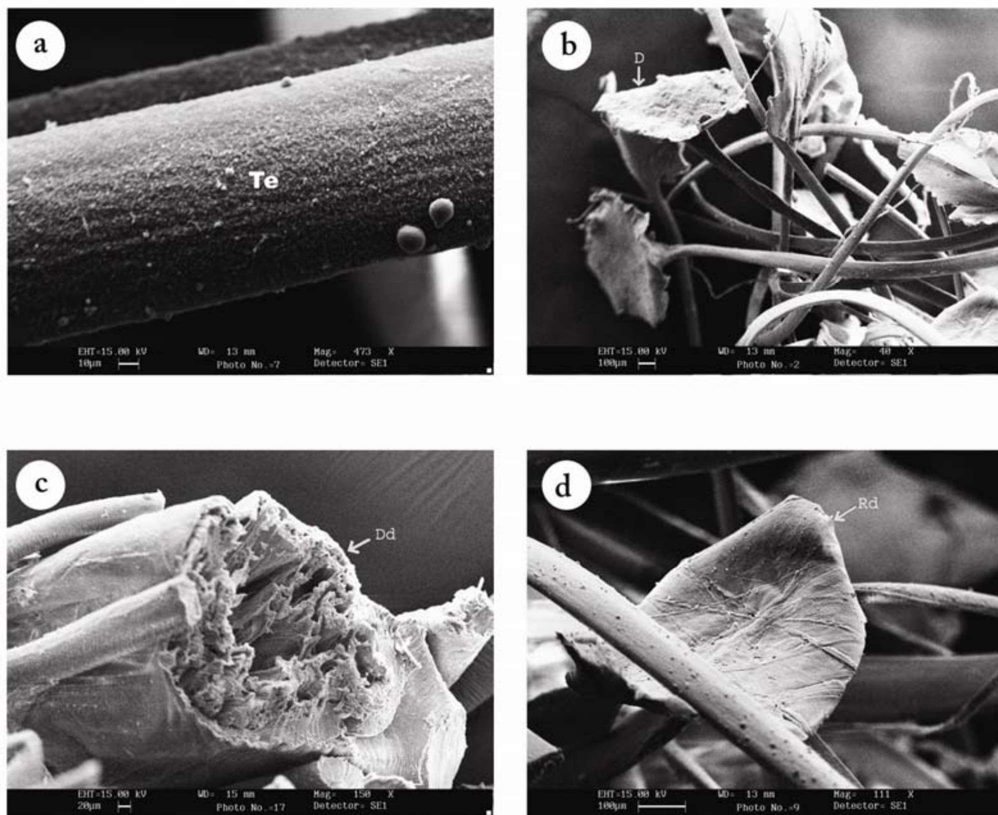


Plate 1.2 : P*erna viridis.* SEM micrograph of a) Byssus thread in extended state-absence of surface corrugations b) Several thread clusters branching off with variable shapes of attachment disc .c) The byssus thread has broken off.

d) Rolling of attachment disc

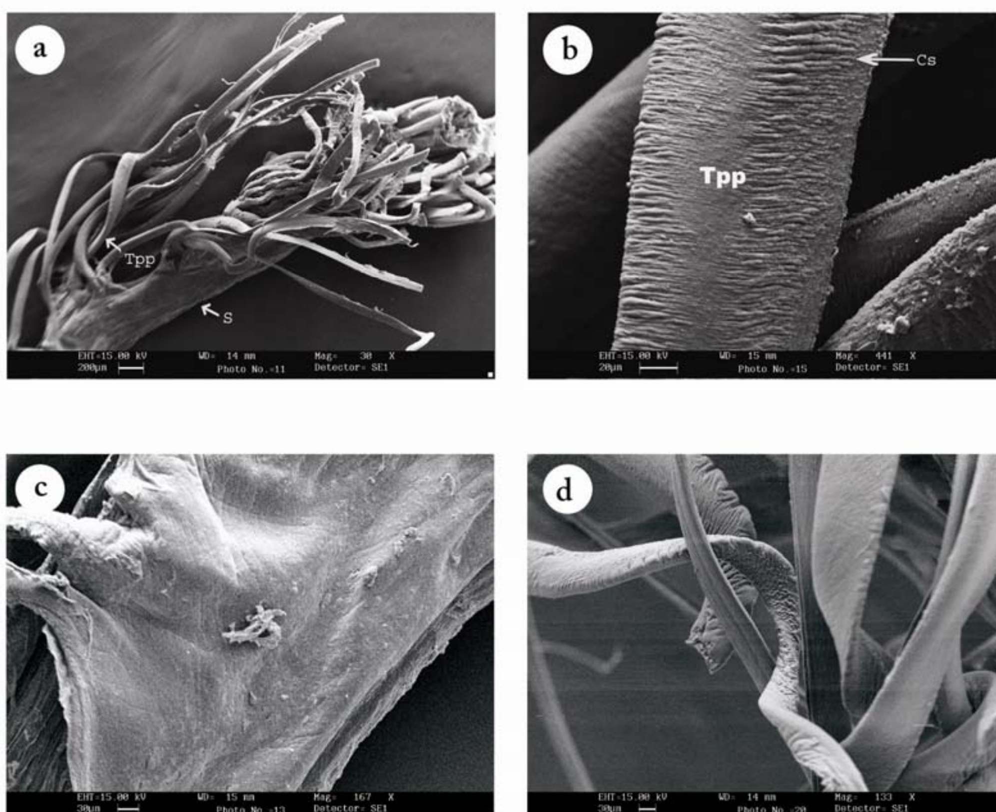


Plate 1.3 : *Perna indica* . SEM micrograph of a) Byssus thread with the stem emerging from a raised structure near the base of the foot

b) Proximal portion of thread evidencing corrugated surface layer. c) Broad flattened portion of thread expanding along wide axis of Plaque d) Basic features of proximal thread portions twisted; note different morphology of thread edges.

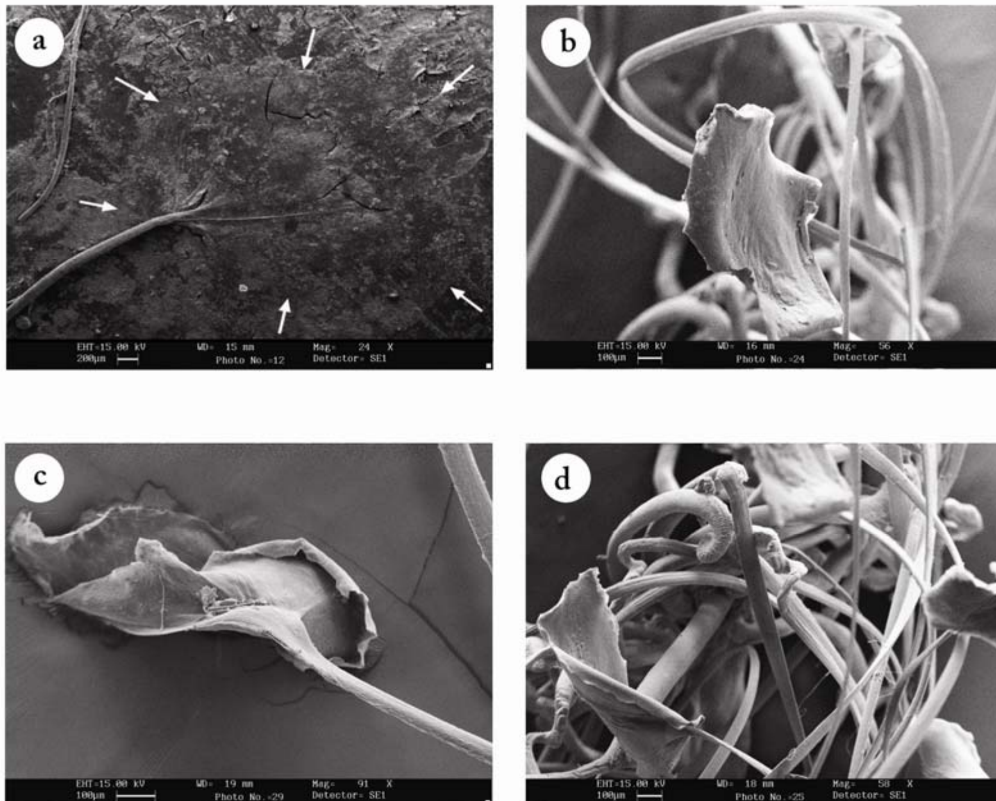


Plate 1.4: P*erna viridis* SEM micrograph a) byssus plaque attached to a mussel shell. b)The byssus thread has broken off and the thin centre of the triangle has collapsed c) Single adhesive disc in destructed condition. d) Proximal portion of threads merge with connecting cuff.

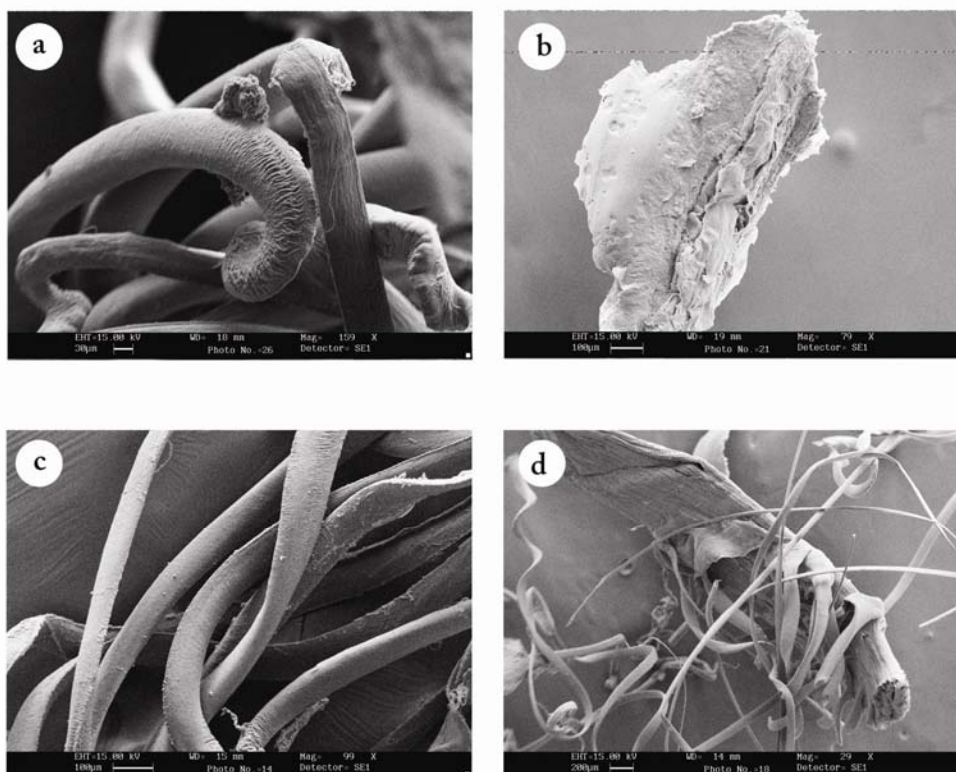


Plate 1.5: SEM micrograph of *Perna indica* thread fragments a) Curled up outer layers and inner portion are more clearly evidenced by dissociation b) Connecting cuff isolated c) Treads in extended state: note absence of surface corrugations.
d) Multiple threads (arrowed) radiating from byssus apparatus showing severe destruction. .

Adhesive Disc

The disc is a flattened plate with an essentially lanceolate shape (Plate1.1b); its size varies considerably. Generally the disc like a leaf, with the thread connection as the stalk and the leaf veins represented by the thread branches, which are more or less sharply outlined over the dorsal face of the disc.

The adhesive plate is thickest where the threads are connected and becomes increasingly thinner towards its edges. In most cases, the thread axis forms a sharp angle with the disc plane. The last portion of the thread is quite stiff and grafted dorsally on the proximal end of the disc. Discs are compact, rigid, often brittle, with a granular structure. After exposure to WAF of PAHs, they curled upwards (Plate1. 2d). A single adhesive disc in damaged condition is visible in (Plate1.4c). The dorsal surface of the disc has a fine granular texture. The morphology of the ventral surface on the other hand, appears quite variable. This part incorporates foreign matter present on the substratum to which the disc is clamped. All foreign matter appears more or less completely embedded in a granular matrix.

Whenever the disc adheres to a smooth surface, ventral face is also smooth, evidencing that it carries the replica of the surface to which it has been clinging (Plate 1.1.c,d).

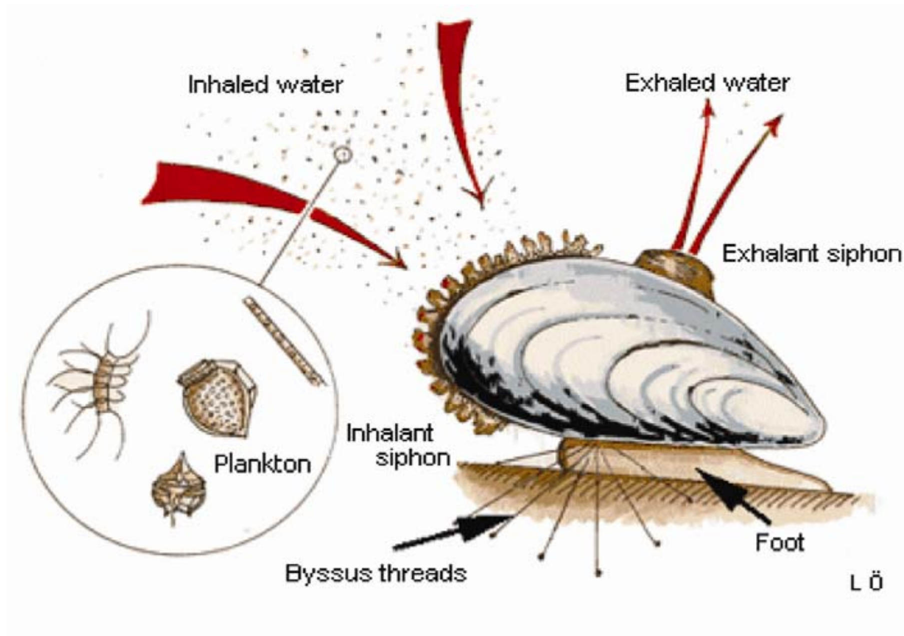


Figure 1.17: Mussel feeding and Respiration (Courtesy : Google).

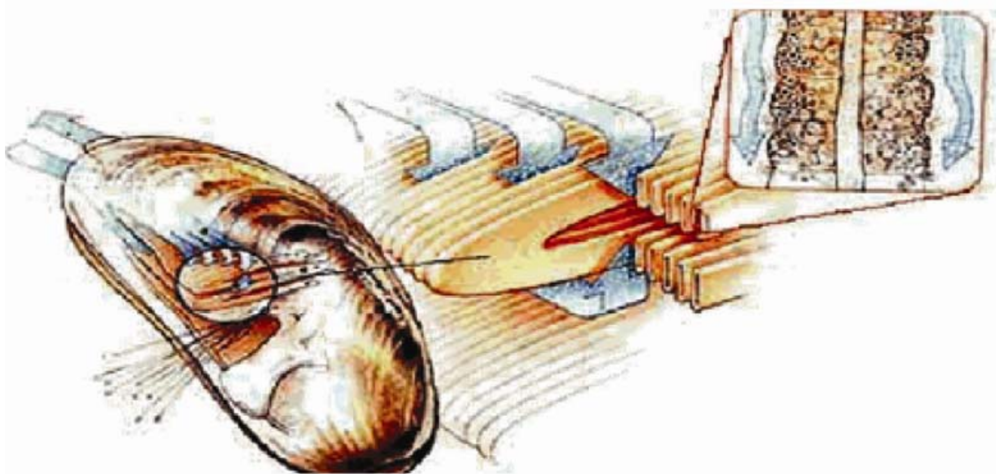


Figure 1.18: Mussel Filtration – Water is pumped into the mussel and across its gills (Courtesy: Google).

1.4 Discussion

Uptake and accumulation of hazardous substances in the tissues of aquatic organisms occur from the sediment, contaminated water and food (Livingstone, 1993) causing biological damages. The uptake and accumulation will depend on the dietary and ecological life style of the organisms (Livingstone 1991; 1998; and 2001)

Invertebrate species were used primarily on the basis of abundance, sampling facility, and wide spectrum of ecological characteristics and sensitivity to chemicals. More recent investigations have clearly highlighted the interest in using invertebrates to link individual responses with changes in populations or communities, as such correlations will be of great value for rapid, early warning assessment of the environmental impact of chemicals (Repetto *et al.,* 2000).

Sublethal alterations in the physiology of bivalves, principally *Perna,* have been used in the evaluation of pollution. Bivalves are able to accumulate numerous chemical compounds in their tissues, which directly or indirectly may produce significant impacts on their biology (Martin *et al.*, 1984 ; Hawkins *et al.*, 1986 ; Widdows *et al.,* 1987; Widdows and Johnson 1988 ; Widdows *et al.*, 1990 ; Widdows *et al.,* 1997). The structural and functional role of bivalves within a community or ecosystem are fundamentally affected by their physiological processes such as feeding, respiration, excretion, and growth (Dame 1996). Among the physiological responses of bivalves, clearance rate is one of the most important since it not only is related to the acquisition of energy necessary for vital functions, but is also sensitive to environmental changes such as organic pollutants (Axiak and George 1987b, Axiak *et al.,* 1988, Bayne and Thurberg, 1988 ; Sunila, 1988 ; Livingstone and Pipe 1992).

The mode of entry of PHCs into an organism is an important factor in determining the accumulation and distribution within the body as well as its excretion from the body (Varanasi and Malins, 1977).

Entry of hydrocarbons into marine organisms both by ingestion of hydrocarbon contained in food (Blumer *et al.*, 1970) and by direct absorption of hydrocarbons from the water are known to occur (Lee *et al.*, 1972), the latter presumably via unmediated transport (Kotyk, 1973, Menon and Menon, 1998,1999).

Although both routes can contribute to the mussels, direct absorption is considered as a major source of hydrocarbon accumulation. The mussels discharge or dispose of some accumulated hydrocarbons upon lowering the exposure level, yet they retain a portion of accumulated hydrocarbons for a few weeks even when none are added to the system.Molluscs took up petroleum hydrocarbons somewhat more slowly, but accumulated them in their tissues to considerably higher concentrations and released them more slowly than did crustaceans (Anderson, 1973; Neff and Anderson, 1974).

In the present study it was proved that the hepatopancreas was most prone to bioaccumulation of hydrocarbons.They showed that hydrocarbons were transported via the haemolymph, and that selective discrimination and accumulation occurs at the basal lamina of the digestive tubles. Within the tubules, PAHs were immobilized in membrane-bound vesicles, which are finally thrown out by way of faeces.

It was also shown that bioaccumulation via particle ingestion depends on the feeding rate and feeding selectivity of organism and the assimilation efficiency and the contaminant concentration of the ingested food particles (Lee *et al.*, 1990, Menon and Menon, 1999).

The rate of oxygen consumption is an index which can be used to assess compensations on energy brought about by environmental conditions. The enhanced rate of respiration in the low concentraion of LDO WAF treated animals may be due to an increase in metabolic demand to excrete hydrocarbons.

Exposure to low concentrations of water accommodated fractions of oil may lead to significant reduction in filteration efficiency. These compunds exert a narcotizing effect on ctenidial cilia, produce morphological alteration of branchial filaments etc.

Exposed animals were found to clear algae at a much slower rate than did the control animals. This is probably an indication of reduced water movement due to reduced ciliary activity.

The fact that no foot protrusion was observed under oil stress explains the lack of thread formation (Barnes, 1964).

In sub lethal tests, there were significant changes in oxygen consumption in the green- mussel between treatments and duration. The clearance rate and byssus production in the test mussels, however, varied significantly with exposure time. The clearance rate of *P. viridis* decreased when the mussels were exposed to WAF of PAHs. Byssus production is sensitive to mechanical agitation (Young, 1985) and water velocity (Dolmer and Svane, 1994). The number of byssus threads produced was initially higher when *P. viridis* was transferred to lower concentrations of WAF of PAHs in the laboratory, and byssus production subsequently decreased. Similar results were reported by (Seed and Richardson, 1999). Newly detached blue mussels *Mytilus edulis* were stimulated to produce more byssus during the first 24 h of attachment (Young, 1983). Similar observations were noted in the present study.

It was reported by way of instantaneous clearance rate of bivalves in a mixture of algae. A clear cut dose-response relationship (Bricelj and Malouf, 1984; Ward and MacDonald,1996; Bacon *et al.*, 1998). In this study, measurements of clearance rate in *P. viridis* and *P. indica* were undertaken in algal solutions after the mussels were exposed to various concentrations of WAF of PAHs treatments. Gill area and pumping rate in mussels are closely related (Jones *et al.*, 1992). Hence, seriously damaged gill

filaments could reduce the effective gill surface area in *P. viridis* and may affect its pumping rate.

Filtration of particles from water is primarily a function of the lateral frontal cirri. Irrespective of species, cirri in bivalves are structures whose tips are reported to serve as nets to capture water-borne food particles and whose motion is thought to deliver particles to the frontal cilia for "further processing" (Silverman *et al.,* 1996). Studies have shown that *P. perna* has 18–23 pairs of cilia per latero-frontal cirrus (lfc) with each pair averaging 18 lm in length (Gregory *et al.*, 1996; Gregory and George, 2000). While our study on filament or cell morphometrics, showed substantial depletion of cirri emanating from lfc, especially two days after exposure to PAHs.

Rapid decrease in the filtration rate of bivalves, when exposed to PAHs was reported by Widdows *et al.*, (1982).

The high degree of negative correlation obtained between the concentrations of PAHs in tissues of *both Perna viridis and Perna indica* and the clearance rates suggested that these organic pollutants might be exerting a deleterious effect on this physiological response, and that the degree of stress was a function of the levels of these xenobiotic chemicals in tissues of the organism. Several ecotoxicological studies have shown that the clearance rate is the component of the energy budget most affected by toxic compounds, (Howell *et al.,* 1984 ; Axiak and George 1987a ; Capuzzo 1988 ; Widdows and Johnson 1988 ; Bourdelin 1996), principally saturated hydrocarbons with low boiling points (GESAMP 1994).

Significant decreases in clearance rate due to increases in pollutant levels have been observed in other field and laboratory studies, such as in the case of the bivalves *Arca zebra* (Widdows *et al.*, 1990), *Macoma balthica* (Stekoll *et al.*, 1980), *Modiolus demissus* (Gilfillan 1975), *Mytilus edulis* (Martin *et al.,* 1984, Widdows and Johnson

1988), *Venus verrucosa* (Axiak and George 1987b) *Crassostrea virginica* (Stegeman and Teal 1973).

Based on the literature and the present results obtained, the clearance rate may be considered as a convenient, non-destructive biomarker useful in evaluation of the effects of pollution because the response time to the pollutant is relatively short; and the parameter can be precisely measured. Mussel may have compensatory mechanisms, such as a temporary increase in ciliary beating rates, in order to cope with impairment of gill structure. Gill area and pumping rate in mussels are closely related (Jones *et al.*,1992). Hence, seriously damaged gill filaments could reduce the effective gill surface area in *Perna* affecting pumping rate.

The present study provides a detailed description of the morphology of the byssus and of its structure in *Perna spp*. The results reported basically agree with previous observations obtained by means of simpler techniques (Field, 1922; Brown 1952). It is interesting to note that the organisation of byssus components (e.g. stem laminae, thread portions, adhesive disc) can easily be interpreted in functional terms: a clear relationship may be drawn between the organization and the mechanical properties of different byssus components.

Although byssal threads seem to have initially evolved to aid in larval dispersal and post-larval settlement, many groups also use the byssus for adult attachment (Yonge, 1962; Stanley, 1972; Sigurdsson *et al.*, 1976; De Blok and Tan-Maas, 1977; Lane *et al.*, 1985). Mussels of the Mytilidae, which includes all marine species, have a byssal complex with three main components: a root that is attached to the byssal retractor muscles; a stem that extends from the root and consists of a series of overlapping cuffs; and fibrous byssal threads that individually project of each cuff (Bell and Gosline, 1996).

Byssus morphology is related to the mechanical events that occur during byssus formation (Waite, 1983; Eckroat *et al.,* 1992), from the ventral groove (Smyth, 1954).

As threads are secreted as a liquid, they are molded to the walls of the ventral groove (Brown, 1952; Smyth, 1954; Bairati and Vitellaro-Zuccarello, 1974; Waite, 1983).

Once a thread has been completed, specimens of *Perna spp* change position and repeat the process to form another thread. Similar observation was noticed in *Mytilus edulis* (Clapp, 1950). *Perna spp*. threads branch from two opposite sides. Our observations agree with those of Brown (1952), who reported that the threads of *Mytilus edulis* are attached to the byssus with overlapping rings that are fused with the fibrous laminae of the byssal root. Brown (1952) explained that in *M. edulis,* as the fibrous laminae of the root increase in length, the stem lengthens and older threads are carried farther from the body of the organism.

The byssi of *Perna viridis* and *Perna indica* were similar in the anchorage of the stem, the pattern in which threads branch from the growing stem, thread surface topography, plaque orientation, and the morphology of the region of the thread that extends into the plaque. Byssal attachment is fundamental to the success of mussels that colonize hard substrata, information concerning structural characteristics of the byssus could be used together with knowledge of thread formation and attachment to develop or adapt mechanisms of controlling biofouling of mussels.

Chapter 2

HISTOPATHOLOGICAL STUDIES IN *Perna spp.*

Contents

2.1 Introduction
2.2 Materials and methods
2.3 Result
2.4 Discussion

2.1 Introduction

A Clear understanding of cause and effect of toxic reactions could be identified only with the help of histopathology. The histopathological studies on mussel is a noteworthy and promising field to understand the extent to which changes occur in the structural organization of the organs due to environmental pollutants. In molluscs, histopathological examination is widely recognized as a reliable method for disease diagnosis and for assessing acute and chronic toxic effects of pollutant. The histopathological studies have gained wider acceptance in the field of aquatic toxicology, mainly because the assessment of the impact of xenobiotics on the tissues could be done easily. The significance of histological studies as a part of toxicological investigation probably lies in the fact that extremely low level of pollutant may fail to produce subtle damages in its metabolically sensitive tissues.The ability of any tissue to regulate its normal physiological function is extensively related to its structural integrity and any damage to this tissue usually results in abnormal metabolic activities.

The study of histology is useful to understand the correlation between structure and functions of an organ. It imparts the knowledge of normal cells and tissues and helps to distinguish them from abnormal ones with deranged functions.

Together with classically recognized biomarkers, histopathology is increasingly used as indicators of pollution effects since they provide a definite biological end-point of historical exposure.

The relationship between concentration of contaminants in tissues and toxic effects measured in organisms is receiving increased attention during the last decades (Chapman, 1997).

Histopathological alterations in selected organs and tissues, mainly gill, hepatopancreas, mantle and adductor muscle, are conceived as histopathological or tissue-level biomarkers.

Histo-cytological responses are relatively easy to determine, and can be related to health and fitness of individuals which, in turn, allows further extrapolation to population/community effects. A wide range of histo-cytological alterations in bivalves have been developed and recommended as biomarkers for monitoring the effects of pollution. Externally visible diseases and histopathological lesions in mussel served as primary indicators of exposure to contaminants, and certain diseases and lesion types have proven to be reliable biological indicators of toxic/carcinogenic effects resulting from such exposure.

The gill and digestive gland of bivalve molluscs play cardinal role in food collection, absorption and digestion. Chronic exposure of bivalves to pollutants in water and sediments may ultimately impair their nutrient absorption ability, and compromise their growth and reproduction. Given the high filtering capacity of bivalves, gill tissues will not only be continuously in contact with pollutants in water, but may concentrate

the pollutants contained therein due to the remarkably high volume of water that they filter.

Compared with certain species of the closely related genus *Mytilus,* and particularly *M. edulis,* where numerous studies describe the normal and abnormal appearance of gill filaments and other soft tissues before and after exposure to PAHs, little was known of these structures in *P. viridis* and *P. indica*.. The cause and effect relationships between histopathological alterations and pollutant exposure in bivalves are not fully understood. This study was undertaken in order to increase our understanding of its response to pollution. Using a sub-lethal concentration of PAH as a test pollutant, experiments were performed to obtain the histopathological response of hepatopancreas, gill , mantle, adductor muscle and pedal disc of mussels exposed to PAH at different realistic concentrations.

Since bivalves constitute an important part of the food of coastal population ,there is a need to study the histopathological changes in various organs in bivalve in response to environmental stress factors including chemical pollutants(George *et al.*, 1976; Axiak and George, 1987; Sasaki *et al.,* 1999; Ibarrola, 2000; Pennec and Pennec, 2001).

A series of works has demonstrated that the digestive gland tubules of molluscs are reduced in thickness in response to a variety of environmental stressors (Lowe *et al.* 1981; Tripp *et al.* 1984; Marigo´mez *et al.* 1986b, 1993; Minniti 1987; Recio *et al.* 1988; Vega *et al.* 1989; Cajaraville *et al.* 1992; Ireland and Marigo´mez 1992). Thus, the measure of such reduction has been proposed as a biomarker of the biological effect of sublethal exposure to pollutants.

Pollution monitoring techniques using mussels include measurement of physiological rates (Watling and Watling, 1982; Marshall and McQuaid, 1993), evidence of bioaccumulation of heavy metals (Regolli and Orlando, 1994) and

estimation of the biotoxicity of metals from pathological changes in soft-tissue morphology (Sunila, 1988a,b; Gregory *et al.*, 1999).

Bayne *et al.* (1980) presented a comprehensive overview of stress parameters in mussels, including histopathological assessment techniques.

There is some evidence of the changes in the distribution and relative occurrence of cell types in response to pollutants (Lowe *et al.* 1981; Cajaraville *et al.* 1990a, 1990b; Marigo´mez *et al.* 1990b, 1996). Moreover, the cell type composition of the mollusc digestive gland may change due to stressing factors other than pollution (Marigo´mez *et al.* 1993). In this context, the examination of changes in the histological organization of the digestive gland tissue is useful to determine the accumulating ability of a sentinel mussel, and even to interpret correctly the data obtained by means of analytical chemistry. The thickening of the blood vessel walls and basal lamina underlining the digestive tubules, the depletion of glycogen reserves and the alteration of the calcium metabolism have been described in a variety of gastropod mollusks on exposure to pollutants (Recio *et al.* 1988b; Ireland and Marigo´mez 1992; Cajaraville *et al.,*1990b; Marigo´mez *et al.* 1990b, 1993, 1996; Beeby 1993).

2.2 Materials and Methods

A 4-mm cross section of each mussel, including hepatopancreas, gills, mantle, adductor muscle, pedal disc, and foot was dissected and fixed in Davidson's solution for 24 h. The tissues were then transferred through graded alcohols (70-95 % [v/v]) before final dehydration in absolute ethanol. The alcohol contained in the tissues were next eliminated by immersing them in xylene. The tissues were then impregnated with paraffin, which is soluble in xylene, at 60^{0}C and embedded in paraffin. After processing, 5µm sections were stained using Haematoxylin-eosin staining procedure (Howard *et al.*, 2004).

Histopathological screening

The cytological quality of the following tissues and organs was assessed on a simple scale of 1-5, 5 being considered as "normal": the hypatopancreas, gill, mantle, adductor muscle tissue, foot and pedal disc.

The general histopathological condition ("health") index used here, is based on the extent and *severity* of cytological abnormalities or lesions in all tissues. This type of index has been used by a number of authors Peters and Yevich (1989). This type of grading system only summarises the health of invertebrates in a very general way according to the criteria in Table 2.1.

Table 2.1.

Score	Criteria
5	An individual showing no apparent tissue abnormalities, well-nourished
4.5	Individual showing no obvious tissue abnormalities but where feeding condition is sub-optimal
4.0	Individual showing slight tissue abnormalities in one organ or part of system, e.g. abnormal vacuolation or slight/local inflammation
3.5	Individual showing moderate abnormalities/lesions in one organ or part of system, e.g. inflammation, necrosis, etc.
3.0	A definite pathological condition is recognisable, i.e. serious abnormalities in one organ, or a more diffuse general condition, e.g. lesions, inflammation, evidence of parasitisation, starvation, etc.
2.0	Individual showing extensive pathology, e.g. lesions, neoplasia, extensive parasite infestation/infection. Condition apparently critical, involving several organs and sometimes coupled with signs of starvation/wasting
1.0	Terminal case, substantial wasting of key tissues, little apparent function remains

The general indices were derived as an average value of the frequency of the lesions measured in each tissue. It is based on the use of six individuals of each species and during every survey both under laboratory and field conditions. To derive the final value we represent the number of organisms that show prevalence in any of the detected lesions using the next expressions and associated number of individuals.

Semiquantification of histological lesions were carried out by means of a light microscope as described by Riba *et al.* (2002). Histopathological alterations were semiquantitatively evaluated by ranking the severity of lesions, taking into account the number of individuals showing a concrete lesion from each treatment (n = 6) as described in detail by DelValls *et al.* (1998). The nomenclature assigned depending on the number of individuals containing a concrete lesion was as follows: 0 individuals (0), 1 individual (0/1+), 2 individuals (1+), 3 individuals (1+/2+), 4 individuals (2+), 5 individuals (2+/3+), 6 individuals (3+).

A general index of damages for each analysed tissue was assigned: DQ (chinitose alteration), AL (lamellae alteration), MC (ciliar alteration),DE (epithelial desquamation), IH (hemocitarian infiltration), DT(tubular alteration), PC (ciliar loss), OT(tubular occlusion), AE (epithelial loss), Ac (conjunctive alteration), EC (ciliar epithelium), MB (basal membrane). An arithmetic average value was obtained from the semiquantitative assessment of the lesions for each treatment and tissue.

Statistical Analysis

Results of total number of pathological changes were analyzed statistically using Wilcoxon Signed Rank test to assess effects of experimental treatments upon the response variables.The statistical software used was Sigmastat 3.5

2.3 Results

Exposure to PAHs produced discernable changes in the hepatopancreas and gills of *Perna viridis* and *Perna indica*.

Visual appraisal by light microscopy

Digestive Diverticula

The digestive diverticula consists of numerous digestive tubules which are linked with the lumen of 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. The tubule epithelium contains two cell types, one acidophilic, columnar and vacuolated, the other pyramidal and basophilic. The acidophilic cells are responsible for intracellular digestion of food, and are usually referred to as digestive cells. The digestive cell is characterized by the presence of numerous cytoplasmic vesicles and by microvilli which project from the cell apex into the tubule lumen. The function of the basophil cell is not known, but it has been regarded by various authors either as an immature digestive cell or as an enzyme secreting cell. Photo micrographs 2.1a and 2.1b show the structure of digestive diverticula of *P. viridis* and *P. indica* of control animals.

In PAHs–exposed mussels, swollen residual bodies were observed in a state of fusion, forming complex structures enclosed by a single membrane .Control mussels were not devoid of these structures, but their presence was considerably limited. Another effect of the PAHs treatment in the basophilic cells was the appearance of dense granules, which probably correspond to secretory vesicles, the number and size of which was much greater compared to the controls. Additionally, in the BHC and LDO treated mussels, alterations in the morphology of the basophilic cells concerning the vacuolization of the rough endoplasmic reticulum was observed .Hepatopancreas of *P.*

indica exposed to high concentration of LDO WAF showed complete loss of tubular structure and necrosis (Plate. 2.1d). Necrosis and exudates formation was also observed in *P. viridis* after BHC exposure (Plate. 2.1c).

Analysis of histological preparations of the control animal group shows that the digestive gland (hepatopancreas) consists of numerous, compressed small channels (tubules).Between the tubules lie loose connective tissues and hemolymphatic spaces. In transverse sections the tubules are seen as circular structures. The cytoplasm of digestive cells is filled with secretory granules of different sizes. Many cells contain vacuoles with phagocytotic content and the nucleus is placed toward the basal membrane. The basophil cells are characterized by the presence of cell granules of calcium carbonate. In comparison with the structure of digestive gland of other species of mussels, the digestive gland of *Perna spp* is simpler, because this gland may have two to four cell types .No pathological changes in the digestive gland of control mussels could be observed.

Changes in the midgut and stomach lining wall were observed in mussels exposed to WAF of BHC and LDO WAFs.(Plates 2.2a-d, Plate 3.3a-b,d) Vacuolation of digestive cells became apparent, and the vacuoles of various sizes were observed , Also, the gathering of amoebocytes in areas between the tubules increased which is characteristic in acute inflammatory processes. The first necrotic changes also occurred in BHC and LDO WAFs exposed mussels on the second and third day of the experiment. These changes manifested as the erosion of cytoplasm of the digestive cells. The lumen of tubules contained exudates and cell remains as well as granules from basophil cells. On the fifteenth day of the experiment, the processes of atrophy went even further and in the spaces between the tubules necrosis of connective tissue took place. In some parts of the gland, the tubules lost cell organization simultaneous with expansion of hemolymphatic areas.In places, total breakdown of tubules occurred, and only aggregation of cell particles remained.

At 5 ppm, cell vacuolation was observed after 5th day of exposure to BHC, but the process of necrosis was slower, so that not until the tenth day did the necrotic changes become apparent.

At a lower concentration of LDO (0.05 and 0.5 ppm*)* vacuolation of cells, corresponding to the first degenerative change, was observed in digestive gland of some animals on the fifth day. Necrotic changes were also established in subchronical conditions of poisoning on the tennth day of the experiment, but the digestive glands of the greatest number of the mussels showed only inflammatory reaction.

Similar histopathological changes in the digestive gland of molluscs, as a result of the influence of phenol, have been reported by Sunila (1987) in the snail *Planorbarius corneus.* Similar disturbances of the normal structure in the molluscan digestive gland are caused by other chemical compounds, (Calabrese *et al.* 1984; Sunila 1984; Minniti 1987).

Necrotic changes which have been described earlier were irreversible and they might be a possible cause of death of mussels in acute and subchronic conditions of exposures. Inflammatory reaction and degenerative changes such as vacuolation were reversible. This is confirmed in our study by histological examination of the some mussels which recovered after depuration.

In several of these cases, degeneration of the digestive gland epithelia was observed. This manifested as a sloughing of epithelial cells to the tubule lumen and a general loss of structure of the organ. The final stages of degeneration were marked by a complete absence of epithelial cells and a remnant musculo-epithelial border, often associated with infiltrating haemocytes .

Summarizing, the digestive gland is the major site for PAH accumulation in mussels and shows histological changes that may be used as biomarkers of the

biological effect of exposure to PAHs. In previous laboratory experiments, mussels were demonstrated to be useful sentinels for PAH pollution when the digestive gland was used as the target tissue for chemical, and histological, analyses.

It is known that digestive gland is a major organ for digestion and resorption of food in mussels. The histopathological changes seen in the present study confirmed that PAHs affected digestive cell function.

Gill

Mussel gill cells are attractive models in ecotoxicological studies because gills are the first uptake site for many toxicants in the aquatic environment; gill cells are thus often affected by exposure to pollutants.In bivalve molluscs such as the mussel *Perna viridis* and *Perna indica*, the gills are key organs involved in nutrient uptake, digestion and respiration.

In paraffin sections stained with haematoxylin–eosin, three zones were distinguished in the long central gill filaments: frontal, intermediate and abfrontal. Various types of ciliated cells were present in the frontal zone, and both ciliated and non-ciliated cells were found in the abfrontal zone. The intermediate zone was comprised of flattened endothelial cells.

Gills were found to consist of filaments that increased in length from the two edges to the centre. Filaments that were located at the edges of the lamella were composed of ciliated cells and were shorter than the filaments at level of the middle lamella. Filaments at the edges were connected to each other by connective tissue. Conversely, the long central filaments were not joined by connective tissue. In the long central filaments, three zones were distinguished: frontal, intermediate and abfrontal (Fig.2.1). The terminology used by Sunila (1986) was adopted to classify the various cells present in the gill epithelium. In the frontal zone, several types of ciliated and non-

ciliated cells were found, the cells possessing a different morphology depending on their position in the epithelium. The apical part of the frontal zone was composed of columnar frontal cells with short cilia. Large eu-latero–frontal cells that possessed cirri lay adjacent to the frontal cells. Postlatero-frontal cells (columnar cells with no cilia) were next observed, followed by lateral cells, which were large cells with relatively large nuclei and long cilia. Finally, small post-lateral cells with no cilia were also distinguished in the frontal zone.

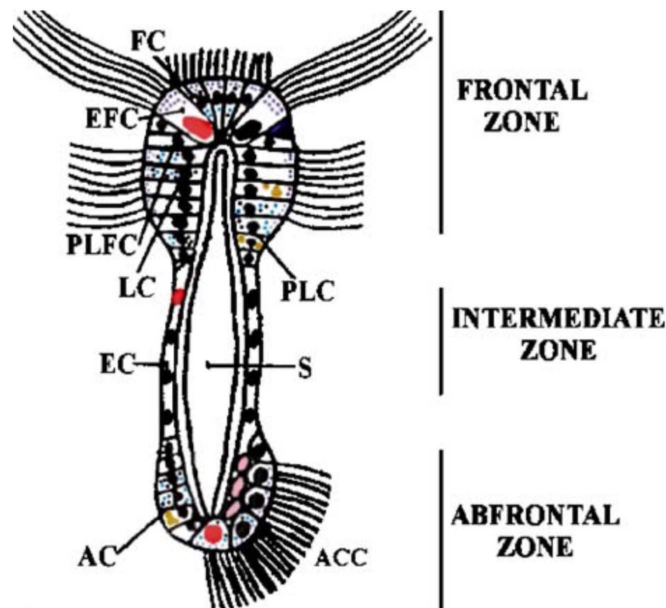


Figure 2.1: General Structure of mussel gill (Sunila,1986)

FC: frontal cells, EFC eu-latero-frontal cells, PFLC postlatero-frontal cells,LC lateral cells, PLC: post-lateral cells,EC endothelial cells,S :haemolymphatic sinus,ACC:abfrontalciliated cells,AC:abfrontal non-ciliated cells

The intermediate zone was comprised of flattened so-called "endothelial" cells . Ciliated and non-ciliated cells were present in the abfrontal zone, each at one side of the filament end .Blood cells or haemocytes were found in the haemolymph sinus of the gill filaments (Plate. 2.4a).

Each gill of a filibranchiate (including Mytilidae) consists of two plate-like demibranchs that are extended anterior-posteriorly on each side of the visceral mass. Each demibranch is comprised of two lamellae, one attached to the gill axis (descending limb) and the other lying free (ascending limb). The two lamellae are held together by connective tissue junctions (interlamellar junctions) that link the ascending and descending limbs .The descending limbs converge at the gill root . Individual lamellae are made up of several rows of filaments through which branchial veins pass . Chitinous rods are seen along with the muscle (abfrontal and frontal) which help to preserve the integrity of the structure.

The arrangement of cilia follow a definite pattern. At the frontal end of the filament are four columnar cells. These cells are provided with cilia which are called frontal cilia. Adjacent to this lies one cell which is large and ciliated latero frontal cell and the cilia associated with it is called latero frontal cilia. A row of lateral cells bear lateral cilia. It is also noted that beneath the ciliated abfrontal cells mucous glands are present.

For respiration, the gills present two functional elements: (1) a peripheral ciliary pump that creates a flow of oxy-genated water over and through the demibranchs and (2) an internal circulatory system that carries the oxygenated haemolymph to the heart. During the evolution of filibranch bivalves, the size of the gills has increased in relation to body mass and mantle cavity. Thus, in addition to their respiratory function, the gill surfaces of filibranchs serve to collect inhaled particles, facilitated by mucous secretion, followed by food-string transport along food grooves. Chemical pollutants in the marine environment, whether in soluble form or associated with sedimentary particles or in suspension in the water, can penetrate into marine organisms in various ways and then easily cross cell membranes via the outer epithelial layer of the gills and other organs located in the paleal cavity (i.e. mantle, gonad and digestive gland).After

absorption, chemical pollutants accumulate and can interact with endogenous molecules thus causing disturbances in cell functions .

There were no obvious gross differences in the histological structure of filaments derived from control animals kept from 1 to 20 days. In brief, each lamella is comprised of numerous parallel filaments, inter-connected by ciliary disks composed of numerous densely packed, interlocking cilia (Plate 2.5d). Individual filaments are characterized by the presence of unique abfrontal, lateral and frontal cilia and sheets of lateral-frontal cirri. The entire lateral surface is covered by a mat of microvilli between which projects occasional groups of up to 4 elongated cilia .

Under the light microscope, some of the PAHs–treated mussels presented structural deformities caused by fusion of the gill filaments (Plate 2.5a). More specifically, fusion of the abfrontal epithelium of adjacent filaments was noted in some of the BHC-treated mussels ,whereas lateral fusion was the main type of structural deformity in the LDO treated mussels.

Other than a moderate reduction in the number of cilia projecting from lateral frontal cells, there were no obvious surface morphological abnormalities of gill filaments in animals exposed to WAF of BHC for up to 5 days (Plate 2.4d). After 5 days, however, occasional areas devoid of microvilli were present on some lateral surfaces.Progressively more severe abnormalities were seen from fifteen to 20 days after exposure. There were extensive areas where abfrontal surfaces were denuded of cilia (Plate 2.4b). In some instances, abfrontal cells appeared necrotic, their internal organelles spilling into the extracellular space (Plate 2.4c) and hyperplasia and hypertrophy of cells of *P. viridis* (Plate 2.4b) were also noticed.

Lateral squamous cell microvilli had become more irregular in shape and had increased in diameter after 20 days exposure to WAF of LDO. The most obvious alteration in filament morphology was the remarkable increase in the number of cilia

projecting from squamous lateral cells. Cilia projecting from these cells on filaments from control specimens and those exposed to LDO for up to 5 days were quite sparse. However, at 15 and 20 days after exposure to LDO, much of the lateral surfaces were covered with cilia . In some instances the cilia appeared to project in clumps from lateral surfaces . 20 days after exposure to LDO, apart from frontal-lateral cirri appearing to have lost most of their `curl' , there were no obvious changes in either lateral frontal or frontal cilia numbers or length. Loss of interfilamental connections were visible in the gills of *P. indica* (Plate. 2.5c).

Adductor Muscle

Photomicrograph of 2.6a show the normal adductor muscle tissue of *P.viridis*. The filaments are of two kinds, thick paramyosin filaments, and much thinner filamernts which very probably contain mainly actin. Both are short, relative to the length of the fibre, lie parallel to fibre. The arrays partly overlap, and in the regions of overlap each thick filament is surrounded by twelve thin ones, and each thin filament is usually 'shared' by two thick ones. The regions of the fibre where the arrays of thin filaments are exposed are identified as I-bands, the intervening regions (array of paramyosin filaments partly overlapping with array of thin filaments) as A-bands. The relative positions of the arrays change with the length of the muscle: the extent of overlap in the A-bands increases as the muscle shortens and decreases on stretching. Thus the muscle shortens by a sliding filament mechanism. although the muscle is identified as a striated muscle because it has A-band and I-bands, it differs from cross-striated muscles in two respects. In the first place, the bands lie at a small angle (about 10 degrees) to the fibre axis, and are arranged helically around the outer part of the fibre, giving rise to the 'double-oblique striation' visible in the light microscope; moreover, the bands branch and anasstomose with each other in the core of the fibre. In the second place, there are no Z-lines, but in the planes where Z-lines would be expected, discrete dense bodies are present. Similar dense-bodies occur in many smooth muscles, and in

the anterior byssus retractor of Mytilus. The contractile apparatus of it closely resembles that of the oyster muscle in many respects, but the filaments are not clearly grouped into separate arrays.This feature , the presence or absence of segregated arrays of filaments , could serve as the main criterion for distinguishing striated from smooth muscles . Where the arrays of thick and thin filaments overlap in the A- bands , the two kinds of filaments are put forward that the paramyosin filaments are comparable to myosin- containing filaments of the cross-striated muscles of vertebrates, and that the cross- links in the oyster muscle are comparable to actin-myosin links.

In a number of mussels, necrosis of the muscle was observed. and no inflammatory reaction was seen in relation to necrotic muscle.

Foot

Between the adductors lies the large muscular hatchet –shaped foot. Two small muscles connect the foot to the shell dorsally and serve to draw the foot inside the shell: the anterior and posterior foot–retractors. The posterior adductor dwarfs the anterior adductor, which lies just left of the beak. Below *Perna's* anterior adductor lies the brown, finger-like foot. The mussel's byssal threads emerge just below the foot. Embedded in the tissue at the base of the foot is the opaque white thread- producing byssal gland. The byssal gland invests the foot tissue just below the grove . A single pair of long , cord- like anterior byssus retractors running anteriorly from the byssal gland and attaching to a small shelf just to the right of the beak..Besides pulling on the byssal threads, these muscle's contractions cause the foot to move forward. Running posteriorly from the foot is a pair of foot retractors; running posteriorly from the byssal gland are two pairs of posterior byssus retractors. These three pairs of muscles attached to the shell in a line just anterior to the posterior adductor. This arrangement of several "lines" (muscles) to a single point (base of byssal threads and foot) by

distributing the waves force into many lines of tension, as a sail's rigging distributes the wind's force. No pathological change observed in foot tissue.

Mantle

Plate.2.7b show the normal mantle tissue of *P.viridis*. The bivalve mantle is a large bilobed structure which covers the animal's body and also secretes the shell. The mantle edge has three folds. The outermost fold, the least conspicuous of the three, actually secretes the shell. Its inner surface secretes the periostracum; its outer surface secretes the calcareous prismatic layer of the shell. The entire outer surface of the mantle secretes the nacreous layer which lines the shell. The middle fold has a sensory function. (in *Perna* it appears that this (the middle) fold actually secretes the periostracum, rather than the outer fold; the middle fold is continuous with the periostracum and ensheaths, the thin outer fold). The outer fold-by far the largest-is supplied with muscles which attach the mantle to the shell. The outer fold mantle in *Perna* is relatively muscular and edged with branching. The mantle edges are fused dorsally.

The space enclosed by the mantle lobes is called the mantle cavity.Cilia lining the mantle and covering the ctenidia (which hang down in the mantle cavity) set up a current that draws water into the mantle cavity. In *perna* exhalant orifice is a simple opening in the fused portion of the mantle just behind the posterior adductor muscle.Behind the exhalant opening are a pair of membranes which can open and close the opening, wiping over the posterior adductor as they do so.

Bioaccumulation and Cellular damages

Tables 2.2, 2.3, 2.4 and 2.5 give the semiquantitative determination of the histopathological lesions in gill, and digestive gland, tissues evaluated at 5, 10, 15 and 20 days of exposure.

The semiquantitative histopathological damage produced by BHC treatment was frequently detected on digestive gland, gill, and mantle tissues of *Perna indica*, although it was lower than with the other treatments using LDO.In the LDO exposed green mussels also the damage was more frequent in digestive gland followed by gills tissues. Following a similar pattern to the other treatments, it increases with time.

Table 2.2: Summarised results of semiquantitative histopathological determination in gill, and digestive gland tissues of *Perna viridis* in controls and in those exposed to BHC

Treatment		Control	0.1 ppm	1 ppm	5 ppm	8 ppm
Gills	AL	0	0/1+	0/1+	1+/2+	1+
	MC	0	0/1+	0/1+	2+	2+
	DE	0	0	0/1+	1+	1+
	DQ	0	1+/0	0/1+	1+	1+/2+
	IH	0	1+	1+	2+	1+/2+
	N	0	0	0	1+	1+
Digestive gland	DT	0	0/1+	0/1+	1+	1+
	DE	0	1+	1+	0/1+	0/1+
	PC	0	1+	1+	0/1+	0/1+
	OT	0	0	0	1+	1+
	IH	0	1+	1+	0/1+	1+/2+
	AE	0	0	0/1+	1+	0/1+
	AC	0	0	0/1+	1+	1+
	N	0	0	1+	1+	1+

Table 2.3: Summarised results of semiquantitative histopathological determination in gill, and digestive gland tissues of *Perna viridis* in controls and in those exposed to LDO

Treatment		Control	0.05 ppm	0.5 ppm	2 ppm	4.8 ppm
Gills	AL	0	1+/2+	1+/2+	3+	2+
	MC	0	1+/2+	1+/2+	3+	3+
	DE	0	1+	1+/0	2+	1+
	DQ	0	0/1+	0/1+	3+	2+
	IH	0	2+	1+	3+	2+
	N	0	1+	1+	2+	1+
Digestive gland	DT	0	1+/2+	1+/2+	2+/3+	2+/3+
	DE	0	1+	1+/0	2+	1+/2+
	PC	0	1+	1+	1+/2+	1+/2+
	OT	0	1+	1+	0	0
	IH	0	1+	1+	1+/2+	1+/2+
	AE	0	1+	1+/2+	3+	2+
	AC	0	1+	1+/2+	2+/3+	2+/3+
	N	0	0	0	1+	1+

Table 2.4: Summarised results of semiquantitative histopathological determination in gill, and digestive gland tissues of *Perna indica* in controls and in those exposed to BHC

Treatment		**Control**	**0.1 ppm**	**0.6 ppm**	**2 ppm**	**5 ppm**
Gills	AL	0	2+	1+/2+	3+	2+/3+
	MC	0	2+	1+/2+	3+	3+
	DE	0	2+	0/1+	3+	2+/3+
	DQ	0	0/1+	0/1+	3+	2+/3+
	IH	0	3+	1+	3+	2+/3+
	N	0	1+	1+	3+	2+/3+
Digestive gland	DT	0	1+/2+	3+	2+/3+	3+
	DE	0	1+	1+/2+	2+	3+
	PC	0	2+	2+/3+	2+/3+	3+
	OT	0	1+	1+	0	0
	IH	0	1+	1+/2+	2+	2+
	AE	0	1+	1+/2+	3+	2+
	AC	0	1+	1+/2+	3+	2+
	N	0	0	0	2+/3+	2+

Table 2.5: Summarised results of semiquantitative histopathological determination in gill, and digestive gland tissues of *Perna indica* in controls and in those exposed to LDO

Treatment		Control	0.04 ppm	0.4 ppm	1 ppm	3.5 ppm
Gills	AL	0	3+	1+	1+	1+/2+
	MC	0	1+/2+	3+	1+/2+	2+/3+
	DE	0	1+/2+	1+/2+	3+	2+
	DQ	0	1+/2+	1+/2+	3+	2+/3+
	IH	0	2+/3+	1+	2+	1+/2+
	N	0	2+/1+	2+/1+	1+/2+	1+/2+
Digestive gland	DT	0	1+	1+	2+/3+	1+/2+
	DE	0	1+	1+	2+/3+	2+/3+
	PC	0	1+/2+	0/1+	2+	1+/2+
	OT	0	1+/2+	0/1+	1+/2+	1+
	IH	0	1+	1+	2+	1+
	AE	0	1+	1+	2+	2+
	AC	0	1+	1+	1+/2+	2+
	N	0	1+	1+	2+	2+

Table 2.6: Inference of Wilcoxon Signed Rank Test for the histopathological parameters observed in the hepatopancreas and gills of *Perna viridis* when subjected to various treatments with BHC WAF

Day of sampling	Treatment	Digestive tubule (Sig.)	Gill (Sig.)
5	Control+0.1 ppm	N.S	N.S
	Control+1 ppm	N.S	N.S
	Control+5 ppm	P<0.01	N.S
	Control+8 ppm	P<0.01	P<0.01
10	Control+0.1 ppm	N.S	N.S
	Control+1 ppm	N.S	N.S
	Control+5 ppm	P<0.01	P<0.001
	Control+8 ppm	P<0.01	P<0.01
15	Control+0.1 ppm	N.S	N.S
	Control+1 ppm	N.S	N.S
	Control+5 ppm	N.S	P<0.001
	Control+8 ppm	P<0.04	P<0.03
20	Control+0.1 ppm	P<0.04	P<0.001
	Control+1 ppm	N.S	P<0.03
	Control+5 ppm	P<0.02	P<0.02
	Control+8 ppm	P<0.01	P<0.04

Table 2.7: Inference of Wilcoxon Signed Rank Test for the histopathological parameters observed in the hepatopancreas and gills of *Perna indica* when subjected to various treatments with BHC WAF

Day of sampling	Treatment	Digestive tubule (Sig.)	Gill (Sig.)
5	Control+0.1 ppm	N.S	N.S
	Control+0.6ppm	N.S	N.S
	Control+2 ppm	P<0.001	N.S
	Control+5 ppm	P<0.02	P<0.01
10	Control+0.1 ppm	N.S	N.S
	Control+0.6ppm	N.S	N.S
	Control+2 ppm	P<0.03	P<0.001
	Control+5 ppm	P<0.01	P<0.02
15	Control+0.1 ppm	N.S	N.S
	Control+0.6ppm	N.S	N.S
	Control+2 ppm	P<0.01	N.S
	Control+5 ppm	P<0.05	P<0.01
20	Control+0.1 ppm	P<0.03	P<0.001
	Control+0.6ppm	N.S	P<0.05
	Control+2 ppm	P<0.02	P<0.02
	Control+5 ppm	P<0.01	P<0.01

Table 2.8: Inference of Wilcoxon Signed Rank Test for the histopathological parameters observed in the hepatopancreas and gills of *Perna viridis* when subjected to various treatments with LDO WAF

Day of sampling	Treatment	Digestive tubule (Sig.)	Gill (Sig.)
5	Control+0.05 ppm	P<0.01	P<0.01
	Control+0.5 ppm	P<0.02	N.S
	Control+2 ppm	P<0.02	N.S
	Control+ 4.8 ppm	P<0.03	P<0.01
10	Control+0.05 ppm	P<0.01	P,0.003
	Control+0.5 ppm	P<0.02	N.S
	Control+2 ppm	P<0.01	P<0.001
	Control+ 4.8 ppm	P<0.01	P<0.01
15	Control+0.05 ppm	N.S	N.S
	Control+0.5 ppm	P<0.04	N.S
	Control+2 ppm	P<0.001	P<0.002
	Control+ 4.8 ppm	P<0.001	P<0.01
20	Control+0.05 ppm	P<0.03	P<0.03
	Control+0.5 ppm	P<0.01	N.S
	Control+2 ppm	P<0.01	N.S
	Control+ 4.8 ppm	P<0.01	P<0.01

Table 2.9: Inference of Wilcoxon Signed Rank Test for the histopathological parameters observed in the hepatopancreas and gills of *Perna indica* when subjected to various treatments with LDO WAF.

Day of sampling	Treatment	Digestive tubule (Sig.)	Gill (Sig.)
5	Control+0.04 ppm	N.S	P<0.01
	Control+0.4 ppm	P<0.01	P<0.01
	Control+1 ppm	P<0.01	P<0.01
	Control+3.5 ppm	P<0.001	P<0.001
10	Control+0.04 ppm	P<0.01	N.S
	Control+0.4 ppm	N.S	P<0.04
	Control+1 ppm	P<0.02	P<0.001
	Control+3.5 ppm	P<0.01	P<0.01
15	Control+0.04 ppm	P<0.04	P<0.001
	Control+0.4 ppm	P<0.04	P<0.03
	Control+1 ppm	N.S	P<0.03
	Control+3.5 ppm	P<0.04	N.S
20	Control+0.04 ppm	N.S	P<0.03
	Control+0.4 ppm	P<0.02	P<0.05
	Control+1 ppm	P<0.01	N.S
	Control+3.5 ppm	P<0.01	P<0.04

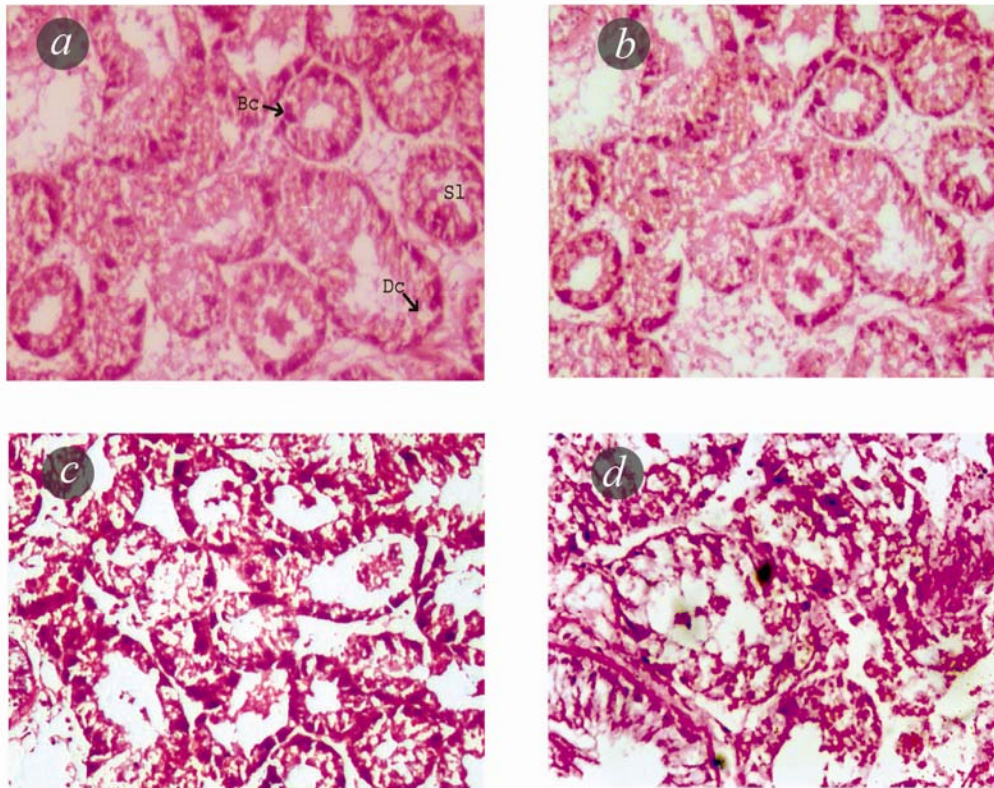
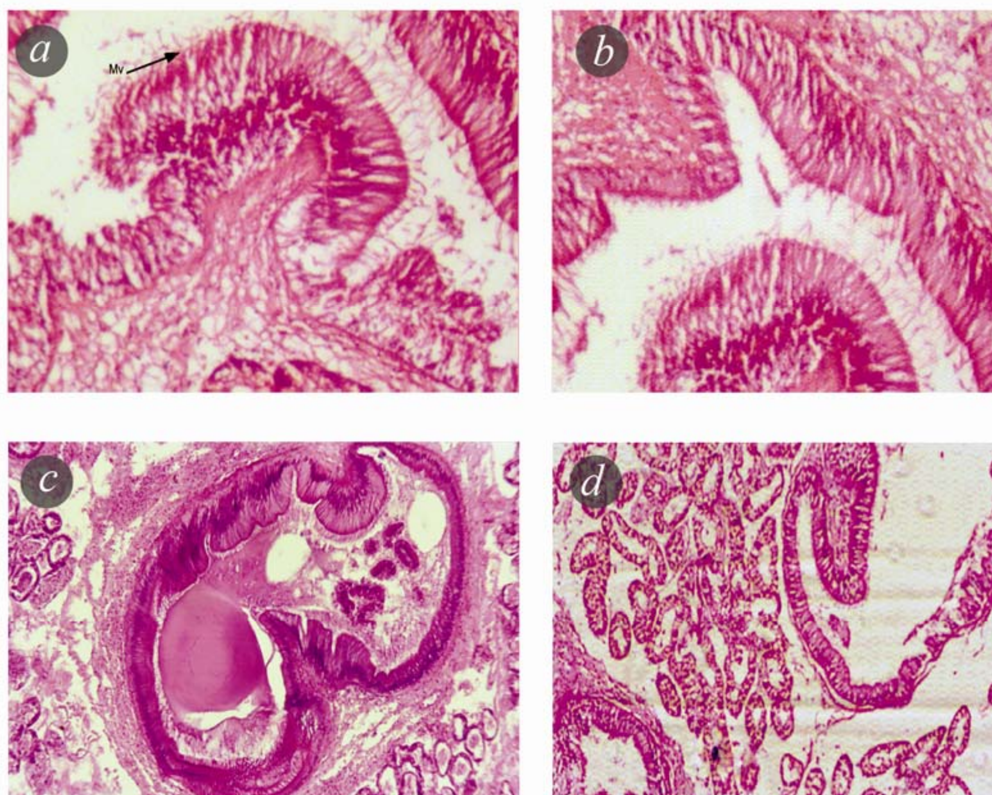


Plate 1. a) Normal structure of digestive diverticula of *Perna viridis* showing the B and D cells. x 200 b) Normal structure of digestive diverticula of *Perna indica* showing the B and D cells. x 200 c) Digestive diverticula of *Perna viridis* exposed to 8 ppm WAF of BHC showing necrosis and exudate formation x 200 d) Hepatopancreas of *Perna indica* exposed to high concentration of LDO WAF showing complete loss of tubular structure, and necrosis. x 200



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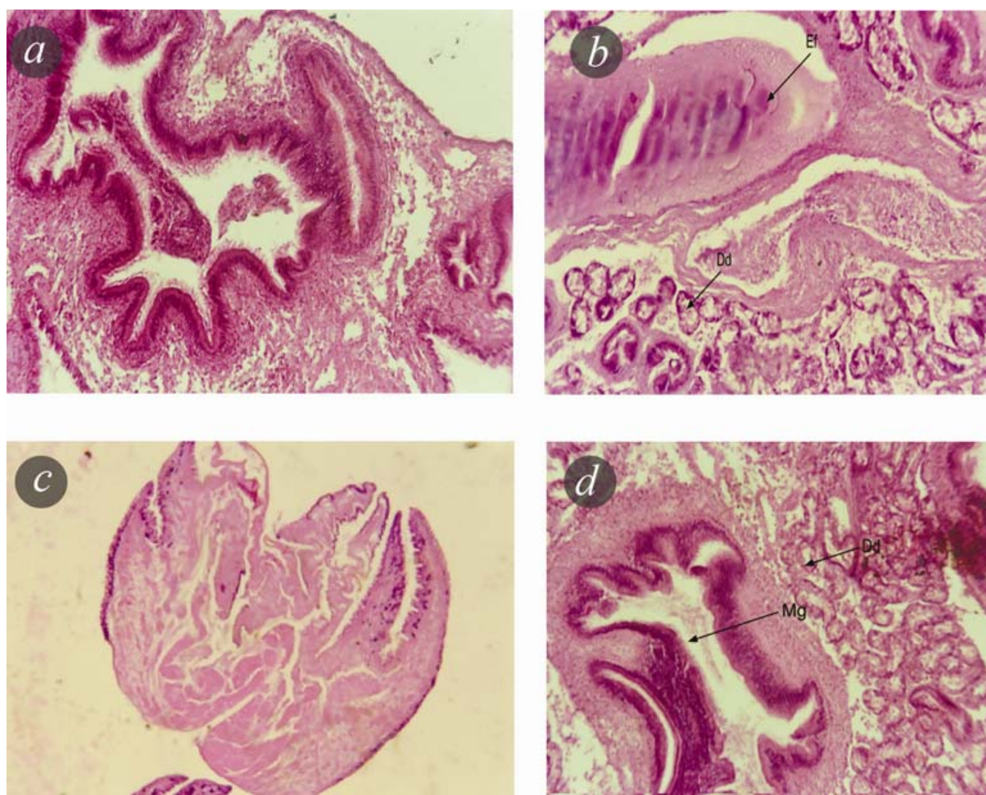

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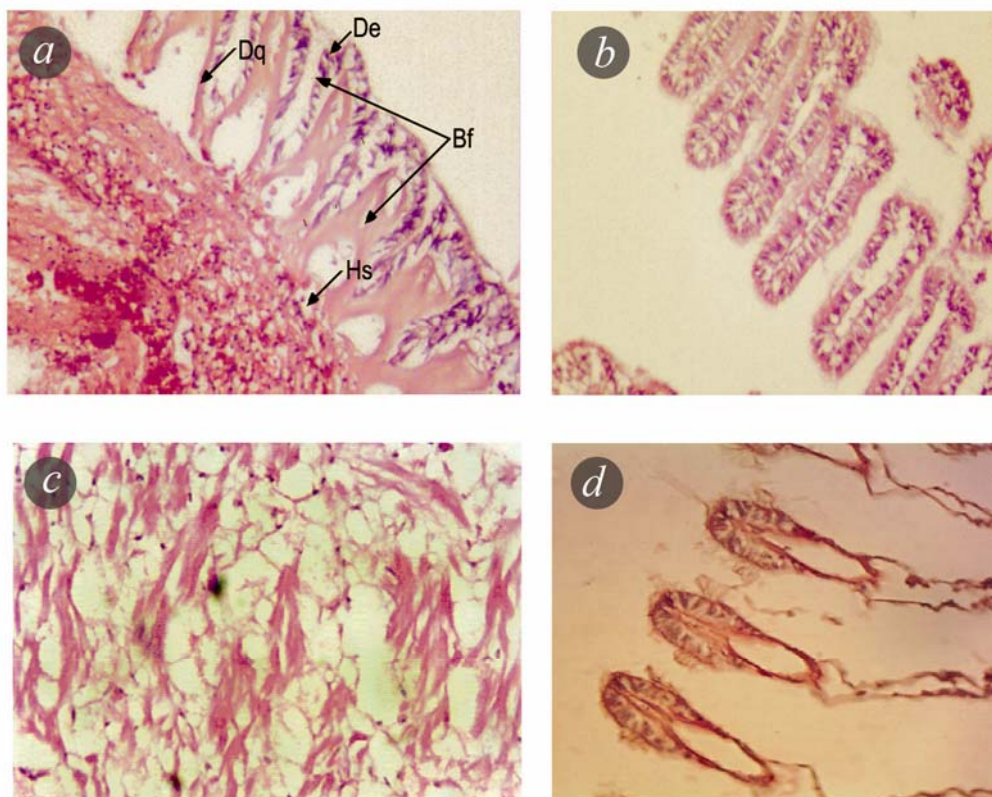

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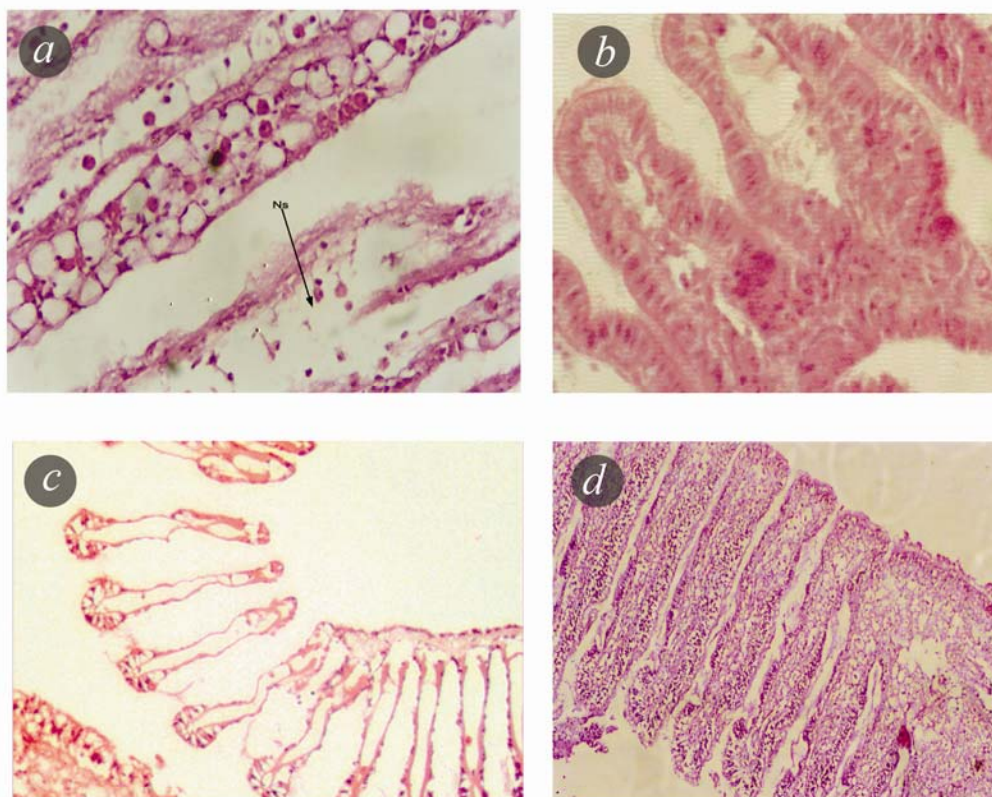

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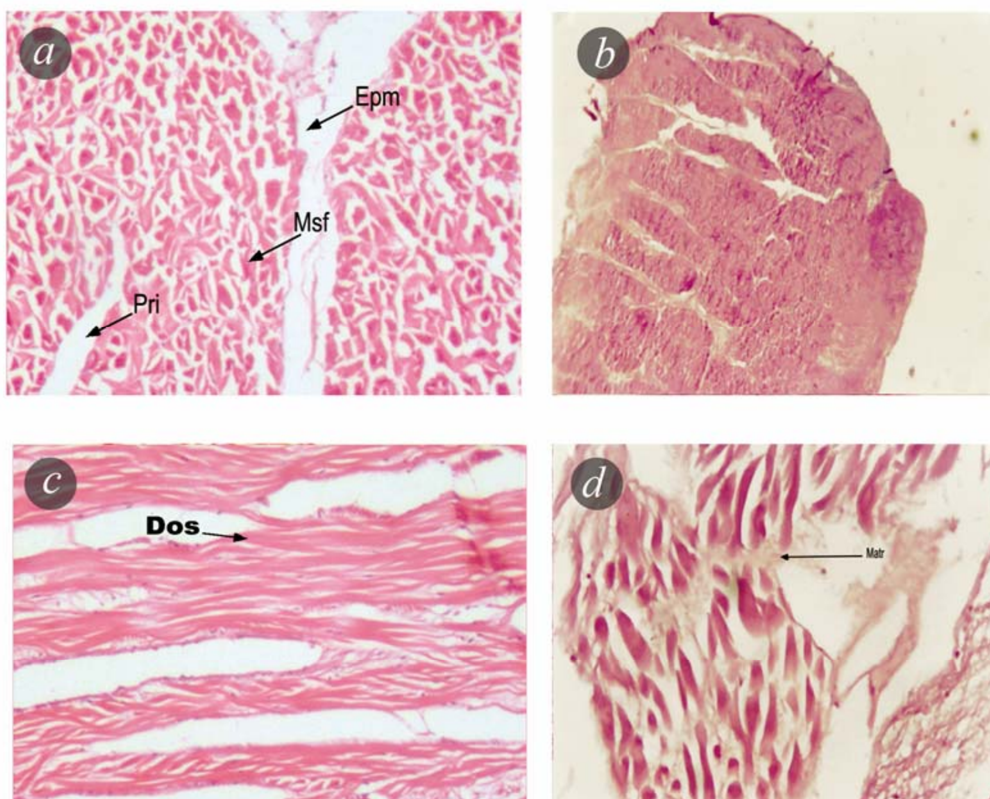

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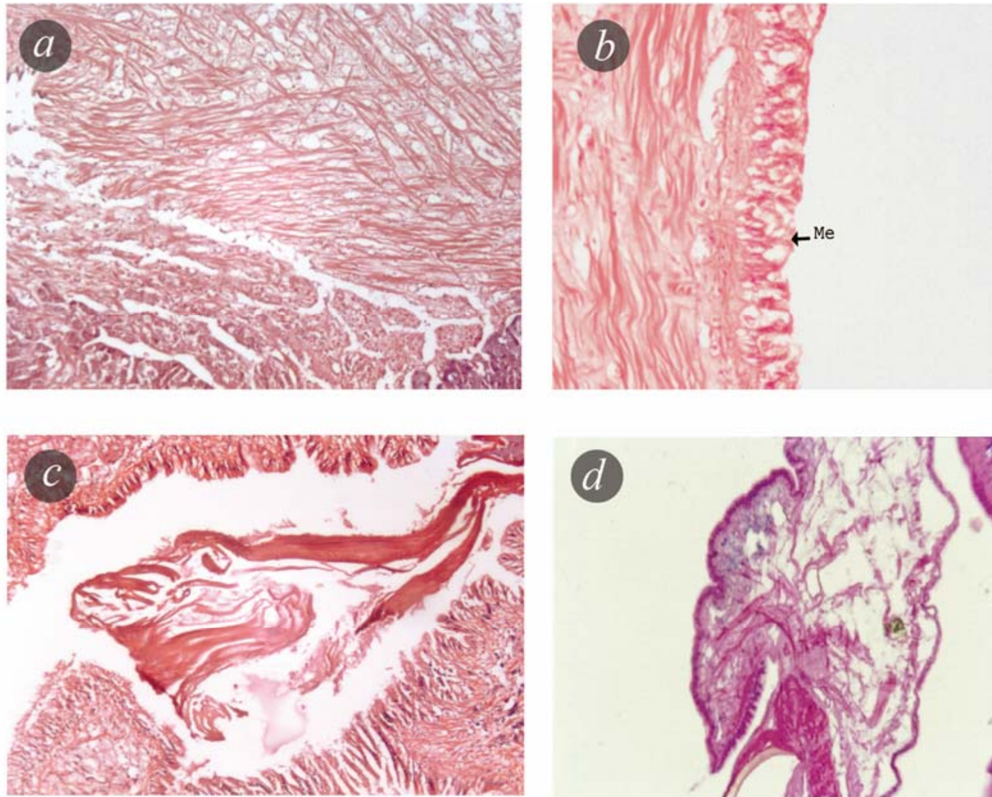

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2.4 Discussion

Earlier studies have shown that mussels imbibe pathogens, PAHs and other toxic substances from their environment and store them in their soft tissues (Goldberg, 1975; Phillips, 1976; Roesijadi and Fellingham, 1987; Lakshmanan and Nambisan, 1989). When stored, they gradually accumulate in the soft tissues, especially the mantle, gills, kidney, foot and digestive gland (Odzak *et al.*, 1994; Regolli and Orlando, 1994; Ward, 1990; Lakshmanan and Nambisan, 1989; Lobel, 1987).

This study has clearly shown that in common with other mussel species, *P. indica* and *P. viridis* were efficient accumulators of PAHs. In spite of a substantial increase of PAHs in tissues, there was, however, no mortality during the experimental period.

Gills of *Perna spp.* consist of several rows of filaments increasing in length from the two edges to the centre. In agreement with Sunila (1986), we have distinguished three different zones in mussel gill filaments: frontal, intermediate and abfrontal. In the frontal zone, both ciliated and non-ciliated cells are present: frontal cells possess short cilia, eu-latero–frontal cells possess cirri, lateral cells possess long cilia and postlatero–frontal and post-lateral cells possess no cilia (Sunila 1986). Frontal cilia transport particulate material to the palps, eu-lateral cilia trap particulated material and lateral cilia take part in driving water between the filaments (Fawcett and Porter 1954; Owen 1974, 1978; Owen and McCrae 1976). Thus, the transport and uptake of nutrients and of contaminants occur in the frontal zone of the gill filaments (Fawcett and Porter 1954; Owen 1974, 1978; Owen and McCrae 1976). The role of the postlateral cells is unknown (Sunila 1986). Both ciliated and non-ciliated cells are also found in the abfrontal zone. The so-called "endothelial" cells are flattened non-ciliated cells in the intermediate zone of the gill filament in which gas exchange and interactions between the external medium and haemolymph occur.

Mucocytes were identified both in the frontal and in the abfrontal zones of mussel gill filaments. Ma et al. (1986) found mucocytes in the laterofrontal and abfrontal zones of mussel gill filaments, whereas Pekkarinen (1991) observed them in the frontal, laterofrontal, postlateral and abfrontal zones. Some cells contain both neutral and sialomucins and some primarily sulphated mucin or neutral glycoproteins (Ma et al. 1986).

Bivalve gills are unique organs that show continuous terminal growth by the addition of new elements in correlation with the lifelong increase in shell size (Sunila 1986). According to Sunila 1986 the growth of bivalve gills proceeds at the posterior end of the gill from an undifferentiated "budding zone". At this zone, cells might actively divide to produce new gill elements. Pekkarinen (1991) have described three zones with enhanced cell division in the gills of mussels: 1) the gill root in which the zone fills the central portion of the root and descends by two arms along the inner margin of the descending limbs, 2) the free end of the ascending limb of the gill filament and 3) the junction point of the descending and ascending limbs. The present work has shown that cell proliferation occurs along the gill filaments in adult mussels.

From 15 to 20 days exposure to PAHs, there was a gradual increase in the number of ruptured cells on abfrontal and lateral surfaces. Lateral cell microvilli became distended and there were large areas where abfrontal cells were denuded of cilia. By 20 days after exposure to PAHs, the cilia comprising the lateral frontal cirri appeared to be separating.The most obvious and perhaps significant change in morphology was the increase in the number of cilia projecting from lateral squamous cells.

Gill filaments from control animals were populated with occasional lateral squamous cells from which projected 2 to 4 elongated cilia. While control animal surface morphology remained normal over the experimental period, 15 days after

exposure to PAHs, there was a marked increase in the number of cilia projecting from the lateral regions of experimental animals. By 20 days after exposure to PAHs, in some instances, the whole lateral surfaces were covered with cilia and there was no apparent division between abfrontal and lateral cilia. In these filaments, abfrontal cilia appeared to continue down lateral surfaces.

Exposure to PAHs unquestionably caused considerable changes in gill filament morphology. Further, pathomorphology varied in nature and apparent degree of severity with time of exposure. Surprisingly, the most obvious changes occurred shortly after exposure and presented as a substantial loss and/or absence of lc, lfc, and by four days, ac cilia. In addition there was a "thinning" of the frontal aspects of the epithelium, generalised intercellular oedema, axon oedema and degeneration, progressive endothelial cell deterioration and swelling and loss of Mv, especially from squamous lateral cells.While there was cilial recovery by ten days after exposure and far less intercellular oedema. From days 15–20, while there was little evidence of intercellular oedema, mucus cells had increased in numbers and secretory droplets and osmiophylic inclusions were present in most cells. In addition, many filaments contained numerous, apparently necrotic ciliated fc, lc and lfc and groups of now "cuboidal" S had either exfoliated or were exhibiting necrotic features. At this time, there was also an increase of cilia projecting from otherwise nonciliated cells lining the abfrontal "shoulder". There was an increase in the number of granulocytes in both the lumen of the branchial vessel and the epithelium throughout the entire period that the animals were exposed to PAHs.

Many of these features have been described in other bivalves exposed to PAHs and other toxins. Changes in the shape and general architecture of gill filaments have been reported together with progressive necrosis of epithelial cells, increased exfoliation and elevated mucus secretion following prolonged exposure to heavy metals (Sunila, 1987, 1988a,b. Hieteman *et al.*, 1988; Axiak *et al.*, 1988). Exposure of

bivalves to low concentrations of PAHs induces the increase in numbers of cilia projecting from lateral cells and cells in abfrontal regions, the change in the shape of squamous cells together with an increase in mucus cells and mucus droplets in otherwise non-secretory cells, suggests that PAHs destabilises the epithelium. While a nonpolluted environment promotes normal differentiation, PAHs encourages a metaplastic response, with non-ciliated cells becoming ciliated and otherwise non-secretory cells taking up a secretory function. In addition, while there is an apparent increase in necrotic cells, there was no evidence to suggest a concomitant increase in exfoliation.

This suggests that PAHs also influences epithelial cell turnover. While most studies focus on the effect that PAHs have on the metabolism of bivalves during exposure to a pollutant, this study also examined their physiology and morphology during a 35 days recovery period. After placement in clean water, there was a dramatic reduction of PAHs in the soft tissues. A reduction of PAHs during the post exposure period has also been reported in the bivalve *Crassostrea virginica*, which when exposed for 45 days showed a subsequent reduction of PAHs after transfer to uncontaminated seawater (Clark, 1996) a phenomenon also reported by Calow (1994). Loss of PAHs from *P. indica* tissues during the recovery period correlated with the recovery of the filtration rate. Within ten days, filtration rates had returned to normal and remained within normal values until sacrifice. Over the 35 days recovery period, the endothelium lining the branchial vein became continuous and endothelial cells appeared morphologically normal. Granulocytes were far less evident in the epithelium and epithelial cell morphology gradually returned to near normal. There remained, however, occasional necrotic cells and myelin figures in nerves, Mv were sometimes swollen.

The digestive gland was the main tissue for PAHs accumulation, with significant differences in tissue PAHs levels between samples from different exposures.

The lysosomes of the digestive cells are the first accumulation site for various PAHs in mussels (Marigo´mez *et al.* 1986a, 1996; Recio *et al.* 1988a) and were proposed as a target compartment to detect the presence of abnormal levels of PAHs in the environment (Marigo´mez *et al.* 1996). On the other hand, exposure to extremely high levels of PAHs may result in an enhancement of excretory activity of digestive cells (Janssen and Dallinger 1991; Dallinger 1993, 1995) and excess xenobiotics are eliminated via feces (Marigo´mez *et al.* 1986a, 1990a; Recio *et al.* 1988a; Ireland and Marigo´mez 1992). Such extreme exposure to toxicants would lead to significant changes in the histological organization of the digestive gland, which affects its accumulative capacity (Marigo´mez *et al.* 1990b, 1996). There is some evidence of the changes in the distribution and relative occurrence of cell types in response to pollutants (Lowe *et al.* 1981; Cajaraville *et al.* 1990a, 1990b; Marigo´mez *et al.* 1990b, 1996). Moreover, the cell type composition of the mollusc digestive gland may change due to stressing factors other than pollution (Marigo´mez *et al.* 1993).

In this context, the examination of changes in the histological organization of the digestive gland tissue is useful to determine the accumulating ability of a sentinel mussel, and even to interpret correctly the data obtained by means of analytical chemistry. Moreover, the same samples might be sufficient to diagnose whether the exposure to pollutants provokes a stress response, with further consequences likely for other levels of biological organization (Marigo´mez *et al.* 1990b, 1996). The thickening of the blood vessel walls and basal lamina underlining the digestive tubules, the depletion of glycogen reserves and the alteration of the calcium metabolism have been described in a variety of gastropod molluscs on exposure to pollutants (Ireland and Marigo´mez 1992; Cajaraville *et al.*1990b; Marigo´mez *et al.* 1990b, 1993, 1996; Beeby 1993).

These effects may be easily detected through routine qualitative observations under light microscopy. Nevertheless, if quantitative data are preferred, histological

techniques may also provide simple, cheap, and quick estimators of the biological effect of the exposure to xenobiotics. A series of works has demonstrated that the digestive gland tubules of molluscs are reduced in thickness in response to a variety of environmental stressors (Lowe *et al.* 1981; Tripp *et al.* 1984; Marigo´mez *et al.* 1986b, 1993; Minniti 1987;; Vega *et al.* 1989; Cajaraville *et al.* 1992; Ireland and Marigo´mez 1992). Thus, the measure of such reduction has been proposed as a biomarker of the biological effect of sublethal exposure to pollutants.The mean epithelial thickness (MET) is reduced in molluscs exposed to sublethal concentrations of toxic substances (Marigo´mez *et al.* 1986b, 1996; Vega *et al.*1989; Ireland and Marigo´mez 1992) and other sources of environmental stress (Lowe *et al.* 1981; Cajaraville *et al.* 1992), thus MET could be used as a tissue-level biomarker of environmental stress.

The digestive tubules of mussels from the control contained abundant digestive cells, scarce to relatively conspicuous excretory cells (varying between individuals), and scarce calcium cells. In treated mussels, excretory and calcium cells increased in relative numbers in comparison with the control and, in addition, digestive cells were active in excretion of cytoplasmic granules .This change is much more marked in *Perna viridis* from the 5 ppm concentrations, where digestive cells were actively excretory and exhibited a conspicuous degree of vacuolization and where calcium cells comprised a large extent of the digestive tubule epithelium .

This study shows the remarkable resilience of *P. indica* and *P. viridis* to high concentrations of toxic, PAHs. Gill filaments exhibited severe pathomorphology during exposure to PAHs, gill filament morphology returned to near normal within 20 days of being returned to clean seawater. While our findings agree with those of other authors that bivalves efficiently accumulate toxic substances in their soft tissues (Olafsson, 1986; De Gregori *et al.*, 1996; Ahn *et al.*, 1996; OConnor and Baliaeff, 1994; OConnor, 1996), our results show that *P. indica* and *P. viridis* were also able to rapidly depurate its soft tissues.

The histopathological effects are very frequently associated with LDO, and sometimes with BHC exposure. These effects were higher in digestive gland followed by gills and lower in mantle and adductor muscle tissues.

It seems that exposure to PAHs does not provoke a degenerative pathological change but an adaptive process that enables mussels to inhabit chronically polluted sites by dramatically modifying the cell and tissue organization of their digestive gland. The digestive gland is the major site for PAHs accumulation in mussels and shows histological changes that may be used as biomarkers of the biological effect of exposure to PAHs. Thus, histological observations and tissue-level biomarkers recorded in this organ might provide a sensitive, quick, and cheap indication of the degree of PAHs pollution in soils in combination with other measures.

Sub-lethal concentrations of PAHs were rapidly accumulated in the soft tissues of *P. indica* and *P. viridis* induced marked changes in gill filament morphology. Gill filaments of mussels are composed of frontal, intermediate and abfrontal zones, each containing characteristic cell types with various functions. These changes were readily reversed on return of the mussels to clean seawater, with near normal conditions being reattained within 20 days. This resilience suggests that caution should be applied in using *P. indica*, and *P.viridis* as biomonitors over long time frames since widely spaced, intermittent releases may not be detected.

Chapter 3

ULTRASTRUCTURE STUDIES IN *Perna* spp.

Contents

3.1 Introduction
3.2 Materials and methods
3.3 Result
3.4 Discussion

3.1 Introduction

Numerous cytochemical and cytological responses have been developed and recommended as potential tests for quantifying and monitoring the environmental impact of xenobiotics in bivalve molluscs.

The hepatopancreas of molluscs is a large digestive gland formed by a vast number of blind ending tubules, the digestive diverticula. This organ is involved in several functions including the extracellular and intracellular digestion of food, storage of lipids, glycogen and minerals; it is also the main site of nutrient absorption and plays a major role in detoxification (Nelson and Morton, 1979; Morton, 1983; Beeby and Richmond, 1988; Henry *et al.*, 1991).

The digestive diverticula consists of an epithelium with a single layer of cells, separated from the surrounding connective tissue and muscle cells by a basal lamina. In several molluscs this epithelium is formed by the digestive and basophilic cells (Pal, 1971; 1972; Owen, 1973; Lobo-da- Cunha, 1997).

Mollusc digestive cells are mainly characterized by the presence of a large number of heterolysosomes, in which the digestion of the food is completed. These

columnar shaped cells are the most abundant in the hepatopancreas of molluscs and their apical surface is covered by microvilli.Lipid droplets and glycogen granules are usually present in the cytoplasm of digestive cells. The basophilic cells, also called secretory or crypt cells, are typical protein secreting cells. These pyramidal shaped cells contain large amounts of rough endoplasmic reticulum, a well-developed Golgi complex and accumulate granules secreted in the cells. They seem to be responsible for the secretion of digestive enzymes, that undertake the extracellular digestion of the food (Pal, 1971; 1972; Owen, 1973; Henry *et al.*, 1991; Franchini and Ottaviani, 1993).

The branched ducts that link the digestive diverticula to the stomach are also important component of the digestive glands. In mollusc hepatopancreas, duct cells can be involved in lipid storage and nutrient absorption (Mathers, 1972). Moreover, some results suggest that these cells also play a role in digestion (Henry *et al.*, 1991).Studies of the hepatopancreas of molluscs are especially interesting because this organ can be useful in ecotoxicology research. The peroxisomes and lysosomes of mollusc hepatopancreas can suffer morphological and functional changes caused by pollutants, and may be valuable as bioindicators of pollution (Moore, 1985; Cajaraville *et al.*, 1989; Fahimi and Cajaraville, 1995). Ultrastructural and cytochemical aspects of lysosomes and peroxisomes have been reported in some molluscs (Owen, 1972a; Pipe, 1986; Cajaraville *et al.*, 1992; Lobo-da-Cunha *et al.*, 1994; Loboda- Cunha, 1997), but it would be interesting to extend those studies to a larger number of species, in order to obtain a more general picture.

The digestive gland of bivalves is a target organ for the bioaccumulation of PAHs; furthermore, the lysosomes of the digestive cells are generally considered as target organelles (Owen 1972; Viarengo 1989; Marigomez et al. 1990a; Chassard - Bouchaud 1996), while the gills have also been shown to accumulate various xenobiotics either in the field (Balogh and Salanki 1987) or in the laboratory (George and Coombs 1977; Marshal and Talbot 1979; Nolan et al. 1984).

The histology and ultrastructure of the digestive gland of bivalves has been studied extensively (Owen 1970, 1972, 1973, Pal 1972, Morton 1983,Pillai and Menon,1996, Menon and Menon,1998). However, light and electron microscopical data concerning the functional morphology of the digestive gland of the common mussel *P. viridis and P. indica* after PAHs exposure are scarce, in spite of the importance of this organism in environmental biomonitoring studies.

To enlarge the knowledge about these marine molluscs, the hepatopancreas. gill, mantle, adductor muscle, foot and pedal disc of *P. Viridis* and *P. indica* were studied by electron microscopy.

3.2 Materials and Methods

Small pieces of hepatopancreas, gill, adductor muscle, mantle and pedal disc of toxicant exposed and control animals were fixed for 2 h at 4°C in 2.5% glutaraldehyde, diluted in 0.4 M cacodylate buffer pH 7.4 with 5 mM $CaCl_2$. After washing in buffer, the fragments were post fixed with 2% osmium tetroxide buffered with cacodylate, dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, before being observed in a Morgagni 268 D Transmission Electron Microscope Fei Netherland and relevant areas photographed (Department of Anatomy, All India Institute of Medical science, New Delhi).

3.3 Results

In semithin sections of both *P. viridis* and *P.indica* hepatopancreas, many digestive cells could be observed in the epithelium of the digestive diverticula. They were columnar cells, but many presented club shape with a round apex projected into the lumen of the digestive diverticula. (Plates 3.1 a-d, and 3.2a-d)

Numerous heterolysosomes were found in these cells, but in some of them the heterolysosomes seemed to be fusing with one another. Other cells were almost

entirely occupied by a single large vacuole, and their cytoplasm was reduced to a very thin peripheral layer. In some cases, a few thin strands of cytoplasm could still be observed in the interior of these cells, suggesting the previous existence of smaller heterolysosomes which must have fused to form the large vacuole .

The apical surface of digestive cells was covered with microvilli, (Plate. 3.2a-d and 3..8a-d). In some digestive cells the apical zone contained many mitochondria and only a few endocytic vesicles but in others the number of endocytic vesicles was very high, and only a few mitochondria were found in the area .(3.12a-d)

These vesicles contained electron dense materials and many presented a cup-shape .These vesicles were more abundant in the apical region of digestive cells, but some could also be found in deeper regions of the cytoplasm .Vacuoles with a clear background, usually containing low amounts of dense material, were also observed near the cell apex (Plates:3.13 a-d).

In the digestive cells of *P.indica*, a large part of the cytoplasm was occupied by heterolysosomes, but the dimensions and the content of these organelles were variable, reflecting different stages of intracellular digestion. Heterolysosomes were more abundant in the middle region of the cell but some could also be found in the apical zone (Plate. 4). In general, heterolysosomes were filled with material of medium density, their shape could be almost spherical or oval. Vacuoles containing dense material were identified as lysosomal residual bodies (Plate. 3.1a), many were spherical but some had an oval shape. The residual bodies were frequent in the middle and basal regions. In some digestive cells the nucleus was oval but in others it had an irregular form adjusted to fit in the free space among the heterolysosomes and their residual bodies

Digestive cells store glycogen granules (and lipid droplets (Plate:3.1d), but large variations in the amounts of these substances were detected between cells.

Golgi stacks were frequently found, each one formed by 4 or 5 cisternae. Most cisternae showed dilated regions filled with an electron dense material in which a pattern of oblique dark lines could be recognized at high magnification .(3.1d).Cells with a single large vacuole were also observed with the electron microscope. In these cells a spherical dense mass was usually found within the vacuole .

Several mitochondria, Golgi stacks, endoplasmic reticulum cisternae, a few peroxisomes and lipid droplets were present in the extremely thin layer of cytoplasm surrounding the vacuole. A flattened nucleus was also localized in the cell periphery Rare images showed cells with a single large vacuole filled with a homogeneous material which seemed about to be extruded into the lumen of the digestive diverticula. A thin layer of cytoplasm with a very high electron density surrounded the vacuole and some mitochondria were the only organelles that could be recognized in these cells that appear to be degenerating (Plate:3.13 a-d).

Digestive Diverticula

The digestive gland of bivalve molluscs is a paired gland consisting of numerous blind ending tubules, the epithelium of which is composed of two cell types, the basophilic cells and the digestive cells, the fine structure of which has already been described (Yonge 1926; Sumner 1966; Owen 1970, 1972; Pal 1972). The basophilic cells possess a well-developed granular endoplasmic reticulum and a cup-shaped Golgi zone above the nucleus that produces membrane-bound vesicles, probably secretory (Sumner 1966; McQuiston 1969; Owen 1973), located mainly in the apical cell part (Owen 1972). Various functions have been attributed to the basophilic cells, although their nature and exact role are uncertain (Owen 1970, 1972; Bush 1986). On the other hand, the digestive cells contain a well-developed lysosomal vacuolar system (De Duve and Wattiaux 1966) that consists of small coated and uncoated tubulovesicular endosomes, irregularly shaped endolysosomes, large heterolysosomes, and

morphologically heterogeneous residual bodies (Cajaraville *et al.* 1995), where intracellular digestion takes place (Owen 1972; Morton 1983). The heterolysosomes of the lysosomal vacuolar system are usually very large vesicles, up to 10 nm, located in the apical or in the mid region of the digestive cells, containing either evenly distributed, finely granular, moderately electron-dense material or clumps of electron-dense material. On the other hand, the residual bodies are smaller lysosomes, up to 3 nm, show an electron-lucid halo and a granular matrix clumped in circles or rings in the center of them. The residual bodies together with the heterolysosomes are involved in the endocytotic uptake and digestion of nutrients in the digestive cells and may appear in different portions of the cell depending on the stage of digestion (Owen 1972; Morton 1983; Cajaraville *et al.* 1995).

Basophilic cells

Basophilic cells (Plate.3.1a) contain a well developed granular endoplasmic reticulum and a cup-shaped Golgi zone above the nucleus that produces membrane bound secretory granules usually located in the apical cytoplasm (Plate 3.1d). Most of the latter granules, depending on the section, present more or less extended electron-dense intra-granular areas or cores.

In some instances, these secretory granules are seen in a close proximity to the microvillous border just prior to their membranes being fused with the plasma membrane and their content secreted into the tubule lumen .The endocytic canal system is not very well-developed as in the digestive cells. Nevertheless, a prominent endocytic activity is evidenced by the formation of pits, coated on their cytoplasmic face at the base of the microvillus border of basophilic cells.

Digestive cells

In the digestive cells (Figure 2a), the elements of a welldeveloped lysosomal vacuolar system, such as heterolysosomes and residual bodies are apparent. Also

evident is the Golgi complex, located in the supranuclear portion .it is cup-shaped and displays characteristic distended regions located in the periphery or the center of the Golgi stacks (not shown). A condensed material, less regularly arranged, is often localized in vesicles located in the apical cell part .sometimes in a close proximity to a Golgi complex. In some cases, elongated membranous structures are found near the apical cell part and arranged parallel to the lateral plasma membranes. Inside the elongated membranous structures, clumps of electron-dense material, probably similar to that found inside the peripheral regions of the Golgi complex and the apical vesicles described above, are localized.

The endocytic canal system, in the apical cytoplasm of the digestive cells, branches through the cytoplasm and in section has an appearance of short, tubular profiles and vesicles .

Gill

The gills consist of various columnar epithelial, squamous endothelial, and mucous cells (Sunila 1986; Cajaraville *et al.* 1990–1991) and deal with gas exchange, excretion, osmoregulation, transport of food material, formation of a protective coat and lubrication of the surface of the epithelium (Cajaraville *et al.* 1990–1991).(Plates:3.2c-d).Plate((3.14a-d) describe in detail about gill epithelium exposed PAHs.WAF treated mussel tissues.Ciliary axonemes appearance occurred in gills of Perna *indica* (Plate3.3a-d)

Election micrograph of the foot tissue of *Perna viridis* exposed to 2ppm WAF of BHC changes was described in (Plate:3.5a-d).

Adductor muscle structural changes were given in (Plates:3.4a,3.6a-d and 3.10).Degenerative changes in the mantle tissue was in (Plates.3.7a-d and 3.9a-d). Autophagic vacuole are clearly visible in exposed mantle tissues (Plates:3.4c-d).

Pedal disc tissue also showed certain degenerative changes like appearance of electron dense materials (Plate:3.11a-d).Pedal disc groove is visible in (Plate 3.4b).

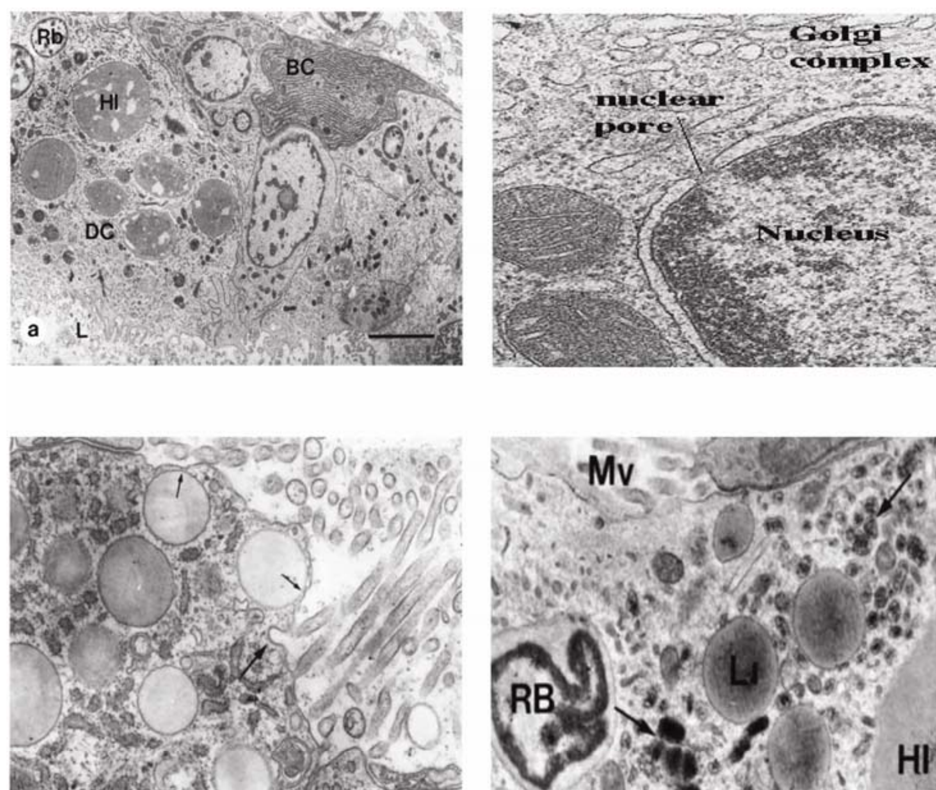


Plate 3.1.a) General ultra structural aspect of a digestive tubule of *P. viridis* showing digestive cells with heterolysosomes (Hl), residual bodies (Rb), and basophilic cells (BC) possessing a well developed granular endoplasmic reticulum:; l, digestive tubule lumen. b)Normal structure of digestive diverticula of *Perna* viridis c) Secretory granules in the apical region of a basophilic cell. Two of them are just prior to their membranes being fused with the plasma membrane (small arrows) and their content secreted into the tubule lumen. In the apical membrane, note the formation of a coated endocytotic vesicle (arrow) d)Electron-dense material is found in small vesicles (arrows) located in the apical part of a digestive cell. Hl:heterolysosome, Li: Lipid inclusion, Mv: microvillous border, RB: residual body 21,000×.

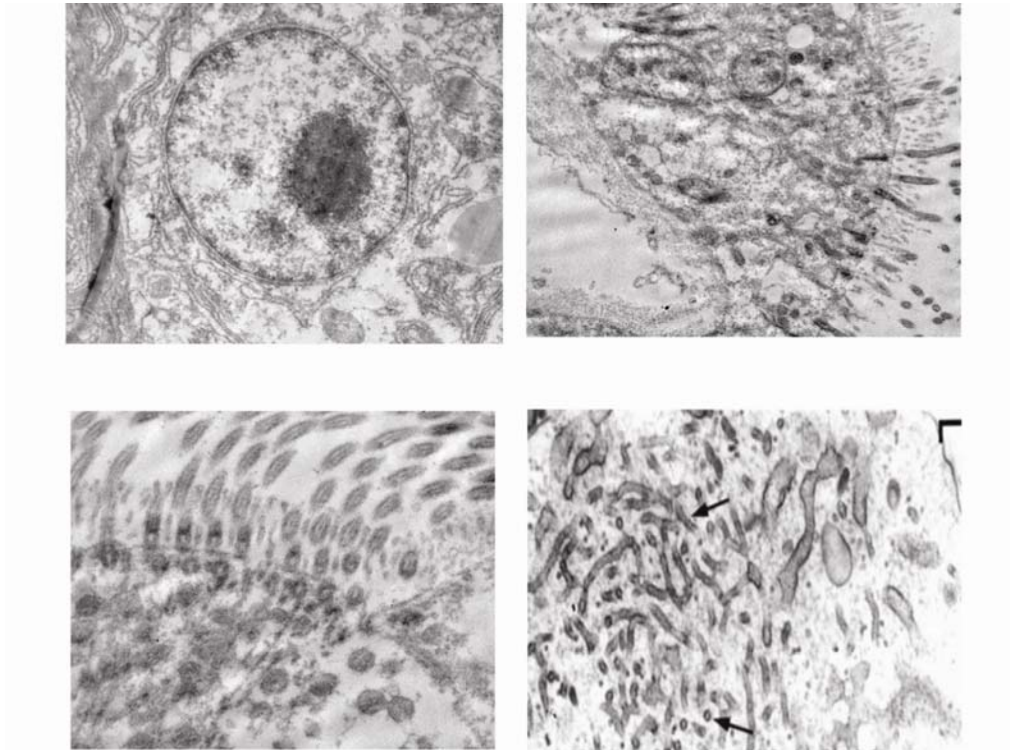


Plate 3.2: a)*Perna indica* Hepatopancreas control b)Electron microscopic view of ciliated epithelial cells of gill filament showing the typical structure; dense bodies(DB) are interspersed throughout the cytoplasm (Ci cilia, Mi mitochondria; Nu nucleus; Go golgi apparatus).c) *Perna indica* gill 1 ppm LDO endocytic vesicle with electron dense content were abundant (d) In the apical cytoplasm of a digestive cell, near the tubule lumen (L), the endocytic canal system, comprising of short tubules and vesicles (arrows), is prominent

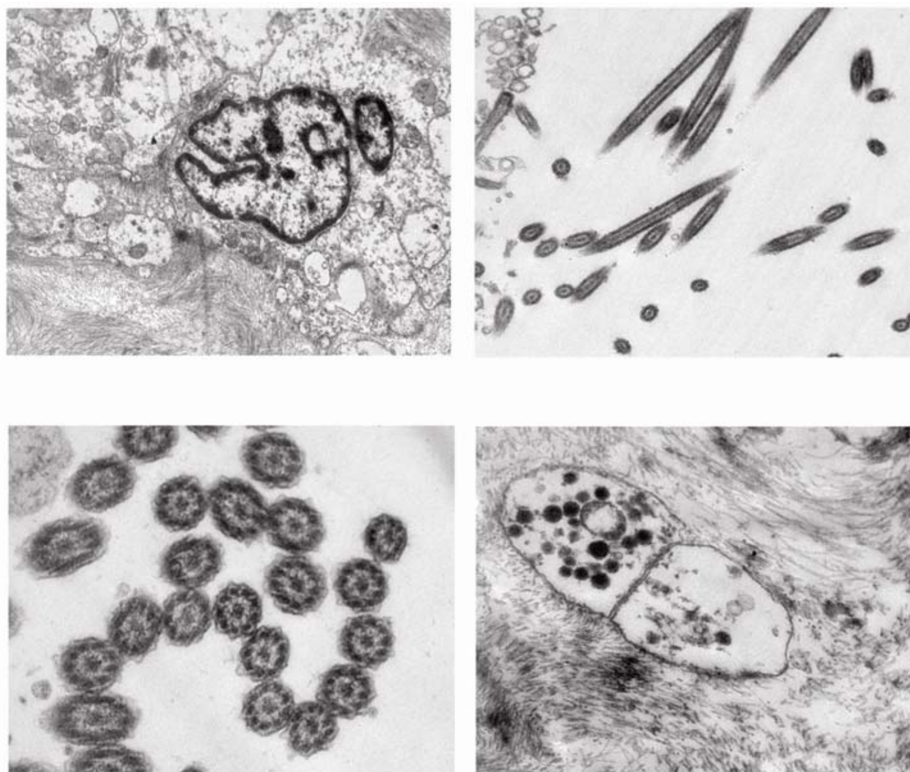


Plate 3.3.a) *Perna viridis* 5 ppm BHC WAF:The irregularly shaped nucleus contains a prominent nucleolus b) Damaged microvillae and cilia of *Perna indica* c) *Perna indica* gill treated with 0.4 ppm LDO These ciliary axonemes from gill of Perna indica d) Digestive cell of Perna indica treated with 5 ppm BHC WAF showing fusion of lysosomes (arrow). H, hetrolysosomes; and filled with electron dense Materials

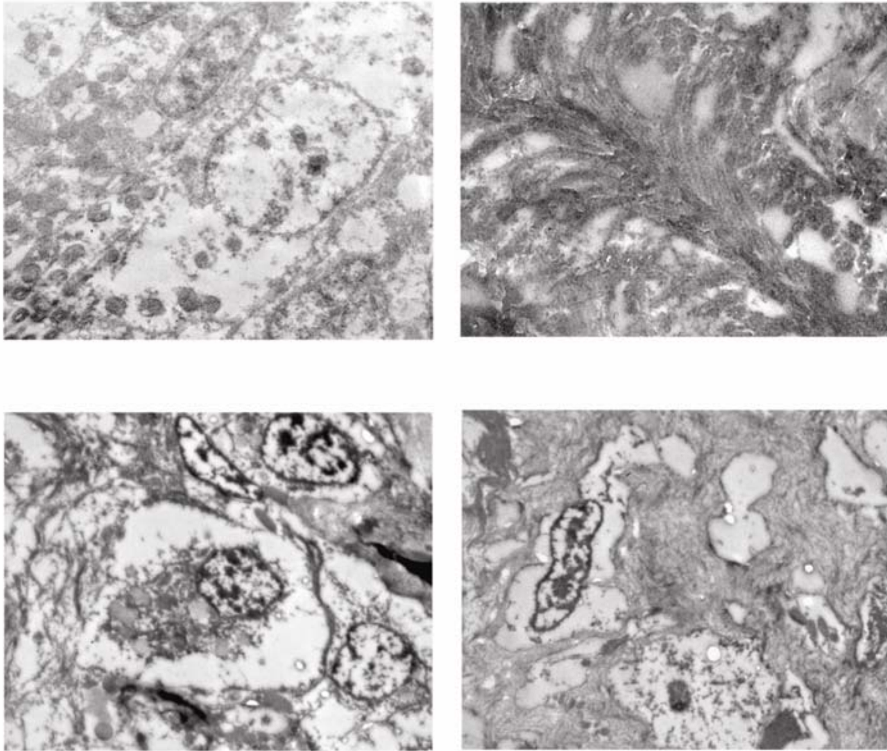


Plate 3.4 :.a) *Perna iviridis* Adductor Muscle 2 ppm LDO

b) *Perna viridis* Pedal disc

c) *Perna indica* 3.5 ppm LDO exposed mantle

d) *Perna indica:* mantle:treated with 2 ppm BHC WAF showing autophagic vacuole containing partially degraded mitochondria

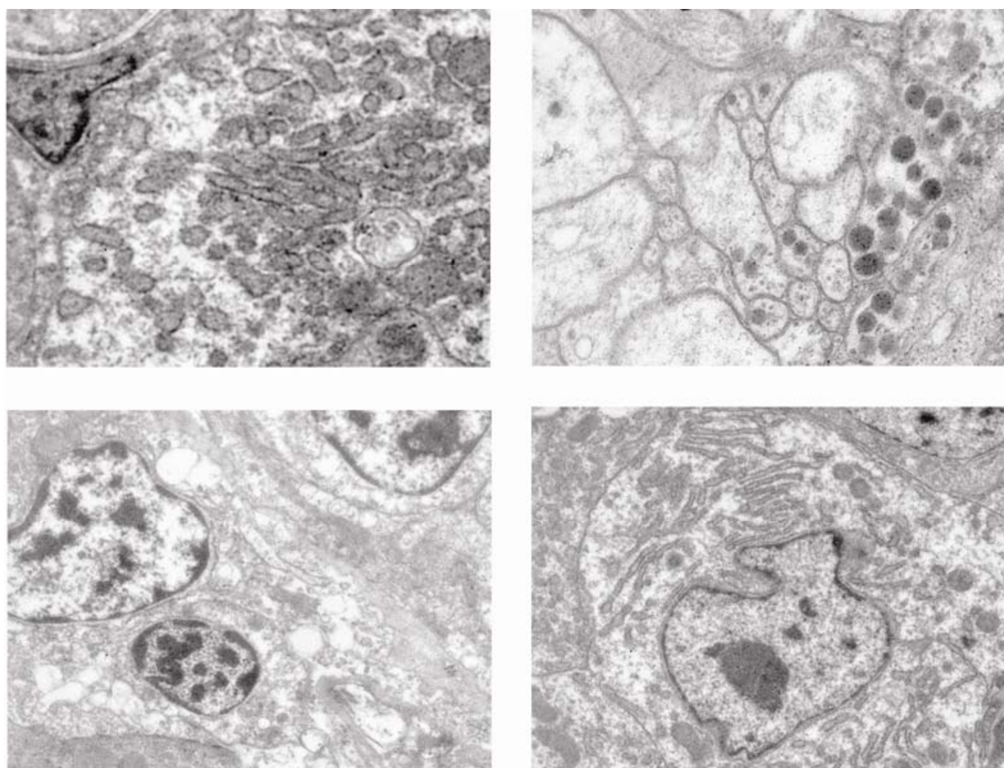


Plate 3.5: Election micrograph of the foot tissue of *Perna viridis* exposed to 2ppm WAF of BHC showing a) Patchy distribution of blebs b) fusion of lysosome c) Residual bodies of mussel showing a dense appearance and granular appearance respectively (scale bar 1μm)

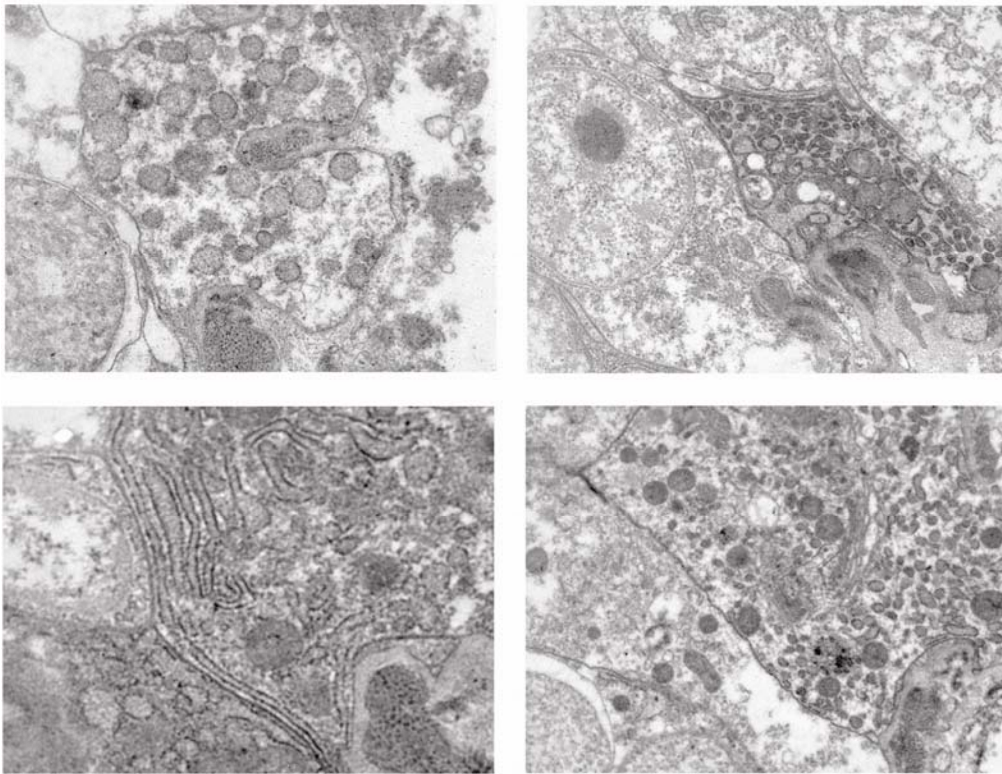


Plate 3.6: Adductor muscle tissue of *Perna viridis* exposed to 5 ppm BHC showing a – b) moderately dense, flocculent substances and dense irregular inclusions in lysosomes c) distorted endoplasmic reticulum d) localization of lipid droplets. (scale bar 2μm)

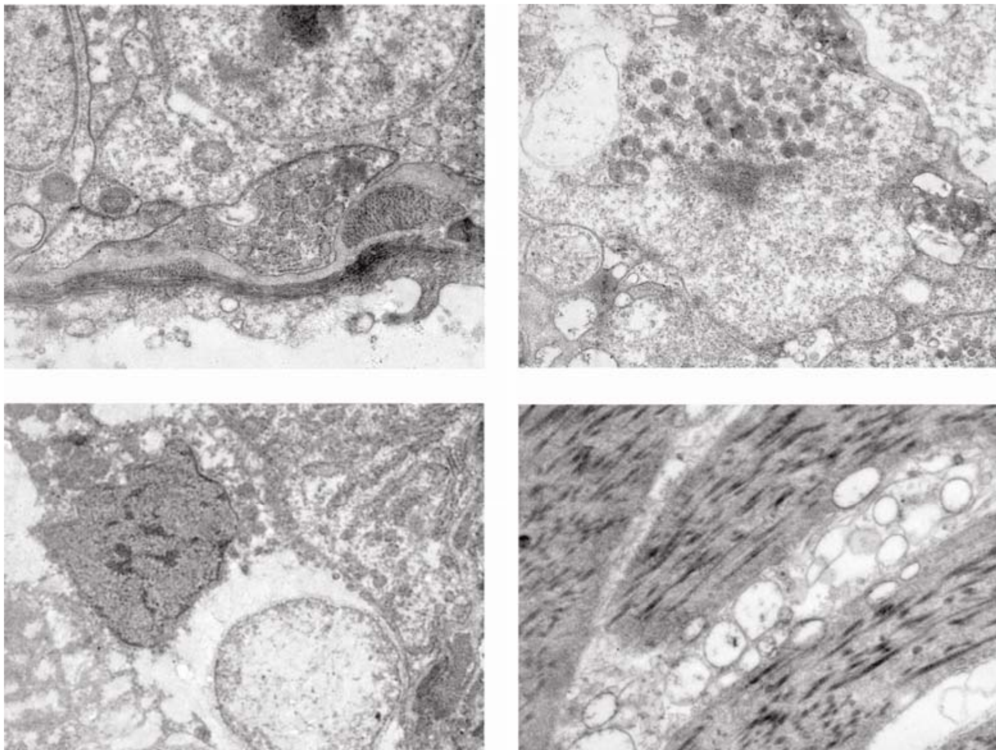


Plate 3.7: Mantle tissue of *Perna viridis* 2ppm WAF BHC exposed animals a) poor development of epithelium b) abundant lipid droplets c) digestive tubule showing sloughed off materials from the cell d) vacuole formation and fusion of cells.

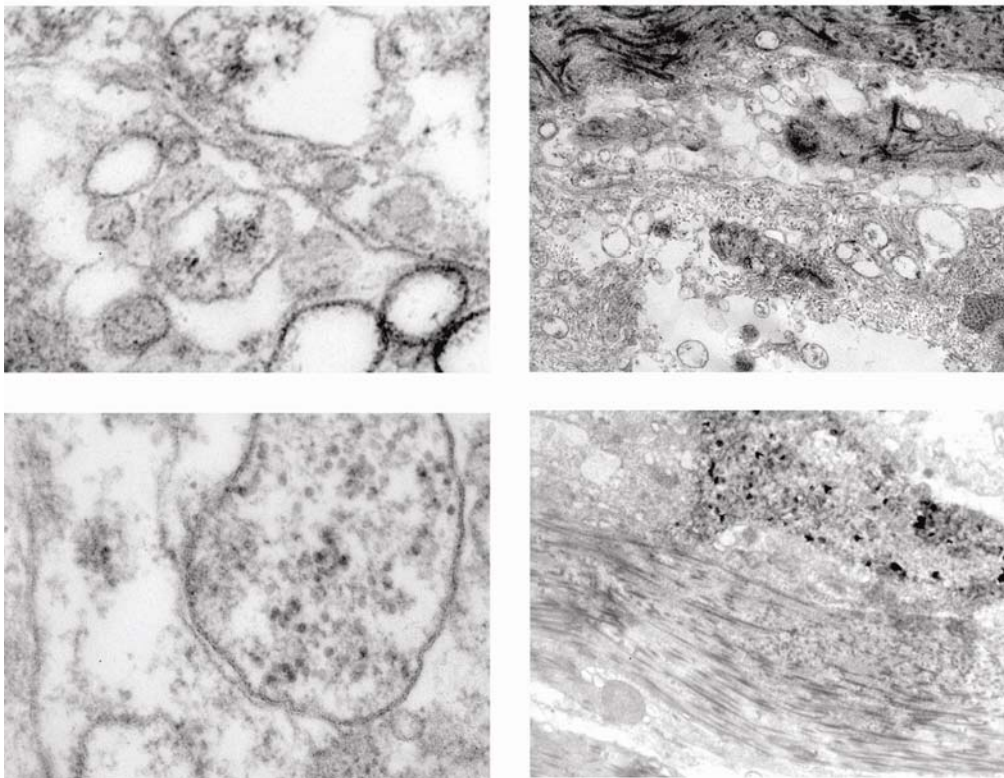


Plate 3.8 Electon micrograph of digestive cell of *p.indica* showing a) fusion of lysosomes b) Endoplasmic reticulum and mitochondria containing electo dense material c) phagolysosomes oval in shape d) electron dense material in mitochondria.

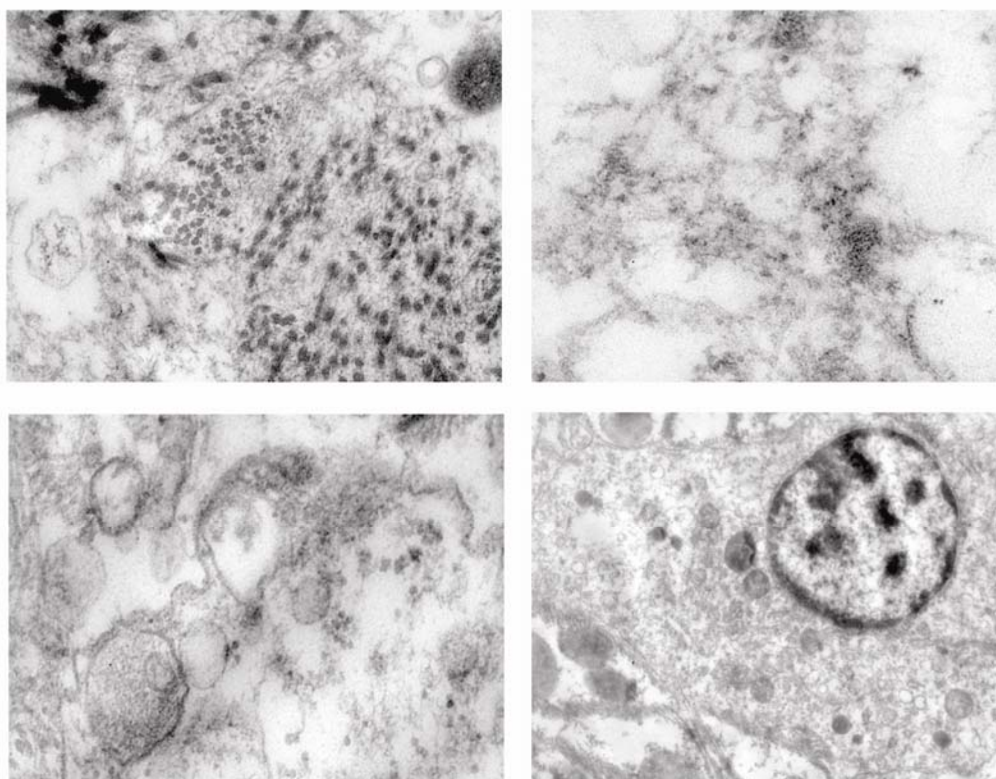


Plate 3.9 Electron micrographs of mantle of *Perna viridis* exposed to 5ppm WAF of BHC a) Group of extracellular dense granules b) marked degenerative changes are apparent c) Numerous vacuolated and degenerating mitochondria are visible in the cells d) appearance of electron dense material in the cell. (scale bar 2 μm)

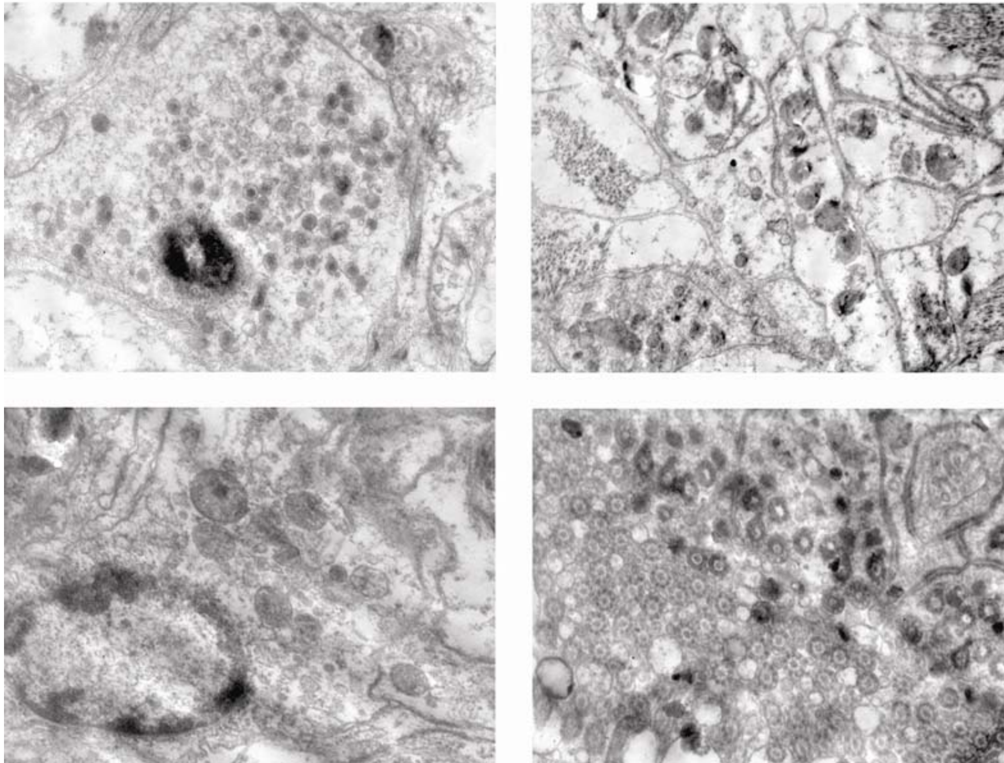


Plate 3.10 : Adductor muscle of *Perna viridis* exposed to 8ppm of BHC WAF a) more electron dense material of a heterolysosomes condensed towards redidual body b) aggregation of flattened vesicles with the residual vacuole of an F/B cell c) Plasma membrane of the microvilli specialized to form hemidesmosomes at the apex of an F/B cell.d) dialated cell enter into the plasma membrane (scale bar 2μm)

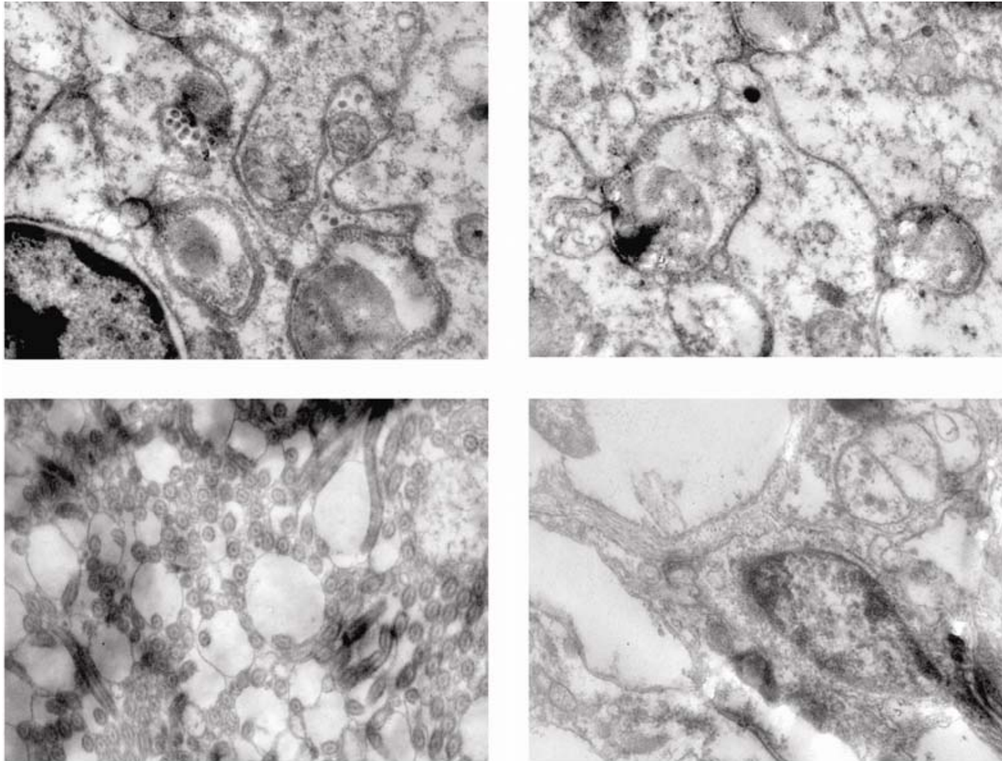


Plate 3.11 Electron micrograph of the apical region of a pedal disc cell showing a) apocrine surface activity with granular endoplasmic reticulum; golgi vesicles b) cells in degenerative condition c) basal region of pedal disc cell Note the much folded basal region with cytoplasmic processes and endocytotic vesicles d) structure of pedal groove filled with electron dense materials. (scale bar 2μm)

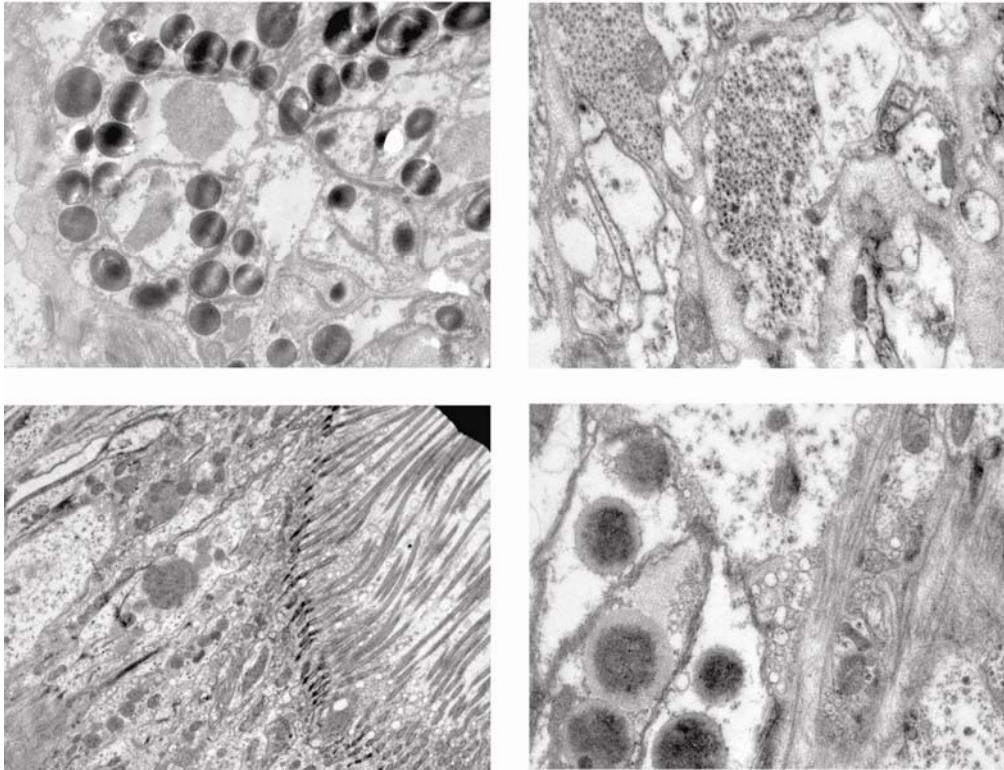


Plate 3.12 : Electron micrograph of digestive diverticula of *P.indica* exposed to 5ppm BHC WAF showing a) lipid droplet accumulation b) The close association between golgi redie , endocytotic vesicle containing fibrous material c) Food particles are apparently being directed between the microvilli d) appearance of irregular resides within the residual vacuole. (scale bar 2 μm)

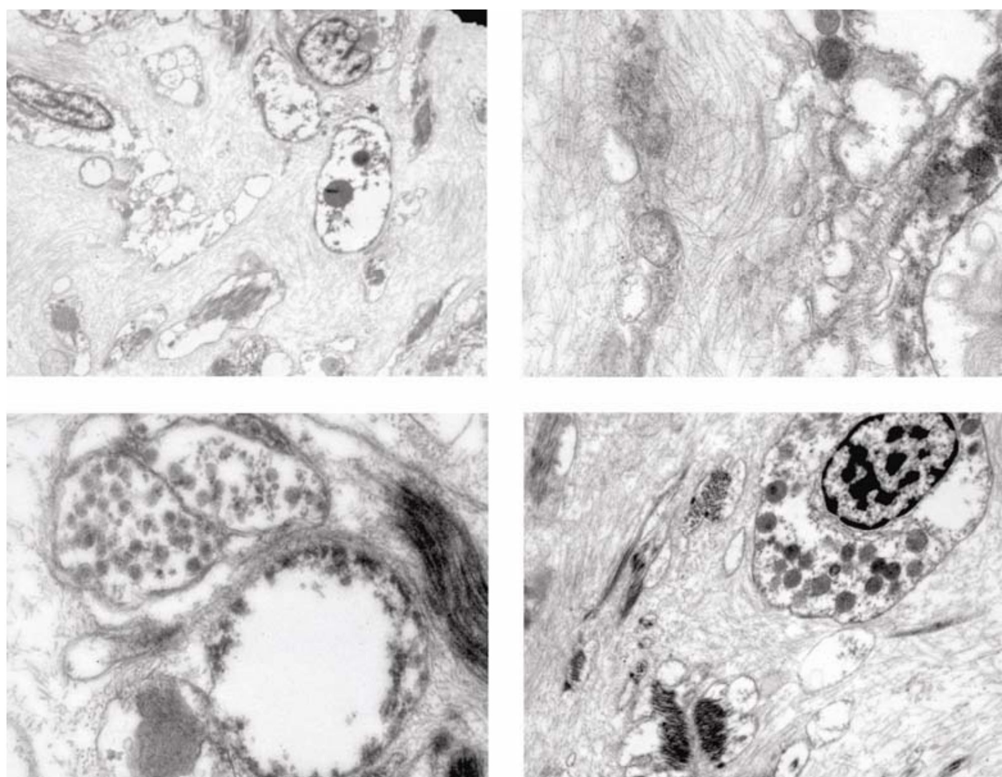


Plate 3.13: Digestive cell of *Perna viridis* exposed to 5ppm of BHC WAF showing a) degenerative mitochondria, loss of material from lysosomes b) digestive tubule showing localization of lipid droplets c) apical region of an F cell in the hepatopancrens. The nuscleus is not visible in this section. d) Residual vacuole of a Bcell in the lumen of the heptopancreas filled with electron dense material. (scale bar 2μm)

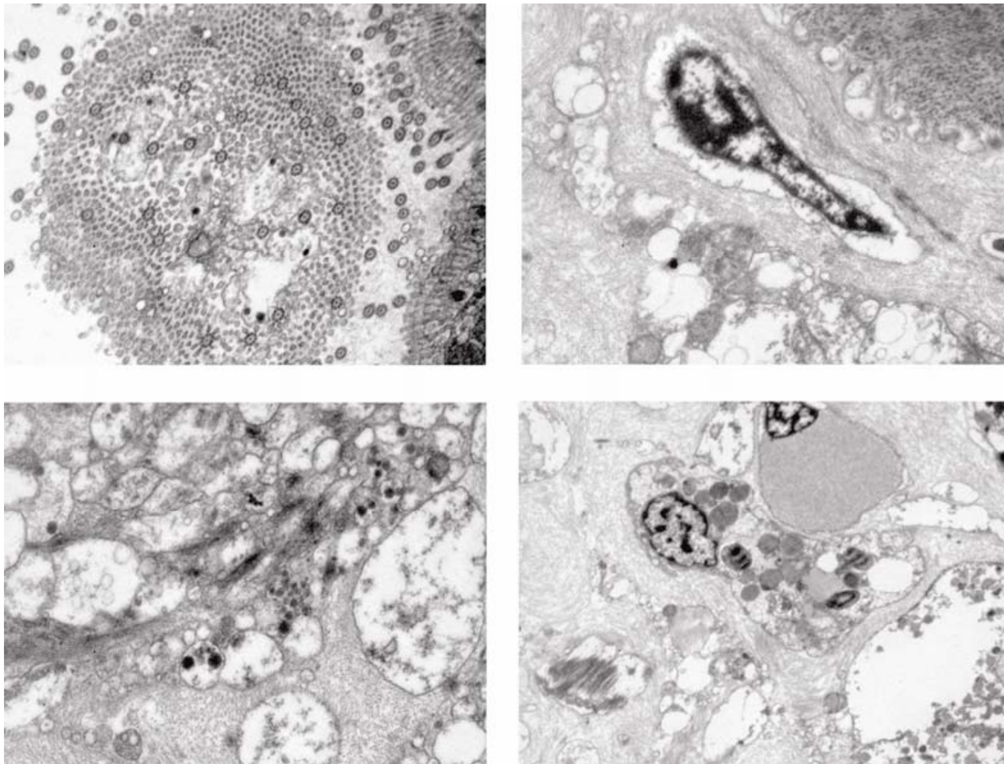


Plate 3.14 a) : Transmission electron micrograph of the distal regions of several cells of the gill epithelium of P.indica exposed to 3.5ppm LDO WAF showing a) sloughed cell fragment b) a membrance bound cytoplasmic protrusion c) Numerous cytoplasmic vacuoles d) Nuclear vacuole (scale bar 1 μm)

3.4 Discussion

Earlier transmission electron microscopic analyses (TEM) of bivalve gill filaments have shown that cilia projecting from various cell types have a unique morphology (Good et al., 1990; Stephens and Good, 1990; Gregory et al., 1996). In the case of *P. perna*, frontal cilia have a well-defined neck while cilia projecting from lateral squamous cells do not (Gregory *et al.*, 1996). It was considered possible that a careful examination of abnormal lateral cilia may enable the cell type to be determined. Unfortunately, the ``undergrowth'' of lateral cilia made it impossible to either conclusively prove or disprove the presence of a cilial neck.

While microvillous swelling, cell necrosis and other pathomorphological alterations might be expected as a consequence of toxic insult, it is more difficult to explain the reason for cilial hyperplasia in lateral cells. Under normal conditions, the few cilia projecting from lateral squamous cells probably help with the circulation of oxygenated water to the epithelium. We postulate that extended exposure to PAHs may deleteriously affect respiration. If this is the case, then the observed increase in lateral cilia may be a response by the animal to increase flow of oxygenated water over the epithelium thereby enhancing the rate of respiration.

The digestive gland of bivalve molluscs is comprised mainly of blind-ending digestive tubules, the epithelium of which is composed of two major cell types: the columnar digestive cell and the pyramidal basophilic cell (Owen 1972, Morton 1983).The digestive cells are subject to cyclical changes and contain a well-developed lysosomal vacuolar system dealing with the uptake and the intra-cellular digestion of endocytosed substances deriving from the extra-cellular digestion of food particles in the stomach (Owen 1972, Morton 1983, Cajaraville *et al.* 1995, Lobo-da-Cunha 2000). The basophilic cells of molluscs are considered mainly as enzyme-secreting cells,

since the presence of enzymes in their secretory granules has recently been reported (Lobo-da-Cunha 1999).

Basophilic cells

The above-mentioned results, in accordance to other reports show that the basophilic cells are responsible for the secretion of digestive enzymes that undertake the extracellular digestion of food particles (Owen 1970, Morton 1983, Henry *et al.* 1991, Lobo-da-Cunha 1999). The secreting nature of basophilic cells of molluscs is partly based on their morphological similarity with the enzyme-secreting cell of the mammalian exocrine pancreas (Owen 1970, 1972).

The electron microscope results in the present study showing granules of the basophilic cells very close to the apical cell membrane, just before their membranes fuse with the plasma membrane and their content is secreted into the tubule lumen, constitutes a further indication of the secretory nature of these granules.

Digestive cells

The Golgi complex of the digestive cells of bivalves is less orderly arranged compared to that in other cell types and commonly displays swollen regions located either in the peripheral or in the central portion of the stacks (Robledo and Cajaraville 1996). These regions contain electron-dense material with characteristic tubular or filamentous substructure, with different degree of condensation (Pal 1972, Owen 1973, Robledo and Cajaraville 1996). Concerning the chemical nature of the electron-dense material inside the tubulofilamentous structures of the Golgi complex observed in the present study. The elongated structures containing similar tubulo-filamentous material could belong to (1) the cisternae of the rough endoplasmic reticulum that has lost most of the ribosomes artefactually or (2) could belong to smooth endoplasmic reticulum or even to the elements of the endosomal system.

The canal system observed in the apical part of the digestive cells of *P. viridis* and *P. indica* is also reported in *M. edulis* (Owen 1972), *Nucella* (Dimitriadis and Andrews 2000), *Risoa* (Wigham 1976), *Lasaea* (McQuiston 1969) and *Cardium* (Owen 1970). The canal system has been regarded as the compartment where the endocytosed material enters the lysosomal pathway (Lloyd 1996). In *Perna*, as well as in the above-mentioned molluscs, no connection of the canal system with the apical membrane is reported. In *Patella vulgata*, an association of the tubules of the canal system with heterolysosomes was reported (Bush 1986). Hypotheses about how pinocytotic vesicles can empty their contents indirectly (Owen 1972) or directly (McQuiston 1969, Wigham 1976) into the secondary lysosomes have been stated. In addition, Bush (1986) suggests a temporary connection of the canal system to the lumen, to establish a concentration gradient along the canal/heterophagosome complex.

In molluscs, the products of extracellular digestion found in the lumen of digestive diverticula are collected by digestive cells for further breakdown in the heterolysosomes. In some species digestive cells can collect relatively large food particles by phagocytosis (Morton, 1979), but in others extracellular digestion is more complete and only dissolved substances are captured by small endocytic vesicles (McLean, 1971; Walker, 1972). Many endocytic vesicles filled with electron dense material were observed in the apical region of *P. viridis* digestive cells. Probably, these vesicles contained the products of extracellular digestion that would be transferred to the heterolysosomes of digestive cells, to complete the digestive process. In some digestive cells the number of endocytic vesicles was very high, indicating a very intense endocytic activity, but in others only a few vesicles could be found, suggesting the existence of stages with low endocytic activity. that would be transferred to the heterolysosomes of digestive cells, to complete the digestive process. In some digestive cells the number of endocytic vesicles was very high, indicating a very

intense endocytic activity, but in others only a few vesicles could be found, suggesting the existence of stages with low endocytic activity.

Several studies have proved that shortly after endocytosis the substances captured by endocytic vesicles will appear in early endosomes; sometime later those substances will be present in late endosomes (or prelysosomal compartment) and finally they will be found in the matured heterolysosomes that contain the largest amounts of acid hydrolases (Thilo *et al.,* 1995; Tjelle *et al.*, 1996). Electronlucent structures limited by a single membrane, containing only small amounts of internal material and practically devoid of acid hydrolases were identified as early endosomes (Tjelle *et al.*, 1996). Structures with identical characteristics were observed in the apical region of *A. depilans* digestive cells and probably correspond to early endosomes. The heterolysosomes and their residual bodies observed in the digestive cells of *P.viridis* were very similar to those described in the hepatopancreas of other mollusc species (Owen, 1972a; Henry et al., 1991; Rebecchi *et al.*, 1996). In the basophilic cells of *P.viridis*, bundles of tubular structures were observed inside some cisternae of endoplasmic reticulum (Lobo-da-Cunha, 1999), but these tubular structures were never found in the digestive cells.

Golgi stacks with dilated cisternae containing dense substances were reported in digestive cells of bivalves (Owen, 1973; Robledo and Cajaraville, 1996). In the bivalve *Nucula sulcata*, the dilated regions of golgi cisternae contained electron-dense material forming parallel thin lines (Owen, 1973), and a very similar pattern was observed in the golgi cisternae of *P.viridis* digestive cells.

The excretory cells described in pulmonate gastropods (Sumner, 1965; Walker, 1970) are morphologically very similar to the cells with a large vacuole as that of *P. viridis*. Some authors considered that the excretory cells were degenerated calcium cells (Thiele, 1953; Sumner, 1965), but others view them as a final stage in the cycle of

digestive cells (Walker, 1970; Morton, 1979; Almendros and Porcel, 1992). Other authors did not recognize intermediate stages and concluded that excretory cells were an independent cell type not derived from digestive or calcium cells (Dimitriadis and Hondros, 1992). In *P. viridis* these cells seem to be an advanced stage of digestive cell maturation. This hypothesis is supported by the observation of cells in an intermediate stage, showing a partial fusion of the lysosomes.

According to the observations made in semithin and ultrathin sections of *P.viridis* hepatopancreas, the single large vacuole seems to result from the fusion of all heterolysosomes and residual bodies of a digestive cell. In the end, the undigested substances accumulated in the large vacuole will be excreted to the lumen of digestive diverticula, and after that the cell will probably die. Some basophilic cells of *A. depilans* possess a large number of vacuoles filling a great part of the cell volume, but they maintain a characteristic pyramidal shape, some apical secretion granules and a large oval nucleus (Lobo-da- Cunha, 1999). Because of these morphological aspects, vacuolated basophilic cells seem not to be precursors of the excretory type cells.

Ultrastructural examination of the digestive cells revealed that secondary lysosomes of mussels were wider due to the enhancement of lysosomal fusion.

This role of the lysosomal system may reflect a general metabolic pattern for the detoxification. However, other molluscs with a lysosomal system less developed than mussels could be limited to detoxify cadmium via binding to insoluble compounds (Marigomez *et al.,* 2002).

After progressed PAHs-exposures the digestive cells become vacuolated and reduced in number, while basophilic cells become hypertrophied and are relatively more numerous (Cajaraville *et al.* 1990a, Marig´omez *et al.* 1990b, 1998).The functional equivalence of basophilic cells with calcium cells of terrestrial gastropods (Marigomez *et al.* 1995), which have been reported to accumulate many xenobiotics

(Marigomez *et al.* 1986; Reico *et al.*1988), could account for our suggestion.In the present study, all visualized PAHs were localized mainly in the frontal epithelium, *i.e.* frontal, laterofrontal, lateral, and postlateral gill epithelial cells and endothelial cells (see Sunila 1986 andWright *et al.* 1987. Since the gill epithelium of many molluscs and *Perna spp* are comprised of various cell types (Nuwayhid *et al.*1978; Wright *et al.* 1987; Cajaraville *et al.* 1990–1991), differences in the PAHs deposition probably reflect differences in the physiology of the cells along the gill filament.

In the digestive cells, a morphological alteration caused by the PAHs treatment was the aggregation of complex structures containing residual bodies, enclosed by a single membrane. Membrane fusion is a possible mechanism for the formation of these structures, since PAHs interact with biomembranes leading to a decrease in membrane fluidity (Webb 1979). Similar structures named "compound residual bodies" were commonly found in the digestive cells of some bivalves (Owen 1972), however, their large number observed in treated mussels could probably be a result of PAHs exposure.

Studies in *M. galloprovincialis* suggested that the basophilic cells also undergo important alterations under stress conditions (Cajaraville *et al.* 1990). In the present study, fragmentation or vacuolization of the rough endoplasmic reticulum of basophilic cells was noted after BHCand LDO treatment. Since this histopathological alteration observed in our experiments has also been reported in basophilic cells after petroleum hydrocarbon exposure (Carles *et al.* 1986; Lowe 1988), it probably represents a general and not a specific stress response. So a suggestion of the present study, is the use of the vacuolization of the rough endoplasmic reticulum of the basophilic cells, as a potential general stress indices in marine pollution monitoring studies (Cajaraville *et al.* 1990; Soto and Marigomez 1997a).

Increased number and size of the dense granules in the basophilic cells compared to that of controls was observed. The increase in the number and size of these granules probably represents an increase in the enzyme activity of the basophilic cells after PAHs treatment. The latter hypothesis is supported by Marigomez *et al.* (1990b), who reported that the increase in the number of the secretory granules of the basophilic cells after xenobiotic exposure may be necessary to augment enzyme secretion for extracellular digestion. Thus, this response may be a defense mechanism to compensate the disturbance of intracellular digestion after PAHs exposure.

The main gill lesions observed in some of the treated mussels were fusion of the lateral epithelium of the filaments in the BHC and LDO-treated mussels. Similar abnormalities have been described in the gills of *M. edulis* either after an experimental exposure to Cu or after field sampling (Sunila 1986, 1987). Sunila (1986) observed empty intercellular spaces and detachment of the abfrontal cells after Cu and Ag treatment in *M. edulis.* Detachment of the cells from the chitinous rod may be a sign of enhanced gill regeneration.Despite the fact that regeneration of the gill epithelium is rather a normal cyclic activity, the gill lesions are probably related to regeneration errors of the gill (Sunila 1986) .

In conclusion, by using light and electron microscope observations , the present study provided useful information on PAHs sequestration by the cells of the digestive gland and the gills. When viewed by TEM, intracellular dense granules were observed distributed in the cytoplasm of the digestive gland cells. In addition, these granules are close to endoplasmic reticulum, forming clusters in association with lipid droplets.

Chapman *et al* (1996) related the molluscs capacity in maintain high PAHs concentration in their tissues within discrete ranges. These organisms may accumulate large amounts of PAHs in their tissues mitigating toxic effects by sequestering then in granular form.

The digestive gland of *P.viridis* and *P. indica* and other marine bivalves consists of numerous blind-ending tubules, the epithelium of which is composed of digestive and basophilic cells. In the digestive cells, the existence of a well-developed endocytic lysosomal vacuolar system, mainly consisting of heterophagosomes, heterolysosomes and residual bodies, evidences intra-cellular digestion processes; in addition, the lysosomal content is characterized by the presence of acid hydrolases dealing with the intra-cellular digestion of nutrients. In the abovementioned studies, there are few data concerning the role of basophilic cells, which display a highly developed rough endoplasmic reticulum and numerous secretory granules, in enzyme-production and secretion.

It may be concluded that *P. indica* and *P.viridis* were similar to other bivalves in that it efficiently accumulates PAHs in its soft tissues. In addition, it would appear that chronic exposure to increased PAHs induces significant morphological changes in its gill tissues. While exposure to other pollutants, either singly or in combination may produce other responses, it is hoped that these data will provide an initial comparative baseline for future studies.

Chapter **4**

STUDIES ON OXIDATIVE STRESS IN *Perna spp.*

Contents

4.1 Introduction
4.2 Materials and methods
4.3 Results
4.4 Discussion

4.1 Introduction

In polluted environments and especially in coastal waters, living organisms are most often exposed to complex mixtures of chemical contaminants, resulting in some degree of acclimation of animals to such bad environmental conditions. Because of the diversity and variability of the chemical threat, defence mechanisms exhibit considerable versatility and adaptability. Among the strategies that have been developed by organisms at the cellular level in the course of evolution to protect themselves from the toxic effects of metallic or organic compounds, the major ones are the antioxidant defence systems.

The antioxidant systems protect cells against the deleterious effects of oxyradical generation by maintaining endogenous reactive oxygen species at relatively low levels and attenuating the damages related to their high reactivity. A range of antioxidant defence mechanisms are present in bivalve molluscs, including low molecular weight compounds (Vitamin E, ascorbic acid, reduced glutathione…) and specially adapted enzymes (Winston *et al.*,1991).

Many different mechanisms of toxicity exist for different contaminants, and a single contaminant may effect its toxicity by more than one mechanism (Livingstone *et*

al., 2000). Recently, contaminant-stimulated reactive oxygen species (ROS) production and resultant oxidative stress has been indicated as a mechanism of toxicity in aquatic organisms exposed to pollution (Di Giulio et al., 1995; Kelly *et al*., 1998; Livingstone 2001).

The level of scavenging enzymes has been extensively used as an early warning indicator of marine pollution. Recently, antioxidant enzymes have also been proposed as bioindicators for environmental impact assessment (Livingstone 1991, Winston and Giulio 1991), due to the fact that both metals and certain organic xenobiotics generate oxidative stress (Sies 1991).

Oxidative stress is a consequence of an imbalance between prooxidant and antioxidant status. A variety of aquatic contaminants induce biomarkers of oxidative stress in fish and other aquatic organisms. Reactive oxygen species (ROS) are continuously generated by aerobic metabolism and can damage important biomolecules such as DNA, proteins, and lipids (Halliwell and Gutteridge 1999). Many studies on the toxicological effects of aquatic contaminants have been performed on a variety of bioindicators, specially in fish and bivalve molluscs (Black *et al*., 1996; Cosson 2000; Wilhelm Filho *et al*., 2001; Torres *et al*., 2002).

The free-radical-mediated oxidative stress results in oxidation of membrane lipoproteins, glycoxidation, and oxidation of DNA: subsequently cell death results. Various necrotic factors, proteases, and ROS from damaged cells also attack the adjacent cells, resulting in tissue injury. Furthermore, tissue injury itself has been reported to cause severe oxidative stresses (Halliwell *et al.,* 1999).

The major role of the endogenous production of ROS is an activity of regulation. Indeed, the radicals can interact directly with the molecules containing sulfhydryl groups and thus change their conformation.

Superoxide anion is the first reduction product of O_2. It is a base with the equilibrium with its conjugate acid, the hydroperoxyl radical $HO_2\bullet$, whose pKa is 4.8. In aqueous solution, at neutral or slightly acid pH, $O_2^{\bullet -}$ is a relatively non-reactive species and dismutates to H_2O_2. This reaction either occurs spontaneously or is catalysed by intracellular enzyme SOD.

The superoxide anion radical O_2^- is produced by reduction of molecular oxygen by capture of an electron. This reaction can be spontaneous in aerobic medium. O_2^- is then generated primarily in membranes because of the high solubility of oxygen in hydrophobic medium (Halliwell *et al.,,* 1999). However, it is produced during various reactions. The superoxide anion radical is characterized by a low reactivity. Moreover, it remains limited to the compartment where it was produced. But it is at the origin of the oxidation of lipids. In fact, the deterioration of the membrane structures is carried out by nucleophilic attack between fatty acids and glycerol of phospholipids. O_2^- can act at the same time like an oxidant and a reducer.

The H_2O_2 is produced by the dismutation of the superoxide anion radical. This anion leads spontaneously to H_2O_2 under the conditions of physiological pH. This reaction can be accelerated by action of superoxide dismutases (Fridovich, 1975). The H_2O_2 is moderately reactive but its diffusion is high, having the capacity to cross the membranes. The H_2O_2 holds a significant place among the ROS because it plays the role of intermediate in the production of other reactive radicals.

The hydroperoxyl (ROO·) and alcoxyl (RO·) radicals rise from the peroxidation of the lipids. These radicals allow the propagation gradually of the lipid peroxidation. After degradation, they lead to aldehyde formation organized in three major groups: 2-acetaldehydes (e.g. 2-hexenal), 4-hydroxy-2-acetaldehydes (e.g. 4-hydroxynonenal) and ketoaldehydes (e.g. malondialdehyde)(Uchida, 2003).

The most studied action of ROS is the lipid peroxidation. This reaction is mainly carried out by the hydroxyl radical (Stegeman *et al.*, 1992a; Steinberg, 1997). This process corresponds to reactions in chain.

In a general way, during this reaction, various compounds are produced such malondialdehyde (MDA) and 4-hydroxynonenal (HNE), both able to bind to proteins and to form adducts.

The DNA damages are mainly caused by the hydroxyl radical (OH·). Superoxide anion radical can cause also cuts of DNA and lesions of the bases.

In the normal healthy cell, ROS and pro-oxidant products are detoxified by antioxidant defenses, including low molecular weight free radical scavengers and specific antioxidant enzymes. (Halliwell *et al.,* 1999). The former comprise both water-soluble (e.g. vitamin C, reduced glutathione (GSH), carotenoids) and lipid-soluble (e.g. vitamins A and E) molecules. The antioxidant enzymes include superoxide dismutase (SOD; EC 1.15.1.1 – converts $O_2 \cdot^-$ to H_2O_2), catalase (EC 1.11.1.6 – converts H_2O_2 to water) and glutathione peroxidase (GPX; EC 1.11.1.9 – detoxifies H_2O_2 and organic hydroperoxides utilizing GSH). Thus a balance is though to exist between pro-oxidant production and antioxidant defense, although low levels of oxidative damage, particularly to key biological molecules such as lipid, protein and DNA, are also always present. However, marked increases in ROS production can overcome antioxidant defenses, resulting in increased oxidative damage to macromolecules and alterations in critical cellular processes. The oxidative damage may be spread far from its point of cellular origin by the different ROS and other products of oxidation, resulting in a condition of oxidative stress.

Any process which leads to increases ROS production either directly, or indirectly via organic radical formation or other mechanisms, can potentially result in enhanced oxidative stress and biological damage. (Halliwell *et al.*, 1999).

The first lines of defence against $O_2^{\bullet -}$ and $H_2O_2^-$ mediated injury are antioxidant enzymes: SOD, GPx, and CAT. Superoxide dismutase (SOD), catalse and peroxidases constitute mutually supportive team of defence against ROS. While SOD lowers the steady state level of $\cdot O_2^-$, catalase and peroxidases do the same for H_2O_2. Free radical scavengers (antioxidants) mainly include reduced glutathione (GSH).

Glutathione peroxidase catalyses the reaction of hydroperoxidase with reduced glutathione (GSH) to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide.

The GRd (GRd; EC. 1.8.1.7) is not always recognized as an antioxidant enzyme. It can nevertheless be included in this category because it makes it possible to reduce the oxidized glutathione (GSSG) according to a NADPH-dependent process, and it is thus at the base of the regeneration of reduced GSH necessary to the operation of the GPxs and of many other enzymes of the cell. Balance between GSSG and GSH is capital in the maintanance of cellular homeostasis (Winston and Di Giulio, 1991).

Other enzymes are regarded as having an antioxidant action. It is the case of glutathione S-transferase. Indeed, the glutathione S-transferase presents a peroxidasic activity with respect to organic peroxides and belongs to the group of the Se-independent GPx.

4.2 Materials and Methods

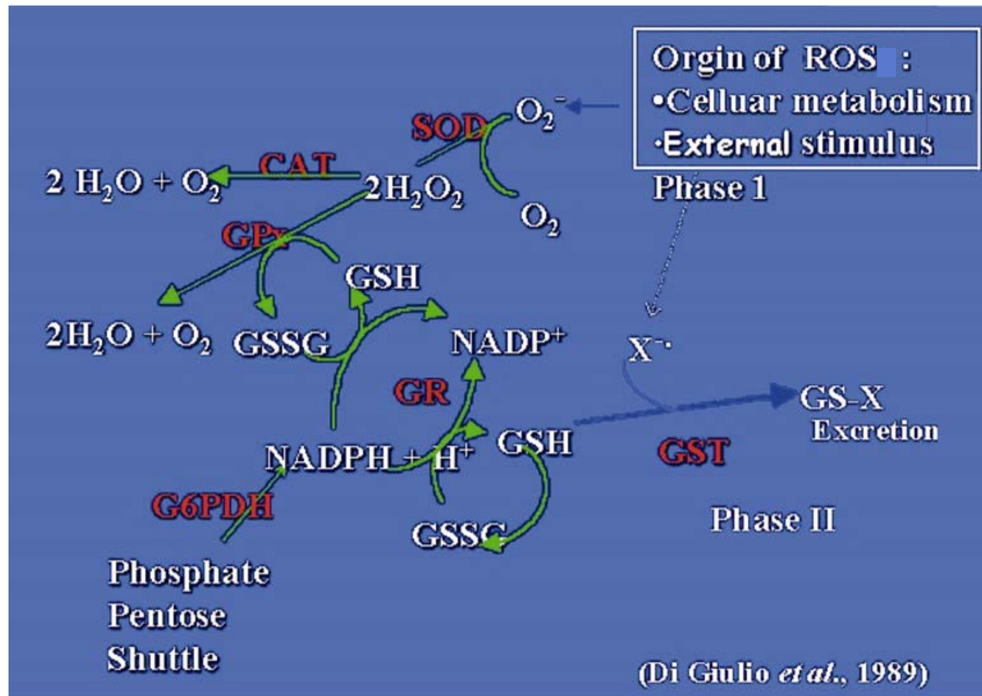
The animals (both *P. viridis* and *P. indica*) were sacrificed after termination of the exposure period of BHC and LDO WAF and their gills, hepatopancreas, adductor muscle tissue, and mantle tissue were dissected out. Enzymatic antioxidants were measured in samples homogenized (1:5 w/v) in 100 mM Tris-HCl buffer pH 8.0, 0.1 mM PMSF, 0.008 trypsin inhibitor units ,0.6 % NaCl, and differential

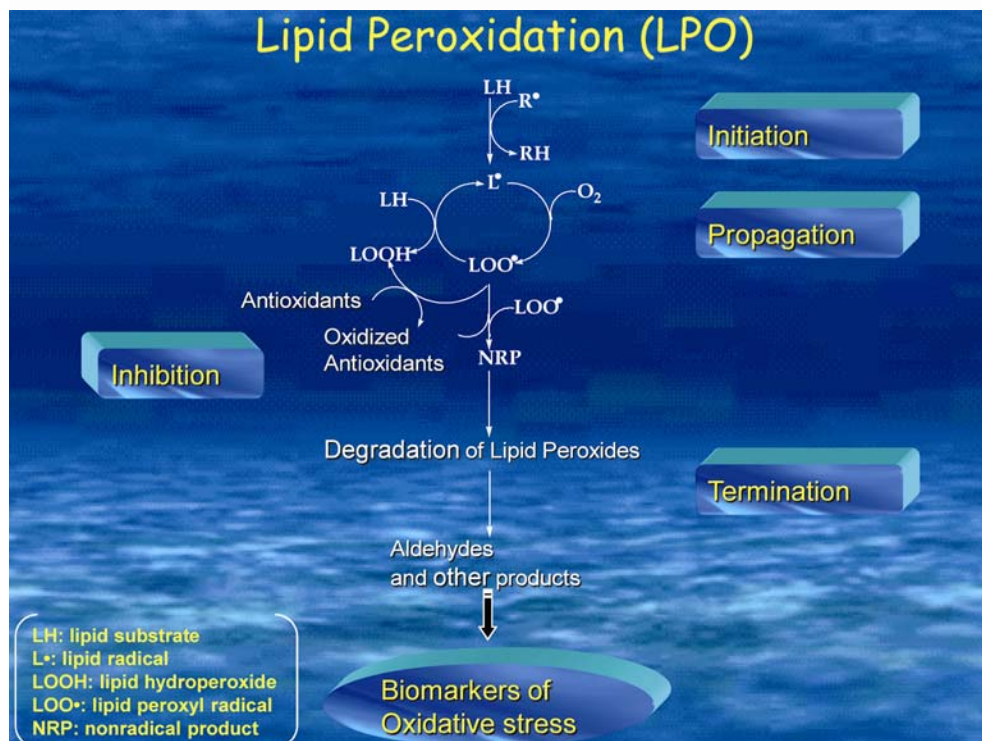
centrifugation was carried out at 4°C to obtain cytosolic fractions. Spectrophotometric measurements were carried out.

- Super oxide dismutase (EC 1.15.1.1) was determined by the method of Mc Cord and Fridovich (1969). One unit of SOD is defined as the amount of enzyme inhibiting 50% the NBT (Nitro blue tetrazolium) per minute per mg protein. Catalase activity (EC.1.11.1.6) was measured by the loss of absorbance due to consumption of H_2O_2 at 340nm according to the method of Aebi (1984). Glutathione peroxidase was analyzed by the method of Rotruck *et al* (1973).Glutathione-S-transferase and Glutathione reductase were estimated by the method of Beutler *et al.,* (1986) and Goldberg (1975) respectively. Total Glutathione was analyzed by the method of Patterson and Lazarrow (1955).
- Lipid peroxidation in tissues were determined by the formation of thiobarbituric acid reactive substances (TBARS) and quantified as MDA equivalents according to the method of Ohkawa *et al.*, (1979).
- Protein was determined by the method of Lowry *et al.,* (1951) using bovine serum albumin as standard.

Statistical Analysis

Data were presented as the mean ± standard deviation. Statistical analysis for all biochemical parameters was carried out using 3-way ANOVA after tested for normality distributions using the Kolomogorov-Smirnov test and homogeneity of variance, complemented by the Levene test. For each experimental exposure 3-way ANOVA were performed to detect the effect of the type of treatment, the duration of treatment and the tissues of treatment. Statistical analyses were carried out with the aid of the Sigmastat 3.5 statistical package.


Orgin of ROS :
•Celluar metabolism
·External stimulus
Phase 1
SOD
O_2^-
CAT
$2 H_2O + O_2$
$2H_2O_2$
O_2
GPx
GSH
$2H_2O + O_2$
GSSG
$NADP^+$
X^-
GR
GS-X
Excretion
$NADPH + H^+$
GSH
GST
G6PDH
GSSG
Phase II
Phosphate
Pentose
Shuttle
(Di Giulio et al., 1989)


Lipid Peroxidation (LPO)
LH
R•
RH
L•
O_2
LH
LOOH
LOO•
Initiation
Propagation
Antioxidants
LOO•
Oxidized
Antioxidants
NRP
Inhibition
Degradation of Lipid Peroxides
Termination
Aldehydes
and other products
LH: lipid substrate
L•: lipid radical
LOOH: lipid hydroperoxide
LOO•: lipid peroxyl radical
NRP: nonradical product
Biomarkers of
Oxidative stress

4.3 Results

The specific activities of SOD, CAT, GSH-Px, GR and GSH were also measured in hepatopancreas, adductor muscle, mantle and gill of *Perna indica* LDO WAF exposed mussels are given in (Table 4.1-4.4) *Perna indica* BHC WAF exposed mussels in(Table4.6-4.9), *Perna viridis* BHC WAF exposed mussels in table (4.11-4.14) *Perna viridis* LDO WAF exposed ones in table (4.16-4.18). GST activities are given in figure 4.1 to 4.16 and 3 way ANOVA in table (4.21 -4.24). The activities of these detoxifying enzymes were highest ($P<0.001$) in the hepatopancreas. This could be related to the fact that the hepatopancreas is the site of multiple oxidative reactions and may, therefore, be the site of maximal free radical generation. The activity of the SOD is high in gill when compared to muscle, indicating an increasing need to destroy O_2^- in gills during respiration.

The statistical significance of the results were tested by ANOVA .Three way ANOVA of *Perna indica* LDO WAF exposed mussels are given in (Table 4.5 and BHC WAF exposed mussels are given in table 4.10. Three way ANOVA of *Perna viridis* LDO WAF exposed mussels are given in Table 4.15 and BHC WAF exposed mussels are given in table 4.20.

The difference in the mean values among the different levels of TISSUES are greater than would be expected by chance after allowing for the effects of differences in PAH CONC and TIME. There is a statistically significant difference ($P = <0.001$). The difference in the mean values among the different levels of PAH CONC are greater than what would be expected by chance after allowing for the effects of differences in TISSUES and TIME. There is a statistically significant difference ($P = <0.001$).

The difference in the mean values among the different levels of TIME are greater than would be expected by chance after allowing for the effects of differences in TISSUES and PAH CONC. There is a statistically significant difference ($P = <0.001$). Lipid peroxidation values were given in table 4.25-4.28.

Table 4.1: Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 0.04 ppm WAF of LDO.

Parameter	Treatment 0.04 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
SOD (units/mg protein)						
Gill	5.69± 0.96	6.59± 0.49	7.42± 0.41	8.18± 1.28	9.54± 0.72	11.65± 1.46
Hepatopancreas	3.62± 0.94	4.57± 1.45	6.83± 0.98	8.64± 0.39	9.61± 0.51	10.54± 0.49
Adductor muscle	4.56± 0.55	5.60± 0.96	6.75± 0.48	7.02± 1.29	10.02± 0.06	12.08± 0.78
Mantle	5.59± 0.35	5.93± 1.34	4.61± 1.24	6.94± 0.53	7.08± 0.44	20.47± 0.33
Catalase (μmol/min/mg protein)						
Hepatopancreas	12.68 ± 0.68	14.51± 1.17	15.49± 1.71	19.28± 1.55	23.48± 1.39	27.43± 1.78
Gill	6.26± 0.97	4.55± 1.42	6.95± 0.78	8.52± 0.95	9.02 ± 0.62	10.22± 0.45
Adductor muscle	4.31± 1.61	5.32± 1.39	8.64± 0.32	11.94± 0.94	12.21± 0.45	13.58 ± 1.53
Mantle	5.32± 1.39	8.42± 1.48	10.32± 1.21	12.68± 0.56	15.41± 1.61	18.81± 1.71
Glutathione peroxidase (μg of GSH/min/mg protein)						
Hepatopancreas	2.75± 1.30	4.62± 0.25	9.65± 0.41	12.84± 0.65	12.96± 0.25	15.32± 0.34
Gill	2.40± 1.25	6.32± 0.62	8.54± 0.32	10.32± 0.54	11.33± 0.47	17.10± 0.22
Adductor muscle	1.69± 0.69	5.34± 0.32	5.67± 0.25	9.66± 1.29	14.65± 1.12	16.54± 0.65
Mantle	2.35± 1.41	5.56± 0.54	6.75± 0.08	8.69± 0.51	9.36± 0.22	12.24± 0.64
Glutathione reductase (GR) (units/mg protein)						
Hepatopancreas	2.77± 0.3	4.87± 1.15	5.07± 1.29	7.39± 1.47	8.47± 1.88	10.27± 2.04
Gill	2.44± 0.1	5.00± 2.12	6.11± 1.02	6.49± 1.38	7.23± 1.88	9.70± 1.75
Adductor muscle	2.32± 0.3	5.01± 4.32	5.55± 1.37	5.72± 1.76	7.39± 1.47	8.10± 2.02
Mantle	2.30± 0.20	5.66± 2.31	6.68± 2.04	6.79± 1.74	7.61± 0.39	8.09± 0.84
Glutathione (GSH) (nmoles/100 g wet tissue)						
Hepatopancreas	0.16± 0.32	14.59± 0.52	16.52± 0.32	18.45± 0.54	22.54± 0.61	25.64± 0.65
Gill	0.14± 0.52	15.21± 0.32	16.55± 0.34	17.10± 0.32	18.64± 0.22	20.54± 0.34
Adductor muscle	0.18± 0.55	12.54± 0.21	13.51± 0.21	15.64± 0.22	18.54± 0.32	23.51± 0.65
Mantle	0.14± 0.32	14.55± 0.25	16.24± 0.22	16.94± 0.24	18.65± 0.44	25.31± 0.54

Table 4.2: Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 0.4 ppm WAF of LDO.

Parameter	Treatment					
	0.4 ppm					
	Control	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)						
Gill	5.69± 0.96	16.58± 0.58	20.42± 0.58	25.18± 0.58	32.16± 0.72	48.65± 0.28
Hepatopancreas	3.62± 0.94	14.57± 0.45	16.53± 0.78	19.64± 0.28	21.42± 0.72	26.54± 0.68
Adductor muscle	4.56± 0.55	15.60± 0.74	16.75± 0.28	17.08± 0.29	19.02± 0.72	22.12± 0.98
Mantle	5.59± 0.35	9.13± 0.34	14.81± 0.24	16.91± 0.58	17.25± 0.34	20.47± 0.33
Catalase (μmol/min/mg protein)						
Hepatopancreas	12.68± 0.68	16.31± 0.08	18.65± 0.45	21.54± 0.81	24.65± 0.50	30.24± 0.56
Gill	6.26± 0.97	6.94± 0.70	7.27± 0.53	10.54± 0.54	11.84± 0.25	12.55± 0.45
Adductor muscle	4.31± 1.61	6.94± 0.65	9.66± 0.22	10.88± 0.65	13.95± 0.36	15.67± 0.70
Mantle	5.32± 1.39	9.65± 1.25	13.11± 1.21	15.72± 1.25	16.32± 1.19	20.54± 1.48
Glutathione peroxidase (μg of GSH/min/mg protein)						
Hepatopancreas	2.75± 1.30	5.94± 0.35	10.65± 0.64	13.64± 0.34	15.99± 0.54	21.73± 0.22
Gill	2.40± 1.25	6.85± 0.51	9.33± 0.75	11.99± 0.45	14.67± 0.58	18.49± 0.58
Adductor muscle	1.69± 0.65	6.77± 0.45	7.94± 0.34	10.95 ± 0.75	16.35± 0.98	19.64± 0.34
Mantle	2.35± 1.41	7.94± 0.45	9.64± 0.72	13.23± 0.51	15.71± 0.58	17.55± 0.75
Glutathione reductase (GR) (units/mg protein)						
Hepatopancreas	2.77± 0.3	10.85± 0.08	15.98± 0.54	21.65± 0.67	27.99± 0.75	28.0± 1.08
Gill	2.44± 0.1	6.21± 0.32	8.46± 0.98	13.11± 0.25	17.17± 0.78	23.25± 0.51
Adductor muscle	2.32± 0.3	6.17± 0.25	7.22± 0.61	11.84± 0.37	16.52 ± 0.22	19.99± 1.50
Mantle	2.30± 0.20	8.64± 1.51	10.25± 0.84	13.57± 0.98	15.19± 0.39	18.02 ± 0.25
Glutathione (GSH) (nmoles/100 g wet tissue)						
Hepatopancreas	0.16 ± 0.32	18.64 ± 0.48	21.88 ± 0.54	23.55 ± 0.25	26.17± 0.43	27.45 ± 0.22
Gill	0.14 ± 0.52	16.21 ± 0.24	17.0 ± 0.25	19.56 ± 0.51	18.64 ± 0.22	20.54 ± 0.34
Adductor muscle	0.18± 0.55	12.54± 0.21	13.51± 0.21	15.64 ±0.22	19.22 ± 0.28	21.55 ± 0.35
Mantle	0.14 ± 0.32	13.55 ± 0.12	15.05 ± 0.42	17.66 ± 0.61	21.84 ± 0.23	24.03 ± 0.31

Table 4.3: Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 1 ppm WAF of LDO.

Parameter	Treatment					
	1 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
SOD (units/mg protein)						
Gill	5.69± 0.96	14.32± 0.03	24.92± 0.58	35.18± 0.28	41.06± 0.79	49.12± 0.68
Hepatopancreas	3.62± 0.94	13.64± 0.94	36.43± 0.88	39.21± 0.48	21.42± 0.72	26.54± 0.68
Adductor muscle	4.56 ± 0.55	11.96± 0.75	27.71± 0.38	36.04± 0.29	43.00± 0.98	52.31± 0.43
Mantle	5.59± 0.35	8.09± 0.45	17.23 ± 1.24	25.72± 1.24	31.33± 1.44	36.26± 0.73
Catalase (μmol/min/mg protein)						
Hepatopancreas	12.68± 0.68	17.64± 0.22	22.91± 0.54	25.61± 0.98	27.12± 0.21	35.84± 0.55
Gill	6.26± 0.97	10.7± 1.3	11.7± 2.7	12.02± 1.4	18.2 ± 1.4	19.54± 0.49
Adductor muscle	4.31± 1.61	16.85± 0.65	18.94± 0.22	24.91± 0.25	29.54± 0.21	33.54± 0.21
Mantle	5.32± 1.39	14.81± 0.51	19.54± 0.25	25.70± 0.31	27.94± 0.20	33.26± 0.32
Glutathione peroxidase (μg of GSH/min/mg protein)						
Hepatopancreas	2.75± 1.30	9.64± 0.22	13.65± 0.64	22.61± 0.54	29.61± 0.79	35.97± 0.94
Gill	2.40± 1.25	10.54± 1.00	12.54± 1.24	15.97± 1.94	19.88± 1.34	23.76± 1.27
Adductor muscle	1.69± 0.65	9.5± 2.0	10.9 ± 2.2	11.10± 3.60	15.30± 3.0	18.50± 4.8
Mantle	2.35± 1.41	11.30± 0.28	14.86± 1.64	15.02± 0.98	16.40± 0.75	18.27± 0.21
Glutathione reductase (GR) (units/mg protein)						
Hepatopancreas	2.77± 0.3	12.24± 0.06	15.00± 0.07	24.94± 0.08	30.22± 0.08	35.64± 1.08
Gill	2.44± 0.1	9.10± 0.04	11.74± 0.35	18.97± 0.27	21.70± 0.08	25.22± 0.09
Adductor muscle	2.32± 0.3	8.97± 0.18	10.00± 0.19	18.07± 0.07	21.00± 0.71	27.94± 0.08
Mantle	2.30± 0.20	9.01± 0.20	10.08± 0.34	11.06± 0.98	19.17± 0.22	21.02± 0.25
Glutathione (GSH) (nmoles/100 g wet tissue)						
Hepatopancreas	0.16± 0.32	20.22± 0.34	23.54± 0.78	26.94± 0.18	28.97± 0.71	34.99± 0.73
Gill	0.14± 0.52	16.97± 0.54	18.90± 0.41	21.70± 0.49	23.17± 0.41	24.10± 0.79
Adductor muscle	0.18± 0.55	13.94± 0.94	14.96± 0.49	16.47± 0.98	21.07± 0.72	22.00± 0.81
Mantle	0.14± 0.32	14.02± 0.43	16.77± 0.35	18.24± 0.53	22.37± 0.25	25.00± 0.27

Table 4.4 : Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 3.5 ppm WAF of LDO.

Parameter	Treatment					
	3.5 ppm					
	Control	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)						
Gill	5.69 ± 0.96	23.58 ± 0.23	31.54 ± 0.79	45.14 ± 0.64	60.26 ± 0.75	68.61 ± 1.25
Hepatopancreas	3.62 ± 0.94	22.04 ± 0.94	27.23 ± 0.43	38.64 ± 0.28	45.31 ± 0.64	52.51 ± 1.17
Adductor muscle	4.56 ± 0.55	19.96 ± 0.75	26.56 ± 0.69	31.55 ± 0.27	39.41 ± 1.74	42.10 ± 1.52
Mantle	5.59 ± 0.35	17.59 ± 0.75	22.21 ± 0.38	34.91 ± 1.24	42.54 ± 1.32	44.47 ± 0.53
Catalase (μmol/min/mg protein)						
Hepatopancreas	12.68 ± 0.68	18.02 ± 0.24	24.00 ± 0.21	30.84 ± 0.94	40.10 ± 0.84	49.33 ± 0.78
Gill	6.26 ± 0.97	12.57 ± 0.74	15.01 ± 0.94	19.70 ± 0.81	23.90 ± 0.94	27.17 ± 0.38
Adductor muscle	4.31 ± 1.61	17.00 ± 0.55	19.64 ± 0.43	21.08 ± 0.30	30.88 ± 0.42	38.05 ± 0.28
Mantle	5.32 ± 1.39	15.01 ± 0.94	18.94 ± 0.73	23.77 ± 0.70	29.01 ± 0.94	34.21 ± 0.78
Glutathione peroxidase (μg of GSH/min/mg protein)						
Hepatopancreas	2.75± 1.30	20.02 ± 0.21	26.94 ± 0.74	33.10 ± 0.84	45.61 ± 0.18	56.04 ± 0.94
Gill	2.40 ± 1.25	15.72 ± 0.94	21.74 ± 0.84	17.97 ± 0.27	18.07 ± 0.18	29.67 ± 0.75
Adductor muscle	1.69 ± 0.65	12.74 ± 0.84	15.21 ± 0.80	18.47 ± 0.70	21.84 ± 0.84	26.21 ± 0.54
Mantle	2.35 ± 1.41	14.93 ± 0.41	16.82 ± 0.71	17.07 ± 0.87	17.98 ± 0.48	20.24 ± 0.40
Glutathione reductase (GR) (units/mg protein)						
Hepatopancreas	2.77 ± 0.30	14.87 ± 0.47	15.84 ± 0.75	25.74 ± 0.84	33.04 ± 0.84	40.84 ± 0.80
Gill	2.44 ± 0.10	10.20 ± 0.47	13.41 ± 0.82	19.47 ± 0.24	22.45 ± 0.41	28.41 ± 0.50
Adductor muscle	2.32 ± 0.30	9.10 ± 0.86	12.10 ± 0.83	21.30 ± 0.54	24.01 ± 0.80	33.21 ± 0.49
Mantle	2.30 ± 0.20	12.40 ± 0.22	16.94 ± 0.27	17.54 ± 0.25	22.61 ± 0.82	27.10 ± 0.24
Glutathione (GSH) (nmoles/100 g wet tissue)						
Hepatopancreas	0.16 ± 0.32	30.64 ± 0.25	42.91 ± 0.45	58.61 ± 0.74	69.73 ± 0.83	82.46 ± 0.71
Gill	0.14 ± 0.52	26.51 ± 0.26	32.64 ± 0.45	38.21 ± 0.42	43.24 ± 0.40	49.61 ± 0.54
Adductor muscle	0.18 ± 0.55	21.43 ± 0.82	27.31 ± 0.24	33.00 ± 0.24	36.20 ± 0.28	39.01 ± 0.73
Mantle	0.14 ± 0.32	18.34 ± 0.16	19.22 ± 0.43	24.16 ± 0.81	27.10 ± 0.21	30.11 ± 0.31

Table 4.5: Three way ANOVA Table showing Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to different concentration of WAF of LDO. (a - e)

a) SOD

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	703.727	234.576	11.478	<0.001
PAH CONC	**3**	7452.345	2484.115	121.550	<0.001
TIME	**5**	8960.337	1792.067	87.687	<0.001
Residual	**45**	919.664	20.437		
Total	**95**	21888.478	230.405		

b) Catalase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	1565.920	521.973	193.106	<0.001
PAH CONC	**3**	1813.985	604.662	223.696	<0.001
TIME	**5**	3798.834	759.767	281.078	<0.001
Residual	**45**	121.637	2.703		
Total	**95**	8355.514	87.953		

c) Glutathione peroxidase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	618.571	206.190	22.388	<0.001
PAH CONC	**3**	1640.992	546.997	59.393	<0.001
TIME	**5**	4135.354	827.071	89.804	<0.001
Residual	**45**	414.438	9.210		
Total	**95**	8235.062	86.685		

d) Glutathione reductase (GR)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	411.441	137.147	59.225	<0.001
PAH CONC	**3**	1861.900	620.633	268.011	<0.001
TIME	**5**	4334.683	866.937	374.374	<0.001
Residual	**45**	104.206	2.316		
Total	**95**	7876.210	82.907		

e) Glutathione (GSH)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	702.026	234.009	2178.48 3	<0.001
PAH CONC	**3**	110.967	36.989	344.344	<0.001
TIME	**5**	86.361	17.272	160.793	<0.001
Residual	**45**	4.834	0.107		
Total	**95**	942.843	9.925		

Table 4.6: Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 0.1 ppm WAF of BH Crude oil.

Parameter	Treatment				
	0.1 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	4.08 ± 0.45	4.12 ± 0.35	4.31 ± 0.22	4.64 ± 0.18	4.95 ± 0.07
Hepatopancreas	3.78 ± 0.50	3.82 ± 0.15	3.91 ± 0.25	4.15 ± 0.22	4.42 ± 0.14
Adductor muscle	3.00 ± 1.10	3.15 ± 1.25	3.26 ± 0.92	3.49 ± 0.14	3.70 ± 0.05
Mantle	3.46 ± 0.14	3.52 ± 0.09	3.68 ± 0.49	3.85 ± 0.06	4.08 ± 0.12
Catalase (μmol/min/mg protein)					
Hepatopancreas	10.01 ± 0.12	1015 ± 0.06	10.29 ± 0.04	10.42 ± 0.01	10.64 ± 0.19
Gill	6.02 ± 0.04	6.19 ± 0.05	6.27 ± 0.03	6.44 ± 0.06	6.68 ± 0.03
Adductor muscle	4.10 ± 0.01	4.34 ± 0.14	4.59 ± 0.22	4.72 ± 0.24	4.98 ± 0.05
Mantle	4.92 ± 0.04	5.01 ± 0.05	5.23 ± 0.16	5.34 ± 0.19	5.46 ± 0.23
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	4.65 ± 1.24	4.69 ± 1.22	4.95 ± 1.05	5.10 ± 1.00	5.24 ± 0.98
Gill	4.19 ± 1.22	4.25 ± 0.35	4.41 ± 0.44	4.86 ± 0.29	5.10 ± 0.08
Adductor muscle	3.10 ± 1.20	3.29 ± 0.49	3.40 ± 0.18	3.59 ± 0.17	3.82 ± 0.08
Mantle	3.58 ± 1.19	3.75 ± 0.95	3.81 ± 0.19	3.92 ± 0.16	4.08 ± 0.13
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.18 ± 0.20	4.29 ± 0.14	4.35 ± 0.26	4.51 ± 0.35	4.62 ± 0.24
Gill	3.28 ± 0.15	3.34 ± 0.09	3.42 ± 0.14	3.50 ± 0.06	3.65 ± 0.21
Adductor muscle	1.92 ± 0.25	2.06 ± 0.14	2.13 ± 0.08	2.25 ± 0.04	2.71 ± 0.03
Mantle	2.64 ± 0.13	2.81 ± 0.08	3.01 ± 0.14	3.19 ± 0.18	3.25 ± 0.04
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	10.19 ± 0.28	10.25 ± 0.22	10.34 ± 0.16	10.46 ± 0.07	10.78 ± 0.05
Gill	9.28 ± 0.14	9.34 ± 0.26	9.49 ± 0.13	9.62 ± 0.14	9.84 ± 0.25
Adductor muscle	8.42 ± 0.04	8.53 ± 0.02	8.64 ± 0.16	8.85 ± 0.25	8.94 ± 0.12
Mantle	9.00 ± 0.16	9.09 ± 0.22	9.14 ± 0.05	9.28 ± 0.12	10.14 ± 0.24

Table 4.7: Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 0.6 ppm WAF of BH Crude oil.

Parameter	Treatment				
	0.6 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	4.24 ± 0.21	4.45 ± 0.06	4.74 ± 0.12	4.90 ± 0.08	5.01 ± 0.08
Hepatopancreas	3.88 ± 0.15	3.90 ± 0.19	4.00 ± 0.06	4.24 ± 0.16	4.51 ± 0.07
Adductor muscle	3.21 ± 0.08	3.20 ± 0.24	3.38 ± 0.01	3.56 ± 0.13	3.82 ± 0.06
Mantle	3.50 ± 0.21	3.64 ± 0.16	3.91 ± 0.06	4.15 ± 0.11	4.29 ± 0.12
Catalase (μmol/min/mg protein)					
Hepatopancreas	10.16 ± 0.08	10.30 ± 0.06	10.51 ± 0.07	10.65 ± 0.03	10.81 ± 0.12
Gill	6.59 ± 0.11	6.64 ± 0.10	6.89 ± 0.08	6.90 ± 0.11	6.95 ± 0.06
Adductor muscle	5.00 ± 0.95	5.16 ± 0.25	5.35 ± 0.14	5.56 ± 0.03	5.73 ± 0.01
Mantle	5.20 ± 0.29	5.31 ± 0.04	5.49 ± 0.03	5.50 ± 0.08	5.68 ± 0.04
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	4.72 ± 0.01	4.85 ± 0.15	4.99 ± 0.22	5.15 ± 0.15	5.35 ± 0.07
Gill	4.26 ± 0.14	4.40 ± 0.16	4.76 ± 0.18	4.92 ± 0.08	5.21 ± 0.05
Adductor muscle	3.25 ± 0.05	3.38 ± 0.04	3.60 ± 0.09	3.79 ± 0.05	3.92 ± 0.06
Mantle	3.68 ± 0.05	3.82 ± 0.02	3.91 ± 0.15	4.10 ± 0.06	4.38 ± 0.03
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.26 ± 0.14	4.39 ± 0.12	4.61 ± 0.13	4.82 ± 0.06	4.95 ± 0.03
Gill	3.41 ± 0.06	3.54 ± 0.14	3.66 ± 0.06	3.81 ± 0.03	3.92 ± 0.04
Adductor muscle	2.08 ± 0.07	2.15 ± 0.07	2.31 ± 0.04	2.75 ± 0.04	2.88 ± 0.11
Mantle	2.76 ± 0.08	2.94 ± 0.05	3.08 ± 0.02	3.21 ± 0.05	3.42 ± 0.16
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	10.26 ± 0.10	10.39 ± 0.12	10.51 ± 0.22	10.74 ± 0.16	10.84 ± 0.02
Gill	10.00 ± 0.06	10.21 ± 0.10	10.39 ± 0.15	10.45 ± 0.22	10.56 ± 0.05
Adductor muscle	8.59 ± 0.03	8.72 ± 0.12	8.89 ± 0.16	8.92 ± 0.05	9.10 ± 0.08
Mantle	9.18 ± 0.10	9.31 ± 0.12	9.45 ± 0.29	10.05 ± 0.13	10.29 ± 0.06

Table 4.8: Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 2 ppm WAF of BH Crude oil.

Parameter	Treatment				
	2 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	4.30 ± 0.08	4.64 ± 0.07	4.82 ± 0.06	4.98 ± 0.05	5.10 ± 0.12
Hepatopancreas	3.92 ± 0.01	3.99 ± 0.02	4.10 ± 0.15	4.30 ± 0.20	4.68 ± 0.06
Adductor muscle	3.41 ± 0.06	3.62 ± 0.01	3.79 ± 0.08	3.85 ± 0.01	3.99 ± 0.11
Mantle	3.69 ± 0.12	3.74 ± 0.10	3.95 ± 0.01	4.29 ± 0.19	4.35 ± 0.14
Catalase (μmol/min/mg protein)					
Hepatopancreas	10.21 ± 0.04	10.35 ± 0.06	10.69 ± 0.05	10.79 ± 0.12	10.92 ± 0.07
Gill	8.14 ± 0.15	8.29 ± 0.05	8.45 ± 0.01	8.64 ± 0.14	8.86 ± 0.01
Adductor muscle	6.00 ± 0.21	6.20 ± 0.18	6.31 ± 0.23	6.39 ± 0.14	6.52 ± 0.18
Mantle	6.49 ± 0.20	6.52 ± 0.21	6.69 ± 0.13	6.75 ± 0.21	6.88 ± 0.06
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	4.86 ± 0.04	4.92 ± 0.12	5.06 ± 0.08	5.21 ± 0.01	5.43 ± 0.04
Gill	4.43 ± 0.08	4.64 ± 0.09	4.83 ± 0.01	5.00 ± 0.06	5.35 ± 0.06
Adductor muscle	3.34 ± 0.06	3.42 ± 0.06	3.71 ± 0.12	3.84 ± 0.05	4.01 ± 0.05
Mantle	3.85 ± 0.05	3.92 ± 0.04	4.21 ± 0.05	4.34 ± 0.11	4.59 ± 0.08
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.49 ± 0.08	4.54 ± 0.07	4.69 ± 0.06	4.85 ± 0.05	5.00 ± 0.02
Gill	3.58 ± 0.03	3.63 ± 0.06	3.79 ± 0.05	3.85 ± 0.03	3.96 ± 0.12
Adductor muscle	2.25 ± 0.15	2.40 ± 0.06	2.59 ± 0.06	2.81 ± 0.09	2.89 ± 0.11
Mantle	2.86 ± 0.02	3.00 ± 0.14	3.19 ± 0.02	3.35 ± 0.05	3.53 ± 0.01
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	10.35 ± 0.19	10.43 ± 0.08	10.56 ± 0.04	10.81 ± 0.02	10.90 ± 0.06
Gill	10.10 ± 0.14	10.29 ± 0.14	10.42 ± 0.15	10.69 ± 0.12	10.83 ± 0.03
Adductor muscle	8.61 ± 0.01	8.73 ± 0.08	8.91 ± 0.02	9.08 ± 0.01	9.24 ± 0.01
Mantle	9.25 ± 0.14	9.34 ± 0.04	9.46 ± 0.01	10.15 ± 0.06	10.33 ± 0.06

Table 4.9: Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to 5 ppm WAF of BH Crude oil.

Parameter	Treatment				
	5 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	4.42 ± 0.07	4.70 ± 0.08	4.92 ± 0.06	5.09 ± 0.04	5.19 ± 0.04
Hepatopancreas	4.00 ± 0.08	4.16 ± 0.05	4.29 ± 0.06	4.35 ± 0.05	4.72 ± 0.01
Adductor muscle	3.59 ± 0.04	3.64 ± 0.08	3.81 ± 0.03	3.90 ± 0.18	4.15 ± 0.08
Mantle	3.72 ± 0.03	3.93 ± 0.10	4.14 ± 0.04	4.36 ± 0.04	4.59 ± 0.16
Catalase (μmol/min/mg protein)					
Hepatopancreas	10.29 ± 0.05	10.40 ± 0.06	10.72 ± 0.08	10.90 ± 0.18	10.99 ± 0.08
Gill	8.30 ± 0.06	8.55 ± 0.01	8.63 ± 0.01	8.84 ± 0.13	8.92 ± 0.16
Adductor muscle	6.19 ± 0.01	6.30 ± 0.03	6.38 ± 0.01	6.54 ± 0.04	6.72 ± 0.75
Mantle	6.58 ± 0.03	6.60 ± 0.12	6.71 ± 0.06	6.85 ± 0.01	7.00 ± 0.14
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	4.93 ± 0.01	5.08 ± 0.08	5.30 ± 0.08	5.32 ± 0.08	5.61 ± 0.02
Gill	4.51 ± 0.04	4.70 ± 0.19	5.00 ± 0.01	5.41 ± 0.09	5.68 ± 0.01
Adductor muscle	3.58 ± 0.01	3.65 ± 0.16	3.91 ± 0.12	4.00 ± 0.05	4.44 ± 0.10
Mantle	3.96 ± 0.08	4.05 ± 0.03	4.25 ± 0.01	4.46 ± 0.14	4.63 ± 0.06
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.52 ± 0.14	4.65 ± 0.06	4.81 ± 0.04	4.93 ± 0.03	5.14 ± 0.01
Gill	3.69 ± 0.02	3.81 ± 0.04	3.92 ± 0.03	4.00 ± 0.12	4.21 ± 0.13
Adductor muscle	2.34 ± 0.16	2.44 ± 0.14	2.62 ± 0.14	2.83 ± 0.14	2.95 ± 0.11
Mantle	2.94 ± 0.18	3.12 ± 0.12	3.26 ± 0.14	3.40 ± 0.13	3.62 ± 0.14
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	10.42 ± 0.14	10.61 ± 0.72	10.83 ± 0.08	10.92 ± 0.12	11.15 ± 0.01
Gill	10.28 ± 0.22	10.39 ± 0.06	10.48 ± 0.06	10.72 ± 0.04	10.92 ± 0.05
Adductor muscle	8.79 ± 0.21	8.85 ± 0.05	8.92 ± 0.01	9.12 ± 0.05	9.32 ± 0.06
Mantle	10.20 ± 0.22	10.29 ± 0.03	10.36 ± 0.04	10.48 ± 0.01	10.63 ± 0.02

Table 4.10: Three way ANOVA Table showing Antioxidant enzyme activity in the different tissues of *Perna indica* exposed to different concentration of WAF of BHC. (a – e)

a) SOD

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	15.569	5.190	1466.658	<0.001
PAH CONC	**3**	1.631	0.544	153.612	<0.001
TIME	**5**	13.190	2.638	745.549	<0.001
Residual	**45**	0.159	0.00354		
Total	**95**	39.807	0.419		

b) Catalase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	656.229	218.743	2025.779	<0.001
PAH CONC	**3**	94.407	31.469	291.436	<0.001
TIME	**5**	164.855	32.971	305.345	<0.001
Residual	**45**	4.859	0.108		
Total	**95**	962.441	10.131		

c) Glutathione peroxidase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	26.896	8.965	2480.904	<0.001
PAH CONC	**3**	1.713	0.571	158.016	<0.001
TIME	**5**	64.420	12.884	3565.295	<0.001
Residual	**45**	0.163	0.00361		
Total	**95**	94.921	0.999		

d) Glutathione reductase (GR)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	45.721	15.240	5952.512	<0.001
PAH CONC	**3**	1.511	0.504	196.711	<0.001
TIME	**5**	17.577	3.515	1373.066	<0.001
Residual	**45**	0.115	0.00256		
Total	**95**	70.666	0.744		

e) Glutathione (GSH)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	27.589	9.196	554.864	<0.001
PAH CONC	**3**	4.217	1.406	84.805	<0.001
TIME	**5**	1260.062	252.012	15205.197	<0.001
Residual	**45**	0.746	0.0166		
Total	**95**	1301.364	13.699		

Table 4.11: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 0.1 ppm WAF of BH Crude oil.

Parameter	Treatment 0.1 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	4.15 ± 0.12	4.26 ± 0.02	4.35 ± 0.01	4.42 ± 0.14	4.59 ± 0.08
Hepatopancreas	3.60 ± 0.05	3.71 ± 0.14	3.78 ± 0.11	3.80 ± 0.12	3.93 ± 0.03
Adductor muscle	2.90 ± 0.06	2.95 ± 0.05	3.06 ± 0.16	3.14 ± 0.13	3.26 ± 0.11
Mantle	3.50 ± .0.05	3.50 ± 0.4	3.61 ± 0.02	3.73 ± 0.01	3.84 ± 0.06
Catalase (μmol/min/mg protein)					
Hepatopancreas	9.25 ± 0.11	9.34 ± 0.15	9.52 ± 0.19	9.69 ± 0.20	9.81 ± 0.29
Gill	5.82 ± 0.14	5.90 ± 0.16	6.00 ± 0.11	6.18 ± 0.22	6.31 ± 0.29
Adductor muscle	4.06 ± 0.16	4.19 ± 0.25	4.32 ± 0.14	4.45 ± 0.08	4.53 ± 0.07
Mantle	4.35 ± 0.14	4.51 ± 0.08	4.60 ± 0.01	4.65 ± 0.06	4.72 ± 0.06
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	4.50 ± 0.04	4.55 ± 0.02	4.60 ± 0.08	4.75 ± 0.09	5.08 ± 0.01
Gill	4.16 ± 0.01	4.25 ± 0.13	4.32 ± 0.12	4.49 ± 0.13	4.58 ± 0.03
Adductor muscle	3.00 ± 0.08	3.20 ± 0.12	3.39 ± 0.14	3.52 ± 0.15	3.60 ± 0.16
Mantle	3.19 ± 0.02	3.25 ± 0.14	3.42 ± 0.15	3.62 ± 0.16	3.81 ± 0.14
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.15 ± 0.11	4.25 ± 0.16	4.41 ± 0.08	4.56 ± 0.07	4.63 ± 0.08
Gill	3.20 ± 0.06	3.39 ± 0.08	3.51 ± 0.01	3.62 ± 0.14	3.78 ± 0.13
Adductor muscle	2.00 ± 0.08	2.14 ± 0.06	2.25 ± 0.04	2.31 ± 0.08	2.42 ± 0.08
Mantle	2.85 ± 0.04	2.96 ± 0.07	3.08 ± 0.02	3.14 ± 0.02	3.35 ± 0.14
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	10.00 ± 0.01	10.20 ± 0.12	10.29 ± 0.14	10.35 ± 0.13	10.55 ± .0.22
Gill	9.12 ± 0.02	9.18 ± 0.08	9.22 ± 0.14	9.30 ± 0.15	9.42 ± 0.15
Adductor muscle	8.14 ± 0.12	8.21 ± 0.08	8.30 ± 0.01	8.45 ± 0.16	8.92 ± 0.05
Mantle	9.00 ± 0.34	9.14 ± 0.12	9.26 ± 0.22	9.39 ± 0.31	10.08 ± 0.16

Table 4.12: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 1 ppm WAF of BH Crude oil.

Parameter	Treatment				
	1 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	4.24 ± 0.02	4.30 ± 0.05	4.41 ± 0.08	4.59 ± 0.18	4.62 ± 0.07
Hepatopancreas	3.81 ± 0.08	3.92 ± 0.01	4.02 ± 0.12	4.16 ± 0.16	4.28 ± 0.14
Adductor muscle	3.01 ± 0.16	3.18 ± 0.14	3.32 ± 0.14	3.35 ± 0.23	3.56 ± 0.16
Mantle	3.62 ± 0.22	3.74 ± 0.24	3.85 ± 0.13	3.92 ± 0.11	4.06 ± 0.13
Catalase (μmol/min/mg protein)					
Hepatopancreas	9.49 ± 0.12	9.54 ± 0.13	9.72 ± 0.14	9.84 ± 0.16	9.99 ± 0.01
Gill	5.85 ± 0.14	5.92 ± 0.21	6.15 ± 0.13	6.21 ± 0.25	6.39 ± 0.16
Adductor muscle	4.15 ± 0.25	4.24 ± 0.29	4.39 ± 0.21	4.52 ± 0.14	4.63 ± 0.21
Mantle	4.42 ± 0.22	4.60 ± 0.14	4.71 ± 0.25	4.80 ± 0.16	4.89 ± 0.14
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	4.65 ± 0.05	4.72 ± 0.04	4.81 ± 0.02	4.98 ± 0.12	5.12 ± 0.11
Gill	4.29 ± 0.16	4.37 ± 0.14	4.52 ± 0.12	4.64 ± 0.13	4.81 ± 0.22
Adductor muscle	3.18 ± 0.15	3.35 ± 0.12	3.51 ± .14	3.61 ± 0.27	4.14 ± 0.12
Mantle	3.35 ± 0.21	3.47 ± 0.05	3.59 ± 0.07	3.78 ± 0.14	3.93 ± 0.22
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.21 ± 0.22	4.39 ± 0.05	4.54 ± 0.01	4.61 ± 0.08	4.70 ± 0.04
Gill	3.50 ± 0.16	3.68 ± 0.08	3.72 ± 0.14	3.89 ± 0.12	3.95 ± 0.04
Adductor muscle	2.19 ± 0.28	2.29 ± 0.14	2.35 ± 0.01	2.48 ± 0.35	2.69 ± 0.15
Mantle	3.10 ± 1.24	3.17 ± 0.01	3.28 ± 0.12	3.39 ± 0.11	3.48 ± 0.22
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	10.15 ± 0.11	10.22 ± 0.14	10.31 ± 0.08	10.49 ± .0.11	10.62 ± 0.18
Gill	9.46 ± 0.12	9.49 ± 0.06	9.55 ± 0.16	9.63 ± 0.12	9.84 ± 0.14
Adductor muscle	8.29 ± 0.14	8.31 ± 0.11	8.49 ± 0.02	8.91 ± 0.12	9.01 ± 0.18
Mantle	9.21 ± 0.22	9.29 ± 0.14	9.45 ± .0.11	9.85 ± 0.24	10.15 ± 0.22

Table 4.13: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 5 ppm WAF of BH Crude oil.

Parameter	Treatment				
	5 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	4.39 ± 0.12	4.48 ± 0.14	4.53 ± 0.21	4.65 ± 0.19	4.81 ± 0.25
Hepatopancreas	4.00 ± 0.11	4.18 ± 0.22	4.23 ± 0.23	4.43 ± 0.18	4.68 ± 0.27
Adductor muscle	3.24 ± 0.21	3.29 ± 0.18	3.39 ± 0.15	3.45 ± 0.18	3.66 ± 0.23
Mantle	3.89 ± 0.11	3.97 ± 0.16	4.07 ± 0.17	4.14 ± 0.12	4.28 ± 0.02
Catalase (μmol/min/mg protein)					
Hepatopancreas	9.64 ± 0.11	9.71 ± 0.25	9.82 ± 0.15	9.98 ± 0.22	10.05 ± 0.34
Gill	5.92 ± 0.18	6.02 ± 0.11	6.22 ± 0.04	6.35 ± 0.14	6.49 ± 0.22
Adductor muscle	4.34 ± 0.22	4.45 ± 0.23	4.59 ± 0.25	4.62 ± 0.08	4.78 ± 0.14
Mantle	4.54 ± 0.23	4.69 ± 0.18	4.72 ± 0.24	4.91 ± 0.12	5.18 ± 0.01
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	4.73 ± 0.04	4.80 ± 0.01	4.95 ± 0.08	5.05 ± 0.01	5.28 ± 0.12
Gill	4.34 ± 0.13	4.51 ± 0.06	4.62 ± 0.07	4.79 ± 0.18	4.95 ± 0.08
Adductor muscle	3.29 ± 0.15	3.42 ± 0.11	3.55 ± 0.15	3.92 ± 0.19	4.21 ± 0.22
Mantle	3.42 ± 0.22	3.58 ± 0.12	3.62 ± 0.16	3.88 ± 0.16	4.05 ± 0.16
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.29 ± 0.18	4.40 ± 0.01	4.59 ± 0.06	4.64 ± 0.14	4.72 ± 0.16
Gill	3.59 ± 0.25	3.69 ± 0.14	3.73 ± 0.15	3.90 ± 0.12	4.01 ± 0.04
Adductor muscle	2.40 ± 0.14	2.51 ± 0.22	2.70 ± 0.25	2.82 ± 0.22	2.95 ± 0.11
Mantle	3.15 ± 0.22	3.26 ± 0.08	3.41 ± 0.08	3.52 ± 0.06	3.71 ± 0.16
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	10.20 ± 0.18	10.25 ± 0.14	10.34 ± 0.08	10.52 ± 0.16	10.71 ± 0.04
Gill	9.50 ± 0.18	9.61 ± 0.01	9.72 ± 0.01	9.85 ± 0.14	10.08 ± 0.11
Adductor muscle	8.42 ± 0.28	8.62 ± 0.14	8.79 ± 0.13	9.08 ± 0.15	9.21 ± 0.22
Mantle	9.41 ± 0.29	9.63 ± 0.04	9.84 ± 0.03	10.05 ± 1.24	10.36 ± 0.25

Table 4.14: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 8 ppm WAF of BH Crude oil.

Parameter	Treatment					
	8 ppm					
		1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)						
Gill		4.52 ± 0.08	4.64 ± 0.14	4.79 ± 0.25	4.85 ± 0.35	5.01 ± 0.34
Hepatopancreas		4.25 ± 0.14	4.39 ± 0.08	4.54 ± 0.31	4.69 ± 0.37	4.82 ± 0.27
Adductor muscle		3.50 ± 0.18	3.59 ± 0.34	3.65 ± 0.14	3.72 ± 0.34	3.95 ± 0.28
Mantle		4.00 ± 0.04	4.19 ± 0.14	4.24 ± 0.18	4.42 ± 0.22	4.61 ± 0.29
Catalase (μmol/min/mg protein)						
Hepatopancreas		10.01 ± 0.14	10.14 ± 0.14	10.29 ± 0.34	10.42 ± 0.42	10.69 ± 0.45
Gill		6.05 ± 0.22	6.15 ± 0.34	6.32 ± 0.14	6.52 ± 0.41	6.81 ± 0.39
Adductor muscle		4.54 ± 0.23	4.58 ± 0.18	4.76 ± 0.49	4.92 ± 0.45	5.08 ± 0.51
Mantle		4.80 ± 0.15	4.92 ± 0.45	5.12 ± 0.34	5.28 ± 0.32	5.42 ± 0.14
Glutathione peroxidase (μg of GSH/min/mg protein)						
Hepatopancreas		4.85 ± 0.14	4.90 ± 0.29	5.10 ± .0.31	5.25 ± 0.35	5.39 ± 0.42
Gill		4.53 ± 0.11	4.61 ± 0.32	4.74 ± 0.41	4.89 ± 0.40	5.05 ± 0.18
Adductor muscle		3.50 ± 0.12	3.64 ± 0.31	3.73 ± 0.17	4.15 ± 0.16	4.39 ± 0.35
Mantle		3.72 ± 0.22	3.80 ± 0.35	3.95 ± 0.34	4.29 ± 0.32	4.45 ± 0.34
Glutathione reductase (GR) (units/mg protein)						
Hepatopancreas		4.52 ± 0.17	4.61 ± 0.31	4.70 ± 0.35	4.85 ± 0.14	5.02 ± 0.16
Gill		3.81 ± 0.24	3.91 ± 0.22	4.08 ± 0.08	4.19 ± 0.18	4.34 ± 0.12
Adductor muscle		2.69 ± 0.22	2.75 ± 0.15	2.92 ± 0.14	3.09 ± 0.14	3.25 ± 0.29
Mantle		3.29 ± 0.25	3.40 ± 0.16	3.65 ± 0.08	3.82 ± 0.14	4.08 ± 0.20
Glutathione (GSH) (nmoles/100 g wet tissue)						
Hepatopancreas		10.53 ± 0.22	10.75 ± 0.29	10.92 ± 0.18	11.01 ± 0.22	11.29 ± 0.18
Gill		10.12 ± 0.12	10.25 ± 0.24	10.42 ± 0.34	10.68 ± 0.37	10.85 ± 0.29
Adductor muscle		9.06 ± 0.14	9.19 ± 0.28	9.31 ± 0.18	9.48 ± 0.22	9.84 ± 0.14
Mantle		9.84 ± 0.29	10.01 ± 0.14	10.26 ± 0.29	10.35 ± 0.27	10.49 ± 0.25

Table 4.15: Three way ANOVA Table showing Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to different concentration of WAF of BHC. (a – e)

a) SOD

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	28.114	9.371	3909.042	<0.001
PAH CONC	**3**	3.453	1.151	480.135	<0.001
TIME	**5**	18.950	3.790	1580.933	<0.001
Residual	**45**	0.108	0.00240		
Total	**95**	58.345	0.614		

b) Catalase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	584.344	194.781	11946.996	<0.001
PAH CONC	**3**	34.366	11.455	702.614	<0.001
TIME	**5**	108.889	21.778	1335.747	<0.001
Residual	**45**	0.734	0.0163		
Total	**95**	761.264	8.013		

c) Glutathione peroxidase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	36.289	12.096	3597.627	<0.001
PAH CONC	**3**	2.037	0.679	201.981	<0.001
TIME	**5**	32.457	6.491	1930.621	<0.001
Residual	**45**	0.151	0.00336		
Total	**95**	73.683	0.776		

d) Glutathione reductase (GR)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	51.673	17.224	6286.814	<0.001
PAH CONC	**3**	2.645	0.882	321.765	<0.001
TIME	**5**	15.971	3.194	1165.844	<0.001
Residual	**45**	0.123	0.00274		
Total	**95**	76.672	0.807		

e) Glutathione (GSH)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	24.184	8.061	983.789	<0.001
PAH CONC	**3**	7.507	2.502	305.380	<0.001
TIME	**5**	1230.527	246.105	30034.549	<0.001
Residual	**45**	0.369	0.00819		
Total	**95**	1269.405	13.362		

Table 4.16: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 0.05 ppm WAF of LDO.

Parameter	Treatment					
	0.05 ppm					
	Control	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)						
Gill	4.27 ± 1.25	5.12 ± 0.95	6.01 ± 0.18	6.61 ± 0.22	7.08 ± 0.85	7.59 ± 0.23
Hepatopancreas	3.47 ± 1.37	4.25 ± 0.18	5.10 ± 0.25	6.18 ± 0.13	6.93 ± 0.39	7.10 ± 0.12
Adductor muscle	1.23 ± 0.5	2.19 ± 0.25	2.28 ± 0.12	2.45 ± 0.29	3.10 ± 0.84	3.65 ± 0.92
Mantle	2.53 ± 1.35	3.14 ± 0.39	3.64 ± 0.14	4.10 ± 0.14	4.29 ± 0.25	5.01 ± 1.50
Catalase (μmol/min/mg protein)						
Hepatopancreas	10.01 ± 1.24	10.14 ± 0.84	10.29 ± 0.23	11.14 ± 0.19	11.35 ± 0.34	11.81 ± 0.39
Gill	4.29 ± 1.36	5.01 ± 0.38	5.30 ± 0.39	5.69 ± 0.94	6.01 ± 0.11	6.35 ± 0.14
Adductor muscle	3.01 ± 1.20	3.24 ± 0.94	3.58 ± 0.80	3.64 ± 0.92	4.16 ± 0.12	4.45 ± 0.84
Mantle	5.16 ± 0.64	5.35 ± 0.14	5.65 ± 0.82	6.10 ± 0.70	6.39 ± 0.75	7.05 ± 0.90
Glutathione peroxidase (μg of GSH/min/mg protein)						
Hepatopancreas	4.01 ± 1.35	5.32 ± 1.39	5.72 ± 1.12	6.11 ± 1.02	6.40 ± 1.43	6.51 ± 1.40
Gill	3.25 ± 1.54	4.31 ± 1.61	4.87 ± 1.15	5.07 ± 1.20	5.59 ± 1.62	5.71 ± 0.61
Adductor muscle	1.69 ± 0.97	2.00 ± 1.37	2.32 ± 3.12	2.60 ± 1.27	2.88 ± 1.28	3.19 ± 0.56
Mantle	2.02 ± 1.24	3.47 ± 1.37	3.53 ± 1.90	4.01 ± 1.35	4.45 ± 1.89	4.29 ± 1.53
Glutathione reductase (GR) (units/mg protein)						
Hepatopancreas	3.87 ± 1.32	4.10 ± 0.25	4.15 ± 0.14	4.58 ± 0.13	5.01 ± 0.65	5.21 ± 0.75
Gill	3.10 ± 1.30	4.00 ± 1.25	4.20 ± 0.12	4.31 ± 0.20	4.68 ± 1.18	5.00 ± 0.29
Adductor muscle	2.00 ± 1.37	2.31 ± 1.05	2.45 ± 0.08	2.51 ± 1.10	2.82 ± 0.95	3.14 ± 0.16
Mantle	1.25 ± 1.31	1.52 ± 0.39	1.62 ± 0.12	1.71 ± 0.45	2.08 ± 0.31	2.29 ± 0.25
Glutathione (GSH) (nmoles/100 g wet tissue)						
Hepatopancreas	0.14 ± 0.01	12.14 ± 0.13	13.12 ± 0.19	13.42 ± 0.25	14.18 ± 0.16	15.10 ± 0.29
Gill	0.12 ± 0.01	11.10 ± 0.08	11.35 ± 0.15	11.48 ± 0.13	12.10 ± 0.12	12.24 ± 0.15
Adductor muscle	0.08 ± 0.02	9.25 ± 0.12	9.56 ± 0.14	9.65 ± 0.15	10.29 ± 0.16	10.25 ± 0.18
Mantle	0.11 ± 0.01	10.15 ± 0.12	11.14 ± 0.16	12.01 ± 0.24	12.85 ± 0.31	13.12 ± 0.16

Table 4.17: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 0.5 ppm WAF of LDO.

Parameter	Treatment				
	0.5 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	5.25 ± 0.19	6.12 ± 0.19	6.35 ± 0.21	7.14 ± 0.12	7.61 ± 0.25
Hepatopancreas	4.40 ± 0.25	5.21 ± 0.18	6.25 ± 0.18	7.20 ± 0.15	7.41 ± 0.16
Adductor muscle	2.31 ± 0.30	2.60 ± 0.29	3.21 ± 0.85	3.49 ± 0.25	3.92 ± 0.16
Mantle	3.28 ± 0.21	3.75 ± 0.15	4.12 ± 0.15	4.31 ± 0.19	5.15 ± 0.09
Catalase (μmol/min/mg protein)					
Hepatopancreas	10.20 ± 0.18	10.41 ± 0.25	11.08 ± 0.19	11.42 ± 0.12	11.61 ± 0.09
Gill	5.18 ± 0.14	5.25 ± 0.07	5.40 ± 0.08	5.96 ± 0.06	6.12 ± 0.07
Adductor muscle	3.42 ± 0.18	3.61 ± 0.05	4.05 ± 0.15	4.29 ± 0.15	4.55 ± 0.15
Mantle	5.49 ± 0.25	5.70 ± 0.82	6.08 ± 0.09	6.25 ± 0.06	7.00 ± 0.09
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	5.35 ± 0.49	5.81 ± 0.06	6.20 ± 1.50	6.50 ± 1.23	6.64 ± 1.15
Gill	4.6 ± 0.31	4.49 ± 0.13	4.52 ± 0.85	4.69 ± 0.12	5.82 ± 0.10
Adductor muscle	2.16 ± 0.45	2.46 ± 0.07	2.65 ± 0.16	2.96 ± 0.84	3.25 ± 0.19
Mantle	3.66 ± 0.35	4.15 ± 0.12	4.25 ± 0.08	4.51 ± 0.16	4.60 ± 0.12
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.46 ± 0.26	4.52 ± 0.02	4.69 ± 0.18	5.12 ± 0.18	5.31 ± 0.25
Gill	4.30 ± 0.12	4.39 ± 0.15	4.45 ± 0.07	4.69 ± 0.18	5.20 ± 0.12
Adductor muscle	2.41 ± 1.05	2.61 ± 0.18	2.72 ± 0.15	2.85 ± 0.12	3.25 ± 0.16
Mantle	1.69 ± 0.25	1.72 ± 0.19	1.81 ± 0.45	2.12 ± 0.18	2.45 ± 0.15
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	12.25 ± 0.14	13.25 ± 0.15	13.44 ± 0.14	14.22 ± 0.29	15.15 ± 0.25
Gill	11.20 ± 0.09	11.34 ± 0.16	11.51 ± 0.16	12.09 ± 1.25	12.34 ± 0.24
Adductor muscle	10.05 ± 0.15	10.19 ± 0.25	10.31 ± 0.24	10.35 ± 0.16	10.44 ± 0.25
Mantle	10.29 ± 0.14	10.39 ± 0.14	12.14 ± 0.25	12.92 ± 0.38	13.19 ± 0.15

Table 4.18: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 2 ppm WAF of LDO.

Parameter	Treatment				
	2 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	5.36 ± 0.13	6.25 ± 0.07	6.55 ± 0.07	7.25 ± 0.05	7.72 ± 0.08
Hepatopancreas	4.52 ± 0.06	5.29 ± 0.09	6.33 ± 0.14	7.48 ± 0.12	8.01 ± 0.26
Adductor muscle	2.65 ± 0.35	3.05 ± 0.14	3.39 ± 0.08	4.01 ± 0.16	4.22 ± 0.15
Mantle	4.01 ± 0.22	4.24 ± 0.19	5.15 ± 0.04	5.29 ± 0.12	6.01 ± 0.25
Catalase (μmol/min/mg protein)					
Hepatopancreas	10.35 ± 0.24	10.52 ± 0.07	11.16 ± 0.29	11.52 ± 0.11	11.85 ± 0.12
Gill	6.12 ± 0.07	6.28 ± 0.06	6.54 ± 0.16	6.83 ± 0.12	7.14 ± 0.01
Adductor muscle	4.01 ± 0.12	4.15 ± 0.13	4.31 ± 0.29	4.49 ± 0.23	5.8 ± 0.01
Mantle	5.88 ± 0.01	5.92 ± 0.07	6.15 ± 0.11	6.35 ± 0.08	6.52 ± 0.13
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	6.01 ± 0.02	6.12 ± 0.19	6.34 ± 0.25	6.81 ± 0.15	7.05 ± 0.08
Gill	5.02 ± 0.15	5.13 ± 0.28	5.31 ± 0.14	5.48 ± 0.07	5.62 ± 0.06
Adductor muscle	2.42 ± 0.02	2.68 ± 0.15	2.75 ± 0.22	2.92 ± 0.25	3.01 ± 0.24
Mantle	3.99 ± 0.01	4.05 ± 0.25	4.11 ± 0.15	4.30 ± 0.14	4.45 ± 0.02
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	4.58 ± 0.35	4.69 ± 0.05	4.75 ± 0.02	4.92 ± 0.15	5.25 ± 0.07
Gill	4.50 ± 0.29	4.61 ± 0.17	4.72 ± 0.15	4.84 ± 0.08	5.00 ± 0.08
Adductor muscle	2.82 ± 0.16	2.95 ± 0.22	3.01 ± 0.15	3.15 ± 0.06	3.29 ± 0.14
Mantle	2.39 ± 0.34	2.42 ± 0.29	2.65 ± 0.20	2.82 ± 0.18	3.15 ± 0.07
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	12.42 ± 0.06	12.61 ± 0.07	12.85 ± 0.15	13.18 ± 0.12	13.34 ± 0.07
Gill	11.90 ± 0.05	11.96 ± 0.12	12.08 ± 0.12	12.25 ± 0.14	13.05 ± 0.08
Adductor muscle	10.41 ± 0.14	10.63 ± 0.22	10.85 ± 0.29	11.10 ± 0.05	11.42 ± 0.18
Mantle	11.10 ± 0.11	11.29 ± 0.18	11.45 ± 0.07	11.88 ± 0.12	12.19 ± 0.09

Table 4.19: Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to 4.8 ppm WAF of LDO.

Parameter	Treatment				
	4.8 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
SOD (units/mg protein)					
Gill	5.44 ± 0.07	6.59 ± 0.06	6.82 ± 0.19	7.34 ± 0.09	8.05 ± 0.12
Hepatopancreas	4.78 ± 0.05	5.30 ± 0.18	6.39 ± 0.12	7.52 ± 0.14	8.12 ± 0.19
Adductor muscle	3.06 ± 0.12	3.25 ± 0.23	3.41 ± 0.05	4.15 ± 0.18	4.33 ± 0.07
Mantle	4.16 ± 0.07	4.29 ± 0.07	5.28 ± 0.12	5.45 ± 0.22	6.16 ± 0.03
Catalase (μmol/min/mg protein)					
Hepatopancreas	10.41 ± 0.08	10.69 ± 0.05	11.20 ± 0.14	11.83 ± 0.18	11.95 ± 0.18
Gill	6.29 ± 0.15	6.42 ± 0.03	6.65 ± 0.01	6.93 ± 0.11	7.29 ± 0.22
Adductor muscle	4.12 ± 0.08	4.32 ± 0.08	4.52 ± 0.05	5.13 ± 0.19	5.35 ± 0.05
Mantle	5.93 ± 0.07	6.02 ± 0.18	6.31 ± 0.12	6.58 ± 0.12	7.00 ± 0.08
Glutathione peroxidase (μg of GSH/min/mg protein)					
Hepatopancreas	6.13 ± 0.01	6.29 ± 0.02	6.42 ± 0.07	6.96 ± 0.08	7.12 ± 0.02
Gill	5.14 ± 0.22	5.28 ± 0.13	5.42 ± 0.06	5.63 ± 0.15	6.15 ± 0.15
Adductor muscle	2.65 ± 0.12	2.81 ± 0.14	3.12 ± 0.05	3.49 ± 0.10	3.61 ± 0.08
Mantle	4.08 ± 0.11	4.25 ± 0.12	4.40 ± 0.19	4.65 ± 0.25	4.81 ± 0.18
Glutathione reductase (GR) (units/mg protein)					
Hepatopancreas	5.16 ± 0.15	5.25 ± 0.06	5.41 ± 0.15	5.69 ± 0.07	6.15 ± 0.08
Gill	5.00 ± 0.29	5.34 ± 0.15	5.52 ± 0.06	6.01 ± 1.24	6.35 ± 0.04
Adductor muscle	3.10 ± 0.19	3.29 ± 0.06	3.56 ± 0.04	3.85 ± 0.12	4.10 ± 0.12
Mantle	3.65 ± 0.25	3.82 ± 0.05	4.11 ± 0.05	4.39 ± 0.09	4.52 ± 0.06
Glutathione (GSH) (nmoles/100 g wet tissue)					
Hepatopancreas	12.65 ± 0.03	12.72 ± 0.02	13.10 ± 0.04	13.49 ± 0.06	14.15 ± 0.07
Gill	12.00 ± 0.01	12.34 ± 0.07	12.46 ± 0.06	13.19 ± 0.03	13.26 ± 0.06
Adductor muscle	10.60 ± 0.09	10.75 ± 0.04	11.29 ± 0.03	11.53 ± 0.01	11.88 ± 0.13
Mantle	11.25 ± 0.15	11.49 ± 0.03	11.92 ± 0.04	12.09 ± 0.04	12.35 ± 0.05

Table 4.20: Three way ANOVA Table showing Antioxidant enzyme activity in the different tissues of *Perna viridis* exposed to different concentration of WAF of LDO. (a – e)

a) SOD

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	164.376	54.792	2522.436	<0.001
PAH CONC	**3**	5.360	1.787	82.248	<0.001
TIME	**5**	120.873	24.175	1112.916	<0.001
Residual	**45**	0.977	0.0217		
Total	**95**	301.079	3.169		

b) Catalase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	615.804	205.268	7022.523	<0.001
PAH CONC	**3**	4.821	1.607	54.977	<0.001
TIME	**5**	39.827	7.965	272.509	<0.001
Residual	**45**	1.315	0.0292		
Total	**95**	667.422	7.025		

c) Glutathione peroxidase

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	143.522	47.841	2633.359	<0.001
PAH CONC	**3**	2.654	0.885	48.690	<0.001
TIME	**5**	56.691	11.338	624.110	<0.001
Residual	**45**	0.818	0.0182		
Total	**95**	208.642	2.196		

d) Glutathione reductase (GR)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	102.484	34.161	1544.299	<0.001
PAH CONC	**3**	17.655	5.885	266.043	<0.001
TIME	**5**	30.513	6.103	275.876	<0.001
Residual	**45**	0.995	0.0221		
Total	**95**	160.396	1.688		

e) Glutathione (GSH)

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	66.628	22.209	191.598	<0.001
PAH CONC	**3**	2.270	0.757	6.528	<0.001
TIME	**5**	1885.489	377.098	3253.211	<0.001
Residual	**45**	5.216	0.116		
Total	**95**	1985.143	20.896		

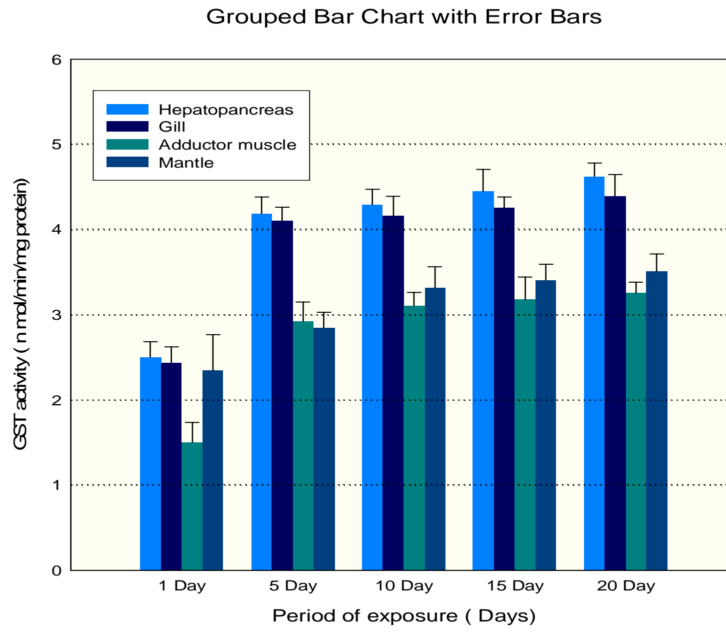


Figure 4.1: GST activity in various tissues of *P. viridis* exposed to 0.1 ppm of BHC WAF

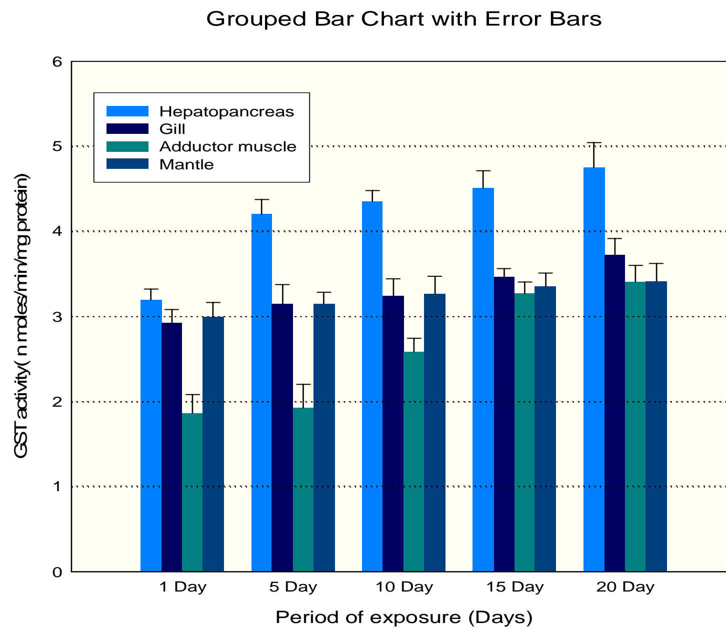


Figure 4.2: GST activity in various tissues of *P. viridis* exposed to 1 ppm of BHC WAF

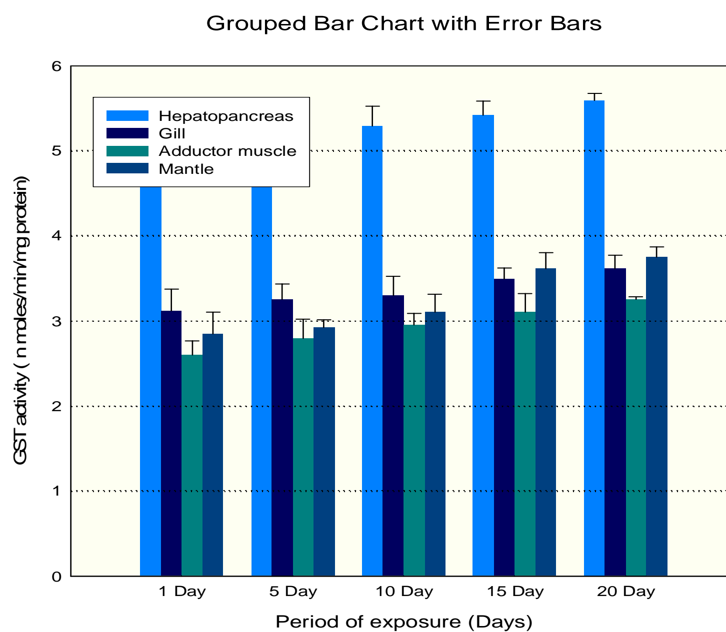


Figure 4.3: GST activity in various tissues of *P. viridis* exposed to 5 ppm of BHC WAF

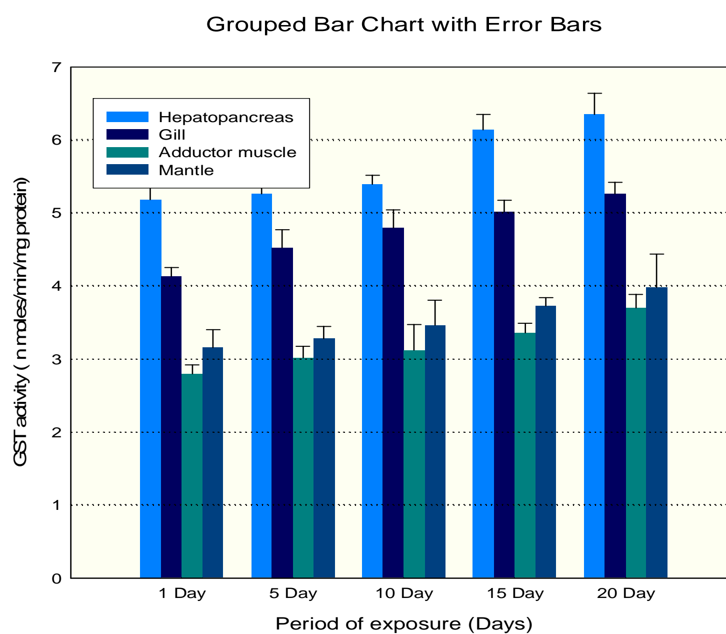


Figure 4.4: GST activity in various tissues of *P. viridis* exposed to 8 ppm of BHC WAF

Table 4.21: Three way ANOVA table showing GST activity in the different tissues of *Perna viridis* exposed to different concentration of WAF of BHC.

Source of variation	**df**	**Sum of Squares**	**Mean Square**	**F**	**P-value**
TISSUES	**3**	34.745	11.582	201.911	<0.001
PAH CONC	**3**	8.987	2.996	52.223	<0.001
TIME	**5**	177.044	35.409	617.301	<0.001
Residual	**45**	2.581	0.0574		
Total	**95**	240.451	2.531		

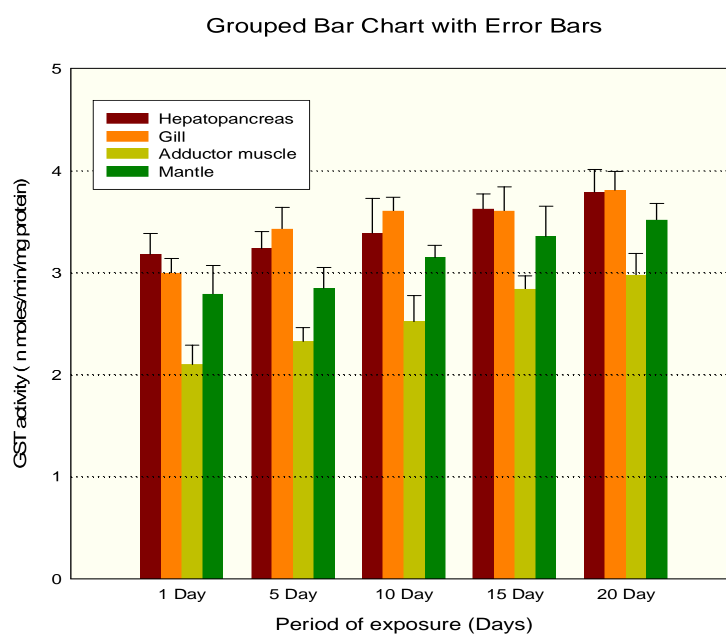


Figure 4.5: GST activity in various tissues of *P. indica* exposed to 0.1 ppm of BHC WAF

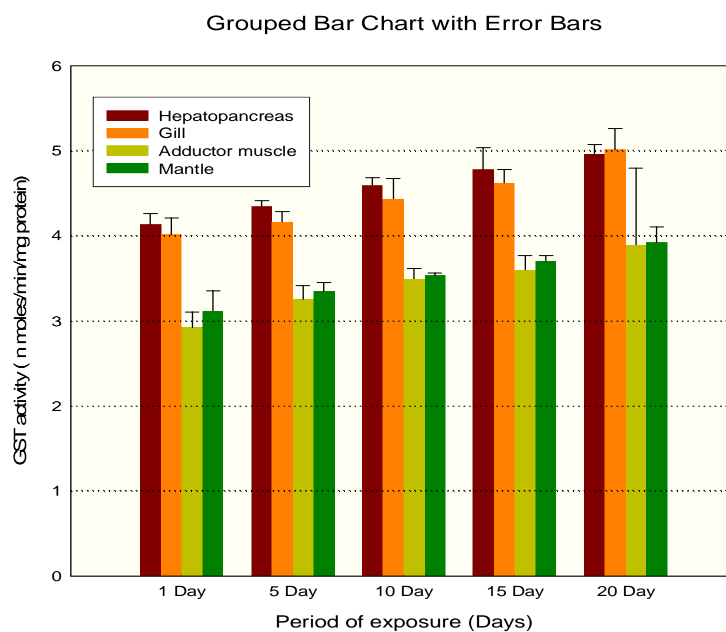


Figure 4.6: GST activity in various tissues of *P. indica* exposed to 0.6 ppm of BHC WAF

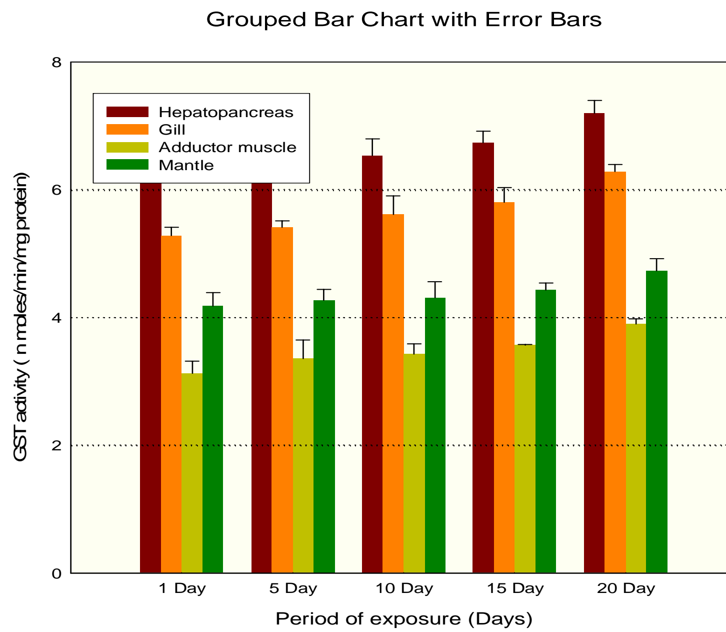


Figure 4.7: GST activity in various tissues of *P. indica* exposed to 2 ppm of BHC WAF

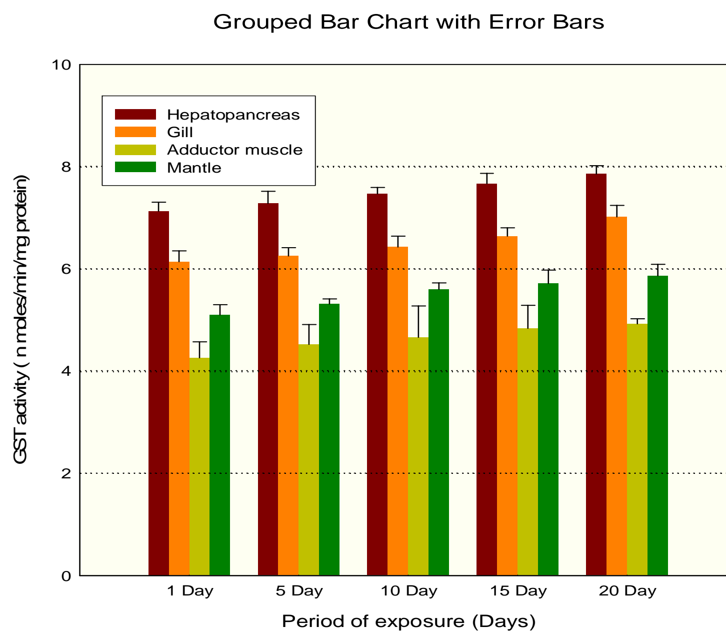


Figure 4.8: GST activity in various tissues of *P. indica* exposed to 5 ppm of BHC WAF

Table 4.22: Three way ANOVA table showing GST activity in the different tissues of *Perna indica* exposed to different concentration of WAF of BHC.

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	42.262	14.087	298.451	<0.001
PAH CONC	**3**	77.857	25.952	549.817	<0.001
TIME	**5**	254.154	50.831	1076.875	<0.001
Residual	**45**	2.124	0.0472		
Total	**95**	407.381	4.288		

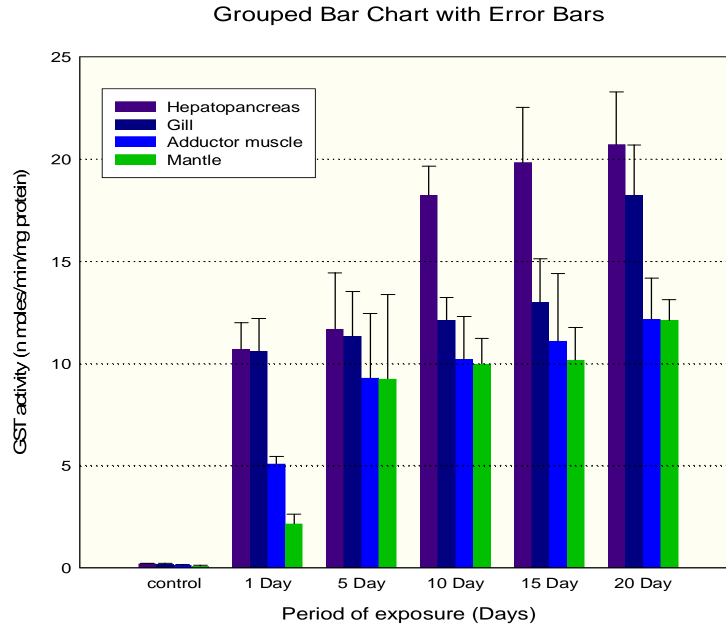


Figure 4.9: GST activity in various tissues of *P. viridis* exposed to 0.05 ppm of LDO WAF

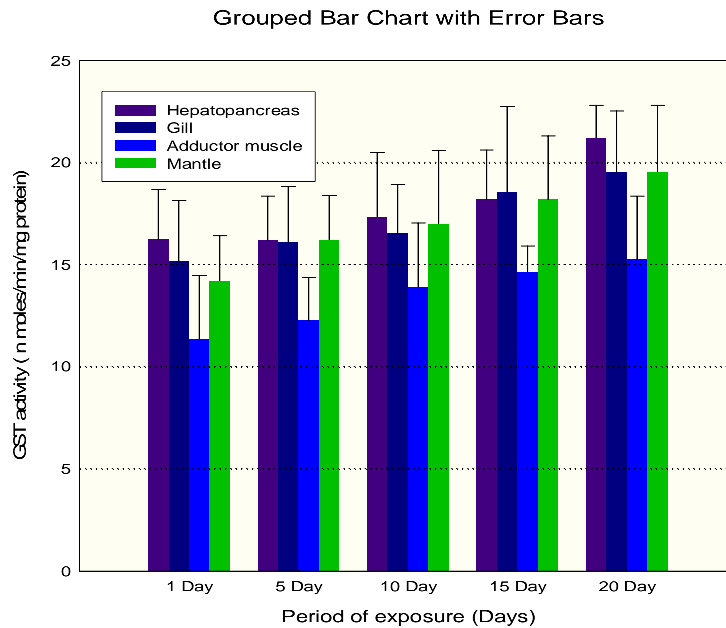


Figure 4.10: GST activity in various tissues of *P. viridis* exposed to 0.5 ppm of LDO WAF

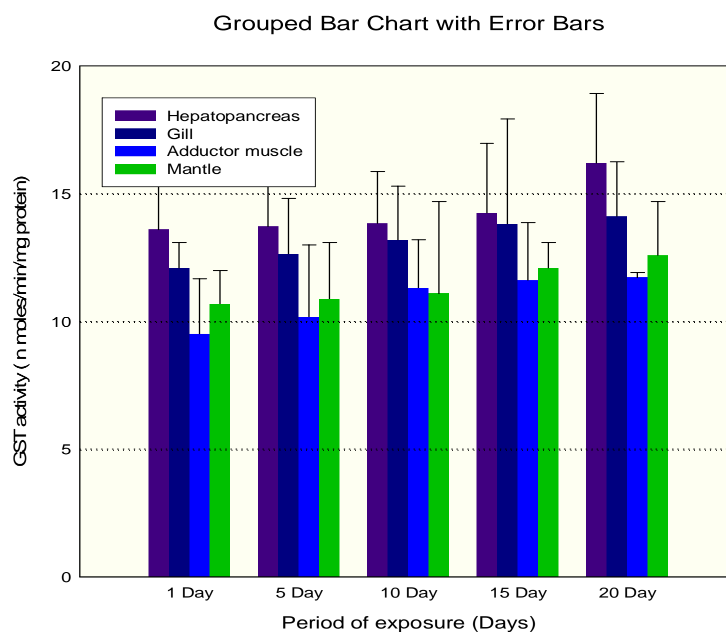


Figure 4.11: GST activity in various tissues of *P. viridis* exposed to 2 ppm of LDO WAF

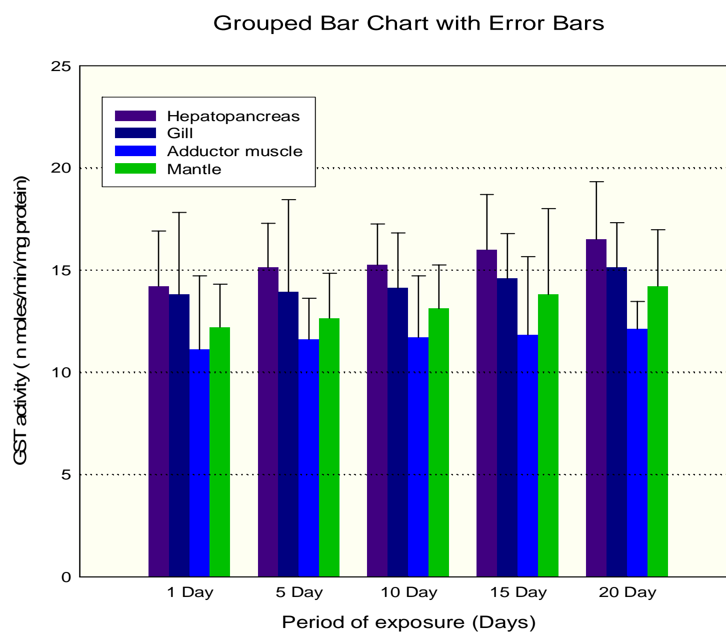


Figure 4.12: GST activity in various tissues of *P. viridis* exposed to 4. ppm of LDO WAF

Table 4.23: Three way ANOVA table showing GST activity in the different tissues of *Perna viridis* exposed to different concentration of WAF of LDO.

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	202.516	67.505	69.242	<0.001
PAH CONC	**3**	198.038	66.013	67.711	<0.001
TIME	**5**	2574.831	514.966	528.216	<0.001
Residual	**45**	43.871	0.975		
Total	**95**	3242.354	34.130		

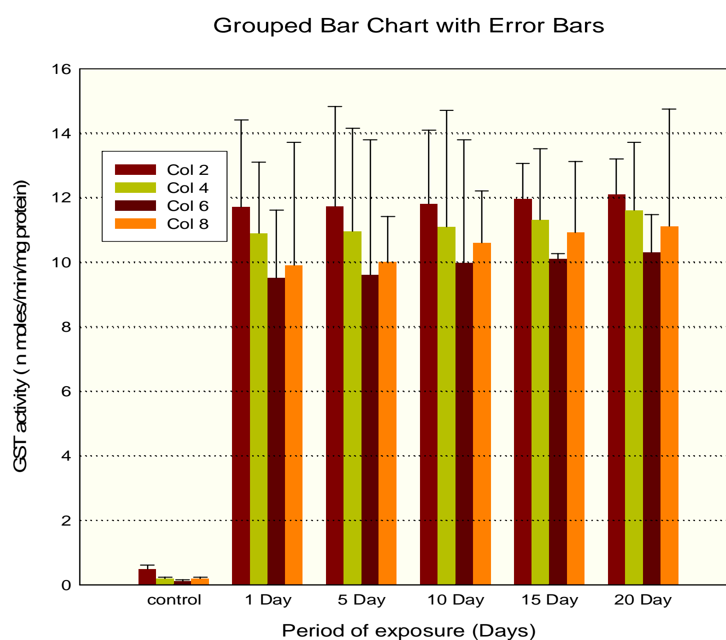


Figure 4.13: GST activity in various tissues of *P. indica* exposed to 0.04 ppm of LDO WAF

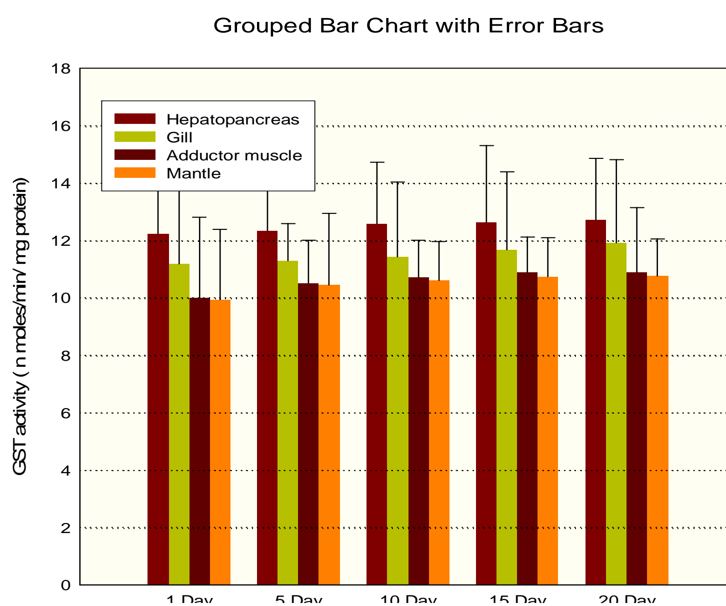


Figure 4.14: GST activity in various tissues of *P. indica* exposed to 0.4 ppm of LDO WAF

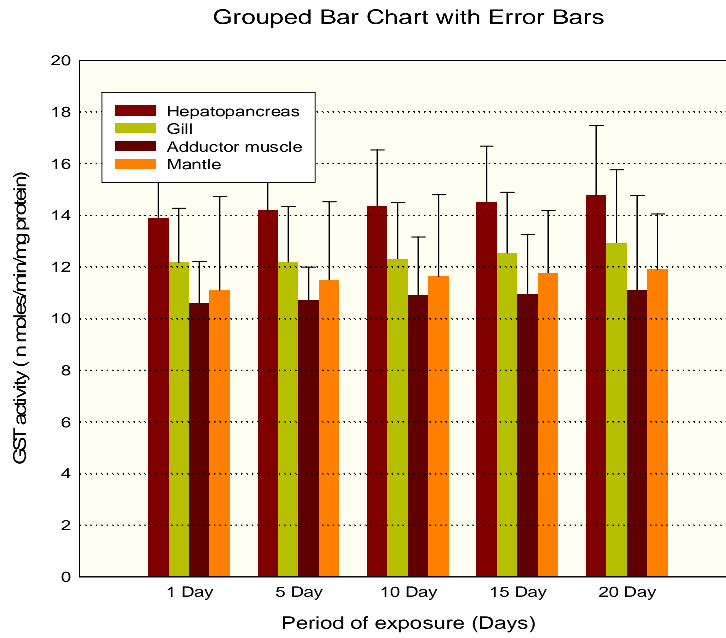


Figure 4.15: GST activity in various tissues of *P. indica* exposed to 1 ppm of LDO WAF

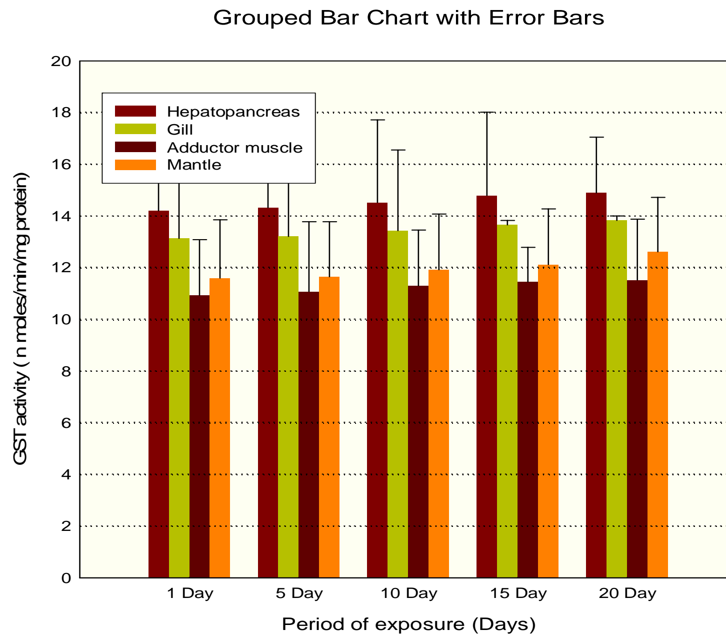


Figure 4.16: GST activity in various tissues of *P. indica* exposed to 3.5 ppm of LDO WAF

Table 4.24: Three way ANOVA table showing GST activity in the different tissues of *Perna indica* exposed to different concentration of WAF of LDO.

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	72.996	24.332	655.288	<0.001
PAH CONC	**3**	39.933	13.311	358.480	<0.001
TIME	**5**	1789.793	357.959	9640.202	<0.001
Residual	**45**	1.671	0.0371		
Total	**95**	1929.475	20.310		

Table 4.25: Levels of Lipid Peroxidation Products in the different tissues of *Perna indica* exposed to different concentration WAF of BH Crude oil.

Parameter	Treatment					
	0.1 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas	11.06 ± 0.62	11.25 ± 0.12	11.64 ± 0.20	11.98 ± 0.16	12.15 ± 0.13	12.49 ± 0.12
Gill	6.72 ± 0.16	6.85 ± 0.15	6.92 ± 0.17	7.01 ± 0.22	7.18± 0.17	7.63 ± 0.12
Adductor muscle	8.18 ± 0.24	8.29 ± 0.22	8.44 ± 0.29	9.01 ± 0.19	10.18 ± 0.12	10.23 ± 0.16
Mantle	10.12 ± 0.18	10.24 ± 0.19	10.32 ± 0.23	10.55 ± 0.19	10.63 ± 0.17	10.72 ± 0.11
Parameter	**Treatment**					
	0.6 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas	11.06 ± 0.62	12.10 ± 0.17	12.21 ± 0.09	12.77 ± 0.07	13.06 ± 0.18	14.16 ± 0.12
Gill	6.72 ± 0.16	8.22 ± 0.09	8.44 ± 0.14	8.76 ± 0.13	8.99 ± 0.17	9.12 ± 0.16
Adductor muscle	8.18 ± 0.24	10.13 ± 0.19	10.25 ± 0.12	10.77 ± 0.09	11.10 ± .13	11.21 ± 0.17
Mantle	10.12 ± 0.18	12.14 ± 0.12	12.22 ± 0.02	12.45 ± 0.01	12.95 ± 0.19	13.01 ± 0.11
Parameter	**Treatment**					
	0.2 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas	11.06 ± 0.62	13.14 ± 0.1	13.22 ± 0.19	14.18 ± 0.18	14.54 ± 0.12	15.02 ± 0.15
Gill	6.72 ± 0.16	8.49 ± 0.12	8.73 ± 0.07	8.91 ± 0.19	9.12 ± 0.16	9.41 ± 0.17
Adductor muscle	8.18 ± 0.24	11.01 ± 0.11	11.44 ± 0.13	11.54 ± 0.17	11.78 ± 0.11	12.15 ± 0.19
Mantle	10.12 ± 0.18	13.08 ± 0.14	13.34 ± 0.04	13.56 ± 0.19	14.12 ± 0.12	14.64 ± 0.34
Parameter	**Treatment**					
	0.5 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas	11.06 ± 0.62	12.20 ± 0.13	12.34 ± 0.14	12.48 ± 0.07	12.74 ± 0.04	12.91 ± 0.05
Gill	6.72 ± 0.16	7.44 ± 0.20	7.56 ± 0.03	7.64 ± 0.01	7.89 ± 0.08	8.01 ± 0.09
Adductor muscle	8.18 ± 0.24	9.19 ± 0.27	9.25 ± 0.04	9.39 ± 0.02	9.52 ± 0.02	9.72 ± 0.01
Mantle	10.12 ± 0.18	11.18 ± 0.14	11.25 ± 0.07	11.36 ± 0.01	11.77 ± 0.07	11.98 ± 0.05

Malondialdehyde Three way ANOVA

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	442.371	147.457	1055.610	<0.001
PAH CONC	**3**	85.380	28.460	203.738	<0.001
TIME	**5**	60.324	12.065	86.370	<0.001
Residual	**45**	6.286	0.140		
Total	**95**	644.900	6.788		

Table 4.26: Levels of Lipid Peroxidation Products in the different tissues of *Perna indica* exposed to different concentrations of WAF of LDO.

Parameter	Treatment					
	0.04 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas		14.14 ± 0.21	14.64 ± 0.18	14.96 ± 0.20	15.01 ± 0.16	15.62 ± 0.17
Gill		6.19 ± 0.72	6.25 ± 0.16	6.44 ± 0.34	6.59 ± 0.24	6.67 ± 0.16
Adductor muscle		11.24 ± 0.16	11.25 ± 0.02	12.14 ± 0.8	12.29 ± 0.14	12.56 ± 0.19
Mantle		12.41 ± 0.13	12.73 ± 0.13	12.83 ± 0.07	13.08 ± 0.11	13.20 ± 0.19
Parameter	**Treatment**					
	0.04 ppm					
	Control	**Control**	**Control**	**Control**	**Control**	**Control**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas		14.23 ± 0.16	14.34 ± 0.22	14.46 ± 0.19	15.16 ± 0.21	15.71 ± 0.31
Gill		6.21 ± 0.13	6.31 ± 0.12	6.52 ± 0.21	6.61 ± 0.23	6.72 ± 0.34
Adductor muscle		11.52 ± 0.24	11.84 ± 0.01	12.28 ± 0.18	12.35 ± 0.24	12.71 ± 0.22
Mantle		12.71 ± 0.19	12.84 ± 0.12	12.94 ± 0.09	13.14 ± 0.19	13.42 ± 0.76
Parameter	**Treatment**					
	1 ppm					
	Control	**Control**	**Control**	**Control**	**Control**	**Control**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas		15.02 ± 0.16	15.19 ± 0.19	15.31 ± 0.18	15.67 ± 0.01	15.74 ± 0.16
Gill		7.14 ± 0.21	7.29 ± 0.28	7.41 ± 0.19	7.52 ± 0.20	7.61 ± 0.19
Adductor muscle		12.14 ± 0.18	12.31 ± 0.19	12.46 ± 0.23	12.59 ± 0.16	12.70 ± 0.17
Mantle		13.18 ± 0.23	13.34 ± 0.23	13.52 ± 0.16	13.64 ± 0.18	13.69 ± 0.20
Parameter	**Treatment**					
	3.5 ppm					
	Control	**Control**	**Control**	**Control**	**Control**	**Control**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas		16.01 ± 0.15	16.20 ± 0.19	16.29 ± 0.18	16.38 ± 0.12	17.08 ± 0.13
Gill		7.20 ± 0.18	7.35 ± 0.20	7.44 ± 0.18	7.52 ± 0.16	7.68 ± 0.16
Adductor muscle		13.28 ± 0.16	13.34 ± 0.13	13.42 ± 0.18	13.54 ± 0.20	13.99 ± 0.07
Mantle		14.15 ± 0.7	14.31 ± 0.10	14.56 ± .13	14.79 ± 0.22	15.21 ± 0.16

Malondialdehyde ANOVA

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	708.110	236.037	742.454	<0.001
PAH CONC	**3**	225.907	75.302	236.864	<0.001
TIME	**5**	871.165	174.233	548.051	<0.001
Residual	**45**	14.306	0.318		
Total	**95**	1973.577	20.774		

Table 4.27: Levels of Lipid Peroxidation Products in the different tissues of *Perna viridis* exposed to different concentration WAF of BH Crude oil.

Parameter	Treatment				
	0.1 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Malondialdehyde (mmoles/100 g wet wt. tissue)					
Hepatopancreas	10.18 ± 0.01	11.06 ± 0.01	11.89 ± 0.01	12.08 ± 0.01	12.49 ± 0.02
Gill	4.33 ± 0.18	4.69 ± 0.17	4.72 ± 0.19	4.95 ± 0.18	5.29 ± 0.12
Adductor muscle	9.16 ± 0.12	9.77 ± 0.18	10.20 ± 0.19	10.97 ± 0.18	11.22 ± 0.17
Mantle	8.10 ± 0.13	8.69 ± 0.14	9.25 ± 0.13	9.67 ± 0.24	10.18 ± 0.23
	Treatment				
	1 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Malondialdehyde (mmoles/100 g wet wt. tissue)					
Hepatopancreas	11.20 ± 0.18	11.49 ± 0.22	12.01 ± 0.01	12.52 ± 0.32	13.06 ± 0.01
Gill	5.16 ± 0.05	5.84 ± 0.12	6.02 ± 0.23	6.18 ± 0.22	6.43 ± 0.18
Adductor muscle	10.06 ± 0.12	10.21 ± 0.14	10.48 ± 0.12	10.76 ± 0.18	12.06 ± 0.17
Mantle	9.48 ± 0.08	9.82 ± 0.16	9.96 ± 0.13	10.01 ± 0.24	10.56 ± 0.36
	Treatment				
	5 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Malondialdehyde (mmoles/100 g wet wt. tissue)					
Hepatopancreas	12.19 ± 0.21	12.64 ± 0.18	13.19 ± 0.20	14.21 ± 0.18	15.16 ± 0.22
Gill	7.16 ± 0.19	8.10 ± 0.20	8.49 ± 0.18	9.08 ± 0.07	10.02 ± 0.19
Adductor muscle	11.10 ± 0.02	12.18 ± 0.08	12.40 ± 0.09	12.56 ± 0.08	14.56 ± 0.18
Mantle	9.18 ± 0.01	9.22 ± 0.07	9.64 ± 0.08	10.16 ± 0.10	12.30 ± 0.09
	Treatment				
	8 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Malondialdehyde (mmoles/100 g wet wt. tissue)					
Hepatopancreas	12.46 ± 0.18	12.74 ± 0.17	14.00 ± 0.19	14.93 ± 0.18	15.30 ± 0.31
Gill	7.31 ± 0.07	8.22 ± 0.19	8.56 ± 0.07	10.26 ± 0.03	10.79 ± 0.08
Adductor muscle	11.68 ± 0.01	12.10 ± 0.20	12.64 ± 0.08	13.18 ± 0.07	14.96 ± 0.08
Mantle	10.17 ± 0.03	10.43 ± 0.17	10.56 ± 0.11	10.97 ± 0.20	14.06 ± 0.19

Malondialdehyde ANOVA

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	14.736	4.912	49.942	<0.001
PAH CONC	**3**	81.714	27.238	276.945	<0.001
TIME	**5**	37.390	7.478	76.033	<0.001
Residual	**45**	4.426	0.0984		
Total	**95**	181.777	1.913		

Table 4.28: Levels of Lipid Peroxidation Products in the different tissues of *Perna viridis* exposed to different concentration WAF of LDO.

Parameter	Treatment					
	0.05 ppm					
	Control	1 Day	5 Day	10 Day	15 Day	20 Day
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas	12.46 ± 0.65	13.21 ± 0.90	14.02 ± 0.18	15.02 ± 0.24	15.73 ± 0.95	16.52 ± 0.85
Gill	6.02 ± 0.54	6.21 ± 0.83	6.98 ± 0.92	7.08 ± 0.28	8.63 ± 0.90	12.00 ± 0.84
Adductor muscle	10.2 ± 0.80	11.50 ± 0.64	12.60 ± 0.90	14.00 ± 0.18	17.15 ± 0.43	19.68 ± 0.18
Mantle	11.0 ± 1.2	12.68 ± 0.32	13.74 ± 0.50	15.24 ± 0.10	16.07 ± 0.60	20.0 ± 0.80
Parameter	**Treatment**					
	0.5 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas		14.00 ± 0.70	14.74 ± 0.19	15.92 ± 0.14	15.98 ± 0.24	16.79 ± 0.13
Gill		7.98 ± 0.56	8.10 ± 0.92	9.14 ± 0.42	9.65 ± 0.33	14.90 ± 0.35
Adductor muscle		12.40 ± 0.35	12.79 ± 0.84	14.64 ± 0.13	18.12 ± 0.14	20.18 ± 0.19
Mantle		13.12 ± 0.18	14.10 ± 0.92	15.64 ± 0.10	16.46 ± 0.18	21.01 ± 0.92
Parameter	**Treatment**					
	2 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas		15.12 ± 0.22	16.14 ± 0.18	16.48 ± 0.13	16.88 ± 0.24	17.42 ± 0.14
Gill		8.01 ± 0.28	8.42 ± 0.94	9.80 ± 0.31	10.12 ± 0.13	16.18 ± 0.13
Adductor muscle		13.52 ± 0.14	14.16 ± 0.18	18.28 ± 0.19	20.16 ± 0.32	22.16 ± 0.13
Mantle		15.13 ± 0.17	15.87 ± 0.35	17.04 ± 0.24	19.28 ± 0.43	23.14 ±0.42
Parameter	**Treatment**					
	4.8 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Malondialdehyde (mmoles/100 g wet wt. tissue)						
Hepatopancreas		16.13 ± 0.16	18.01 ± 0.91	19.04 ± 0.16	20.19 ± 0.19	21.12 ± 0.90
Gill		10.64 ± 0.22	11.44 ± 0.22	12.18 ± 0.31	14.64 ± 0.22	19.19 ± 0.24
Adductor muscle		14.20 ± 0.17	18.19 ± 0.18	21.16 ± 0.24	23.05 ± 0.18	24.17 ± 0.19
Mantle		16.46 ± 0.13	17.09 ± 0.23	19.44 ± 0.18	23.14 ± 0.20	25.96 ± 0.17

Malondialdehyde ANOVA

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	91.967	30.656	98.498	<0.001
PAH CONC	**3**	1142.699	380.900	1223.842	<0.001
TIME	**5**	734.465	146.893	471.971	<0.001
Residual	**45**	14.005	0.311		
Total	**95**	2443.157	25.717		

4.4 Discussion

Reactive oxygen species (ROS) are produced by organisms in the metabolism of polycyclic aromatic hydrocarbons (PAHs), which are considered the most toxic components present in crude oils (Anderson *et al.*, 1974; Cheung *et al.,* 2001). Enhanced production of ROS may lead to oxidative stress, possibly resulting in damage such as mutagenesis, carcinogenesis, protein oxidation and degradation, carbohydrate damage, and lipid peroxidation (Livingstone *et al.*, 1990; Winston and Di Giulio, 1991; Sole *et al.,* 1995). Peroxidation of unsaturated lipids, by ROS and their breakdown products, results in the formation of unstable hydroperoxides, part of the cascade of events caused by oxidative stress which may lead to cell death.. Antioxidant systems act to prevent oxidative damage by eliminating the ROS and they may be induced as an adaptive response after exposure to PAHs, allowing an organism to partially or totally overcome oxidative stress in a polluted environment (Livingstone *et al.,* 1990; Di Giulio *et al.,* 1989; Winston and Di Giulio, 1991). Aromatic hydrocarbons have the potential to enhance oxyradical formation in invertebrates, through redox-cycling and impairment of cellular antioxidant system (Livingstone 2001; Regoli *et al.,* 2003).Prolonged exposure to petroleum contaminants may also, however, result in depletion of components of the antioxidant response as a toxic effect. When the pro-oxidant forces overwhelm the antioxidant defences a cellular oxidative stress is established (Bainy *et al.,* 1996; Doyotte *et al.,* 1997). Induction or depletion of antioxidant defence components (including the nonenzymatic glutathione and enzyme glutathione peroxidase, GPx) and oxidative damage (including lipid peroxidation) reflect responses to organic pollutants and they have been proposed as early warning biomarkers of exposure and effect (Cossu *et al.,* 1997; Cheung *et al.,* 2001).

Altered antioxidant enzyme activities, on exposure to organic xenobiotics, have been detected in a number of molluscan species (Livingstone *et al.,* 1990; Sole *et al.,* 1995). Studies have demonstrated the potential for glutathione antioxidant system

responses to act as biomarkers in bivalves exposed to PAHs. However, results have varied between studies, with both significantly enhanced (Cheung *et al.,* 2001) and depleted (Cossu *et al.,* 1997; Doyotte *et al.,* 1997) glutathione and GPx levels correlated with contaminant body burdens, depending on the particular species employed, pollutant concentration, and exposure period. Generally, the inducible enzymes initially increase on exposure to pollutant, then synthesis declines with deteriorating animal condition caused by chronic pollutant-mediated stress (Winston and DiGiulio, 1991). Increases in lipid peroxidation for bivalves exposed to PAH contaminated sediments have also shown potential as biomarkers (Cossu *et al.*, 1997).

The activity of SOD is high in gills, which indicates the greater potential of O_2^- production in gills during respiration (normal oxygen metabolism).

The specific activities of CAT and GSH-Px were found to be positive in all tissues (hepatopancreas, gill, adductor muscle and mantle). The highest activities of enzymes were observed in hepatopancreas followed by gill, mantle and adductor muscle. This result is in agreement with the notion that hepatopancreas is the major site for xenobiotic update and oxyradical generating biotransformation enzymes. It is the site of multiple oxidative reactions and may, therefore, be the site of maximal free radical generation.

During normal physiological conditions, antioxidant enzymes get activated to protect cells and tissues from oxidative injury. Superoxide dismutase scavenges superoxide radical to H_2O_2, which further gets neutralized by catalase or glutathione peroxidase. Exposure to contaminants generate ROS which may induce or inhibit the antioxidant defense mechanism of the animals. The increased values shown by the antioxidant enzymes in the present investigations could help in controlling the oxidative damage caused by WAF of PAHs and thereby protect the cell from further injury.

The importance of CAT as an antioxidant lies in its involvement with Haber-Weiss reaction, which removes H_2O_2 to generate extremely reactive molecules such as

OH (Winston and DiGiulio, 1991).The increased CAT activity might indicate that cells were subjected to ROS.

There was a significant dose-dependent induction of GPx in all the tissues exposed to WAF dilutions in the laboratory, reflecting a compensatory adaptation of the species to a contaminated environment.There were no significant changes in glutathione levels in animals exposed to higher WAF dilution concentrations.Increases in GPx defence response reflect that animals were under oxidative stress as the result of exposure to pro-oxidant compounds present in the WAF. Induction of the enzymatic catalyst (GPx) indicates that the existing pool of glutathione is being utilized in defence against oxidative stress at a greater rate than usual. This compensatory response appeared to be adequate to stave off oxidative damage.

The elevated glutathione reductase activity can be related to the thiol status of cells which leads to regenerate GSH from GSSG, resulting in increased level of GSH observed during experimental period.

Reduced glutathione (GSH) is one of the main soluble antioxidants acting both as direct scavengers of oxyradical and as a cofactor for several antioxidant enzymes

On the other hand, the exposure of mussels to PAHs resulted in the concentration- dependent increase of GST activity. Presumably, the enhanced conjugation activity indicates that this route may be one of the ways of elimination PAHs in Bivalves. Previously it was shown that sulphate and glucuronide conjugations are the major detoxification pathways for xenobiotics in fish (Stehly and Hayton, 1989).

Glutathione-S-transferase, phase II detoxifying enzyme systems has been used as a biomarker of organic industrial pollutants (Fitzpatrick *et al.,* 1997).The increased activity of GST- a multifunctional protein suggests that it increases the conjugation of epoxides of PAHs with the tripeptide glutathione, the resulting conjugates being water

soluble and thus more easily excretable, thereby minimizing the cellular damages caused by petroleum hydrocarbons .

The measurement of lipid peroxides, in the tissues is an indicator of the amount of free radical damage to lipids in the body tissues at a given time.

All marine organisms are known to contain high levels of polyunsaturated fatty acids, which are the substrates for lipid peroxidation, and the presence of antiooxidants elevates the resistance of their cell membranes to oxidative stress (Rudneva, 1999).

The present study has shown that the level of lipid peroxidation has significantly increased in tissues of different concentrations of WAF when compared with control animals.The increased level of lipid peroxidation might have lead to the production of malondialdehyde (MDA) enhancing the formation of free radicals on polyunsaturated fatty acids in the cell membranes.

Lipid peroxidation (LPx), an indicator of damage to cell membranes, occurs when free radicals react with lipids, and is a source of cytotoxic products that may damage DNA and enzymes.Increased lipid peroxidation has been demonstrated in response to ischemia-reperfusion events in mammalian tissues, paraquat and contaminant exposures in bivalves, cadmium and PCB exposures in mullet, and exposures of catfish to PAH contaminated sediments (Wenning *et al.,* 1988;; Regoli, 1992; Di Giulio *et al.,* 1995; Livingstone, 2001). Laboratory exposures to copper have shown increased lipid peroxidation levels in digestive gland tissues from *Crassostrea virginica* (Ringwood *et al.,* 1998a; Conners and Ringwood, 2000).

In addition, the extensive lipid peroxidation especially of the highly unsaturated fatty acids, altering the ratio of polyunsaturated to other fatty acids could have resulted in decreased membrane fluidity and membrane disorganization (Chen and Yu, 1994).

The formation of MDA is one of the oldest and most frequently used test for measurement of lipid peroxidation by ROS (Gederass *et al.,* 2000).

Considerable and significant rise in these biomarkers provide direct evidence that WAF of PAH exposure is a relevant source of oxidative stress in which local production of oxidative radicals play important roles. The cell's permeability is associated with their membrane stability. Alteration of membrane phospholipids through lipid peroxidation by oxidants that we reported as MDA elevation could cause a loss of membrane stability and integrity leading to increase trans-membrane permeability (Ozyurt *et al.,* 2004).

From the results obtained, it is discernible that significantly elevated level of antioxidant enzymes, found in *Perna* may be a self-protection mechanism against the toxic effect of reactive compounds derived from oxygen metabolism. Thus basal activites of antioxidant enzymes, present in *Perna* play an important role to remove or detoxify the oxyradicals which generated during normal oxygen metabolism.

The glutathione S-transferase family (GST) belongs to the Phase II detoxification enzymes and play an important role in the detoxification and metabolism of many xenobiotics. GST catalyses the combination of a wide variety of substances (including PAH) with glutathione to facilitate their solubility and excretion. CAT are powerful antioxidant enzymes acting against various potentially harmful oxidising agents, the reactive oxygen species (ROS, e.g. HO, H_2O_2), which are produced as intermediates during the detoxification processes. CAT promote the conversion of ROS to water and molecular oxygen. MDA is a metabolite produced by lipid peroxidation of unsaturated fatty acids resulting from the effects of ROS and a marker for the oxidative degradation of cellular membranes.

A marked increase in GSH-Px activity was observed at the exposure of oil effluent to remove the organic hydroperoxides.

In *Perna*, the elevated level of antioxidant enzymes indicate the occurrence of ROS which were generated from phase 1 metabolism.

With these information, it is clear that, an elevated level of antioxidant enzymes, in aquatic organisms could be used as a biomarker for the detection of both ROS and environmental pollution.

Nowadays, interestingly, the induced level of antioxidant enzymes in response to pollutants, are used as a biomarker for the early detection of environmental pollution.

Herein, we have successfully used the molluscan species, *Perna viridis* and *Perna indica*, as the indicator organisms and confirmed that induced level of antioxidant enzymes in response to oil pollution could be used as a biomarker for the early detection of environmental pollution.

Chapter 5

ACETYL CHOLINESTERASE AND LYSOSOMES AS TOXICITY INDICATORS IN *Perna spp.*

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- 5.B.1 Materials And Methods
- 5.B.2 Results
- 5.B.3 Discussion

5.A Acetylcholinesterase

5.A.1 Introduction

Acetylcholinesterase (AChE), is responsible for hydrolysing acetylcholine into choline and acetic acid. Its inhibition is linked directly with the mechanism of toxic action of xenobiotics. AChE activities in blood and tissues emerged as a diagnostic tool in the biomedical area. Subsequently, quantification of this enzyme was applied to laboratory and field studies with both vertebrates and invertebrates to assess exposure to xenobiotics (Mineau, 1991).

In aquatic organisms, there is considerable diversity in the biochemical properties and distribution of cholinesterases, as well as in their sensitivity to anticholinesterase agents (Habig *et al.*, 1988; Bocquene *et al.*, 1990).

Acetylcholinesterase (AChE) catalyzes the hydrolysis (and hence inactivation) of the neurotransmitter acetylcholine (ACh) in cholinergic nerves .Inhibition of AChE resulting in over-accumulation of ACh and prolonged electrical activity at nerve endings comprises a key mechanism of toxicity for xenobiotics. AChE inhibition occurs through the formation of a covalent bond between the active site of the enzyme and the PAHs. The consequences of AChE inhibition depend upon the nervous tissue affected and include cardiovascular, respiratory and gastrointestinal dysfunction (parasympathetic autonomic tissue), hyperactivity, lethargy and unconsciousness (central nervous system), and muscle weakness, respiratory collapse and paralysis (somatic motor nerve fibers).

Cholinesterases are a ubiquitous class of serine hydrolases that hydrolyze choline esters with various efficiency.

The research has now been addressed to Bivalvia, a class of Mollusca showing a well-developed organ system and a worldwide distribution, mostly in marine habitats.

Measurement of AChE in brain tissue and of cholinesterase activity in blood has been employed to monitor exposures to fish and wildlife to xenobiotics .The utility of this metric is enhanced by its relationship between mode of toxic action and by the difficulty of measuring tissue levels of these compounds themselves, which is complicated by the number of compounds that may occur at a given field site and by their frequent rapid metabolism in animals.

The important role of acetylcholine in neurological function, disruption of its breakdown can be utilized to assess neurological and behavioral endpoints. Reductions of acetylcholinesterase activity in fish from polluted sites was observed in muscle tissues of brown trout and flounder in Newfoundland, Canada (Payne *et al.,* 1996). Muscle activities in the three-spined stickleback from parathion polluted streams Germany was also diminished.

5.A.2 Materials and Methods

Protein concentrations were measured according to Lowry *et al.,* (1951) using bovine serum albumin as a standard.

The enzyme activity was measured following the Ellman method (Ellman *et al.*, 1961). In a typical assay, 1050 ml of 0.1 M phosphate buffer, 50 ml of 0.008 M dithiobisnitrobenzoate (DTNB), 50 ml of enzyme solution and 50 ml of 0.045 M thiocholine esters as substrate were successively added. The initial conditions of temperature and pH were respectively 25°C and 7.4 for both molluscs. One enzyme unit (IU) was defined as the amount of enzyme, which catalyzes the hydrolysis of 1 mmol of substrate per min at saturating substrate concentration. The enzymatic reaction rate was quantified using a spectrophotometer against a blank without substrate for each activity measurement. In order to subtract the spontaneous hydrolysis of substrate a second blank was performed without sample. Each ChE activity measurement was duplicated.

5.A.3 Results

Significantly higher AChE activity was detected in the hepatopancreas, followed by the gill and the least levels of AChE were observed in the mantle and adductor muscle, when the values of all measurements from all exposures, were considered.

AChE activity was also significantly higher in mussels $P<0.001$). The results obtained are presented in figure 5.A1 to 5.A16. Increased AChE activity in the hepatopancreas as a function of duration of exposure was recorded in BHC WAF exposed animals (Fig. 5.A1). Slight elevation in the WAF levels in the medium did result in reduced AChE activity in *Perna indica* (Fig. 5.A2). Both hepatopancreas and gills recorded high levels of activity when retained in the experimental media containing 2 ppm and 5 ppm BHC WAF (Fig. 5.A3 and 5.A4).

In the case of animals exposed to 5 ppm of BHC WAF also hepatopancreas and gills showed more activity than adductor muscle and mantle, although comparatively these two tissues did record more AChE activity in comparison to these *P. indica* individuals exposed to 2 ppm.

In the case of *P.viridis* AChE activity was elevated in hepatopancreas and gill, while mantle and adductor muscle recorded reduced activity. Here also increase was time dependent (Fig. 5.A5 and 5.A6).

P. viridis exposed to 1 ppm of BHC WAF recorded rather fluctuating trend in AChE activity. Although hepatopancreas reacted maximum with oil insult adductor muscle was least affected (Fig. 5.A6). Duration of exposure resulted in increase in AChE activity in individuals of *P. viridis* subjected to 5 ppm BHC WAF in the medium. The activity in the adductor was the least (Fig. 5.A7).

A high concentration of 8 ppm BHC WAF resulted in a declinsion in activity between 15 to 20 days. A steady increase recorded from 5th to the 15th day therefore was followed by a reduced AChE activity (Fig. 5.A8). *Perna indica* when exposed to four concentrations of LDO WAF ranging between 0.04 ppm to 3.5 ppm increase in AChE activity was uniformly high and time of exposure dependent. However, with adductor muscle and mantle recorded less activity. It is clear that retained oil can bribg about drastic change in AChE activity in the cardinal tissues like hepatopancreas and gill (Fig. 5.A9-5.A12).*Perna viridis* which is a more tolerant euryhaline mussel also reacted comparably with the species *P. indica* to LDO WAF exposure. Then even a low concentration of 0.05 ppm of LDO WAF resulted in increase AChE activity in hepatopancreas and gills (Fig. 5.A13). Although the activity of AChE was comparable in animals exposed to 0.5 to 4.8 ppm of LDO WAF a clear cut increase in activity occurred in the hepatopancreas and gills of *P.viridis* when encountered with 4.8 ppm of LDO WAF (Fig. 5.A16).

Table 5.A1: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 0.1 ppm WAF of BH Crude oil.

Parameter	Treatment					
	0.1 ppm					
	Control	**1 Day**	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Acetylcholinesterase (AchE) (µmoles of acetyl choline hydrolysed/min/mg protein)						
Hepatopancreas	4.01 ± 0.03	4.21 ± 0.14	4.53 ± 0.13	4.68 ± 0.01	4.91 ± 0.01	5.34 ± 0.10
Gill	3.16 ± 0.02	3.23 ± 0.13	3.60 ± 0.10	3.74 ± 0.01	4.14 ± 0.10	4.34 ± 0.20
Adductor muscle	1.49 ± 0.16	1.64 ± 0.22	1.83 ± 0.16	2.01 ± 0.18	2.49 ± 0.34	2.84 ± 0.14
Mantle	2.10 ± 0.21	2.41 ± 0.16	2.50 ± 0.01	2.84 ± 0.18	2.42 ± 0.13	3.14 ± 0.03

Table 5.A2: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 0.6 ppm WAF of BH Crude oil.

Parameter	Treatment				
	0.6 ppm				
	1 Day	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Acetylcholinesterase (AchE) (µmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	4.40 ± 0.20	4.63 ± 0.01	4.87 ± 0.01	4.96 ± 0.06	5.19 ± 0.08
Gill	3.58 ± 0.12	3.65 ± 0.08	3.84 ± 0.02	4.01 ± 0.15	4.31 ± 0.13
Adductor muscle	2.01 ± 0.13	2.24 ± 0.02	2.53 ± 0.01	2.90 ± 0.04	3.34 ± 0.14
Mantle	2.65 ± 0.23	2.80 ± 0.04	2.91 ± 0.03	3.18 ± 0.01	3.54 ± 0.01

Table 5.A3: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 2 ppm WAF of BH Crude oil.

Parameter	**Treatment**				
	2 ppm				
	1 Day	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	4.54 ± 0.18	4.72 ± 0.01	4.99 ± 0.08	5.34 ± 0.01	4.64 ± 0.01
Gill	3.93 ± 0.02	3.96 ± 0.01	4.51 ± 0.01	5.08 ± 0.08	4.84 ± 0.01
Adductor muscle	2.64 ± 0.11	2.83 ± 0.01	2.95 ± 0.13	3.10 ± 0.12	2.84 ± 0.11
Mantle	2.92 ± 0.01	3.14 ± 0.10	3.24 ± 0.02	3.43 ± 0.01	3.31 ± 0.04

Table 5.A4: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 5 ppm WAF of BH Crude oil.

Parameter	**Treatment**				
	5 ppm				
	1 Day	**5 Day**	**10 Day**	**15 Day**	**20 Day**
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	4.70 ± 0.01	4.83 ± 0.01	5.01 ± 0.02	5.34 ± 0.18	5.20 ± 0.01
Gill	4.00 ± 0.19	4.19 ± 0.14	4.34 ± 0.1	4.58 ± 0.14	4.43 ± 0.13
Adductor muscle	3.08 ± 0.14	3.14 ± 0.13	3.35 ± 0.01	3.64 ± 0.19	3.40 ± 0.10
Mantle	3.25 ± 0.01	3.45 ± 0.08	3.72 ± 0.9	3.84 ± 0.14	3.64 ± 0.12

Table 5.A5: Three way ANOVA Table showing Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to different concentration of WAF of BH.

Source of variation	**df**	**Sum of Squares**	**Mean Square**	**F**	**P-value**
TISSUES	**3**	69.912	23.304	953.830	<0.001
PAH CONC	**3**	4.867	1.622	66.400	<0.001
TIME	**5**	19.375	3.875	158.606	<0.001
Residual	**45**	1.099	0.0244		
Total	**95**	99.297	1.045		

Table 5.A6: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 0.04 ppm WAF of LDO.

Parameter	Treatment					
	0.04 ppm					
	Control	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)						
Hepatopancreas	6.30 ± 0.03	6.49 ± 0.14	6.56 ± 0.12	6.72 ± 0.07	6.95 ± 0.08	7.24 ± 0.24
Gill	5.10 ± 0.04	5.25 ± 0.01	5.43 ± 0.02	5.64 ± 0.08	5.85 ± 0.11	6.01 ± 0.12
Adductor muscle	3.18 ± 0.12	3.23 ± 0.19	3.25 ± 0.12	3.35 ± 0.15	3.64 ± 0.04	3.85 ± 0.01
Mantle	4.29 ± 0.14	4.40 ± 0.13	4.63 ± 0.01	4.85 ± 0.10	5.20 ± 0.12	5.25 ± 0.13

Table 5.A7: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 0.4 ppm WAF of LDO.

Parameter	Treatment				
	0.4 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	6.63 ± 0.01	6.85 ± 0.13	6.92 ± 0.15	7.01 ± 0.20	7.14 ± 0.13
Gill	5.56 ± 0.13	5.63 ± 0.07	5.75 ± 0.06	5.93 ± 0.13	6.5 ± 0.04
Adductor muscle	4.15 ± 0.01	4.28 ± 0.12	4.46 ± 0.35	4.70 ± 0.46	4.89 ± 0.33
Mantle	4.65 ± 0.14	4.80 ± 0.13	4.92 ± 0.05	5.21 ± 0.01	5.35 ± 0.16

Table 5.A8: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 1 ppm WAF of LDO.

Parameter	Treatment				
	1 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (µmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	8.29 ± 0.25	8.35 ± 0.22	8.59 ± 0.13	8.85 ± 0.25	8.79 ± 0.20
Gill	7.20 ± 0.01	7.39 ± 0.18	7.56 ± 0.29	8.16 ± 0.20	7.65 ± 0.16
Adductor muscle	5.18 ± 0.14	5.35 ± 0.03	5.72 ± 0.15	5.85 ± 0.07	6.25 ± 0.13
Mantle	6.12 ± 0.05	6.30 ± 0.18	6.54 ± 0.29	7.08 ± 0.13	6.62 ± 0.19

Table 5.A9: Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to 3.5 ppm WAF of LDO.

Parameter	Treatment				
	3.5 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (µmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	10.01 ± 0.13	10.25 ± 0.01	10.52 ± 0.16	11.08 ± 0.19	11.22 ± 0.28
Gill	8.13 ± 0.11	8.42 ± 0.16	9.72 ± 0.22	8.16 ± 0.16	8.01 ± 0.13
Adductor muscle	6.08 ± 0.03	6.79 ± 0.16	6.80 ± 0.13	7.12 ± 0.05	6.72 ± 0.05
Mantle	7.14 ± 0.06	7.25 ± 0.07	7.53 ± 0.06	7.85 ± 0.25	7.24 ± 0.13

Table 5.A10: Three way ANOVA Table showing Levels of Acetylcholinesterase activity in the different tissues of *Perna indica* exposed to different concentration of WAF of LDO.

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	129.347	43.116	610.591	<0.001
PAH CONC	**3**	103.015	34.338	486.286	<0.001
TIME	**5**	49.220	9.844	139.407	<0.001
Residual	**45**	3.178	0.0706		
Total	**95**	311.441	3.278		

Table 5.A11: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 0.1 ppm WAF of BH Crude oil.

Parameter	Treatment					
	0.1 ppm					
	Control	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)						
Hepatopancreas	4.41 ± 0.02	4.60 ± 0.14	4.91 ± 0.21	5.26 ± 0.18	5.61 ± 0.21	5.93 ± 0.16
Gill	3.63 ± 0.02	3.81 ± 0.04	4.20 ± 0.18	4.73 ± 0.20	4.90 ± 0.18	4.98 ± 0.23
Adductor muscle	1.21 ± 0.03	1.62 ± 0.01	1.65 ± 0.34	1.72 ± 0.16	1.85 ± 0.21	1.92 ± 0.26
Mantle	2.30 ± 0.01	2.63 ± 0.02	2.64 ± 0.14	2.70 ± 0.18	2.81 ± 0.16	2.90 ± 0.17

Table 5.A12: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 1 ppm WAF of BH Crude oil.

Parameter	Treatment					
	1 ppm					
		1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)						
Hepatopancreas		4.71 ± 0.18	4.85 ± 0.22	4.96 ± 0.18	5.01 ± 0.23	4.82 ± 0.18
Gill		3.92 ± 0.22	3.98 ± 0.12	4.13 ± 0.11	4.29 ± 0.16	4.39 ± 0.22
Adductor muscle		2.01 ± 0.13	2.17 ± 0.16	2.25 ± 0.23	2.39 ± 0.16	2.63 ± 0.14
Mantle		2.92 ± 0.14	3.10 ± 0.10	3.22 ± 0.12	3.34 ± 0.11	3.59 ± 0.20

Table 5.A13: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 5 ppm WAF of BH Crude oil.

Parameter	Treatment				
	5 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	5.15 ± 0.17	5.29 ± 0.23	5.31 ± 0.17	5.49 ± 0.20	5.37 ± 0.17
Gill	4.00 ± 0.23	4.10 ± 0.18	4.26 ± 0.12	4.46 ± 0.12	4.20 ± 0.12
Adductor muscle	2.73 ± 0.10	2.84 ± 0.16	2.92 ± 0.14	3.15 ± 0.14	2.32 ± 0.19
Mantle	3.15 ± 0.23	3.49 ± 0.29	3.67 ± 0.12	3.98 ± 0.12	3.50 ± 0.16

Table 5.A14: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 8 ppm WAF of BH Crude oil.

Parameter	Treatment				
	8 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	6.47 ± 0.12	6.62 ± 0.17	7.18 ± 0.26	8.14 ± 0.12	7.02 ± 0.19
Gill	5.00 ± 0.14	5.44 ± 0.18	6.12 ± 0.13	7.08 ± 0.14	6.1 ± 0.24
Adductor muscle	3.10 ± 0.16	3.59 ± 0.24	4.14 ± 0.12	4.69 ± 0.14	4.01 ± 0.13
Mantle	4.16 ± 0.12	4.56 ± 0.14	5.18 ± 0.16	6.14 ± 0.12	5.25 ± 0.10

Table 5.A15: Three way ANOVA Table showing Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to different concentration of WAF of BHC.

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	122.045	40.682	1063.401	<0.001
PAH CONC	**3**	40.951	13.650	356.812	<0.001
TIME	**5**	28.472	5.694	148.847	<0.001
Residual	**45**	1.722	0.0383		
Total	**95**	208.985	2.200		

Table 5.A16: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 0.05 ppm WAF of LDO.

Parameter	Treatment				
	0.05 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	6.14 ± 0.12	6.25 ± 0.42	6.39 ± 0.40	6.67 ± 0.18	7.18 ± 0.24
Gill	5.22 ± 0.11	5.29 ± 0.18	5.42 ± 0.20	5.63 ± 0.10	5.79 ± 0.20
Adductor muscle	2.16 ± 0.18	2.30 ± 0.10	2.35 ± 0.18	2.51 ± 0.05	2.70 ± 0.10
Mantle	4.10 ± 0.21	4.19 ± 0.11	4.36 ± 0.01	4.74 ± 0.08	4.98 ± 0.06

Table 5.A17: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 0.5 ppm WAF of LDO.

Parameter	Treatment				
	0.5 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	6.29 ± 1.20	6.40 ± 0.12	6.96 ± 0.03	7.12 ± 0.14	7.39 ± 0.09
Gill	5.46 ± 0.18	5.69 ± 0.20	6.12 ± 0.01	6.40 ± 0.01	6.82 ± 0.03
Adductor muscle	3.18 ± 0.07	3.40 ± 0.18	3.56 ± 0.29	4.08 ± 0.01	4.29 ± 0.08
Mantle	5.01 ± 0.12	5.19 ± 0.34	5.33 ± 0.14	5.63 ± 0.11	5.84 ± 0.05

Table 5.A18: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 2 ppm WAF of LDO.

Parameter	Treatment				
	2 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	7.28 ± 0.03	7.49 ± 0.01	7.53 ± 0.01	7.62 ± 0.08	7.38 ± 0.01
Gill	6.10 ± 0.18	6.49 ± 0.14	6.64 ± 0.02	6.79 ± 0.18	6.53 ± 0.13
Adductor muscle	3.30 ± 0.01	3.59 ± 0.05	3.69 ± 0.13	3.85 ± 0.18	3.52 ± 0.01
Mantle	5.64 ± 0.12	5.92 ± 0.13	6.41 ± 0.16	6.87 ± 0.09	6.50 ± 0.05

Table 5.A19: Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to 4.8 ppm WAF of LDO.

Parameter	Treatment				
	4.8 ppm				
	1 Day	5 Day	10 Day	15 Day	20 Day
Acetylcholinesterase (AchE) (μmoles of acetyl choline hydrolysed/min/mg protein)					
Hepatopancreas	8.16 ± 0.01	8.27 ± 0.13	8.49 ± 0.17	9.01 ± 0.10	8.61 ± 0.13
Gill	7.30 ± 0.12	7.42 ± 0.08	7.76 ± 0.05	8.19 ± 0.06	7.84 ± 0.16
Adductor muscle	4.19 ± 0.03	4.33 ± 0.12	4.83 ± 0.13	4.96 ± 0.22	4.74 ± 0.01
Mantle	6.23 ± 0.14	6.54 ± 0.08	6.81 ± 0.01	6.99 ± 0.03	6.76 ± 0.13

Table 5.A20. Three way ANOVA Table showing Levels of Acetylcholinesterase activity in the different tissues of *Perna viridis* exposed to different concentration of WAF of LDO.

Source of variation	df	Sum of Squares	Mean Square	F	P-value
TISSUES	**3**	180.933	60.311	523.507	<0.001
PAH CONC	**3**	41.621	13.874	120.425	<0.001
TIME	**5**	117.410	23.482	203.827	<0.001
Residual	**45**	5.184	0.115		

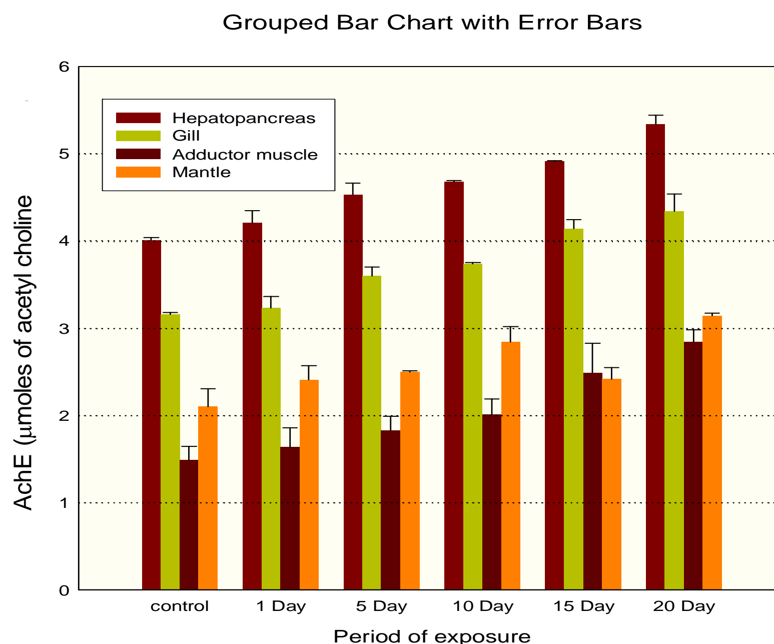


Figure 5.A1: AChE activity of different tissues of *P. indica* exposed to 0.1 ppm of BHC WAF

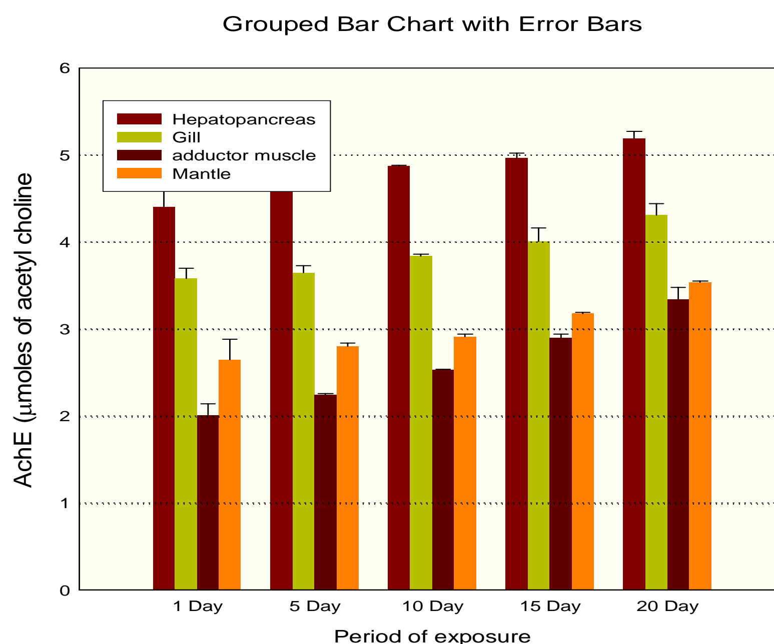


Figure 5.A2: AChE activity of different tissues of *P. indica* exposed to 0.6 ppm of BHC WAF

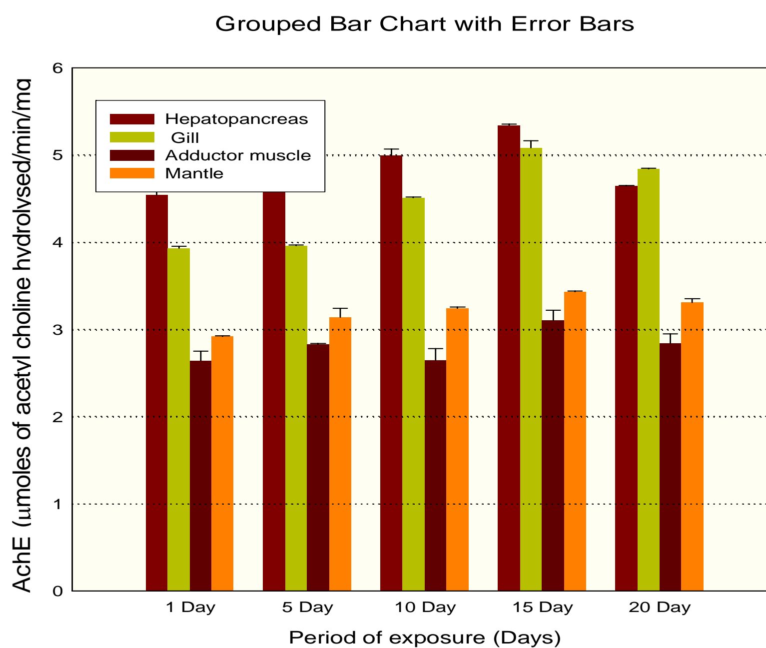


Figure 5.A3: AChE activity of different tissues of *P. indica* rxposed to 2 ppm of BHC WAF

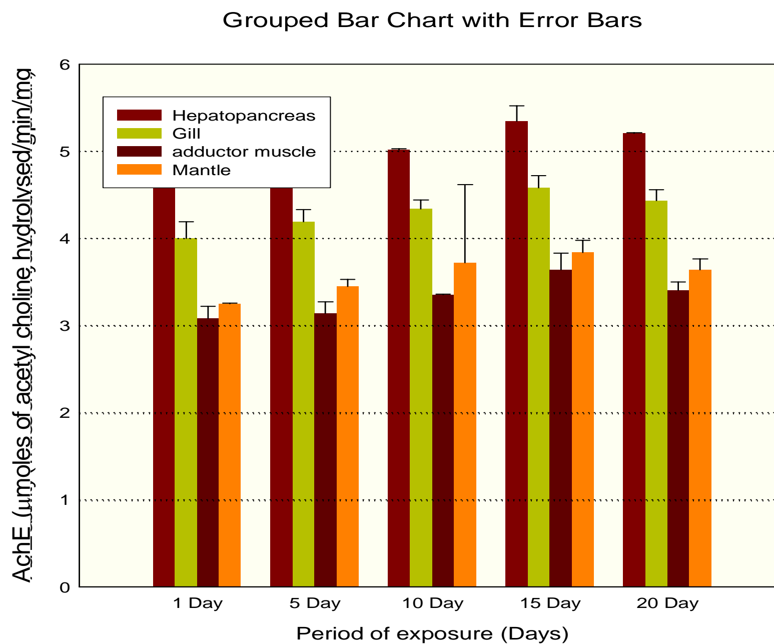


Figure 5.A4: AChE activity of different tissues of *P. indica* exposed to 5 ppm of BHC WAF

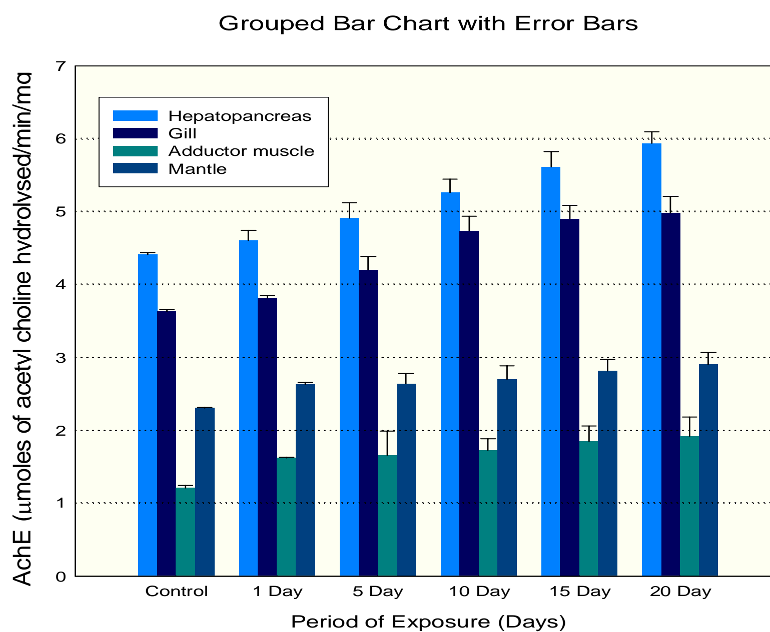


Figure 5.A5: AChE activity of different tissues of *P. viridis* exposed to 0.1 ppm of BHC WAF

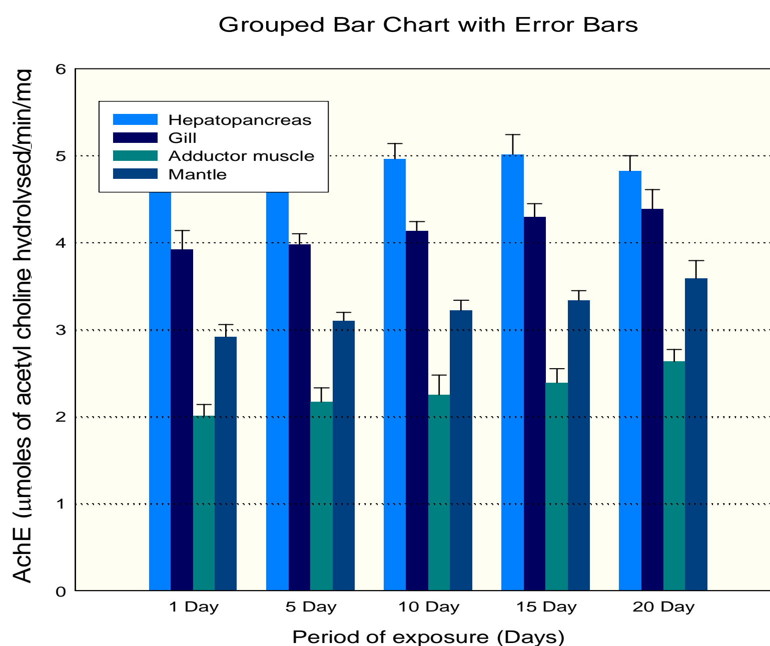


Figure 5.A6: AChE activity of different tissues of *P. viridis* exposed to 1ppm of BHC WAF

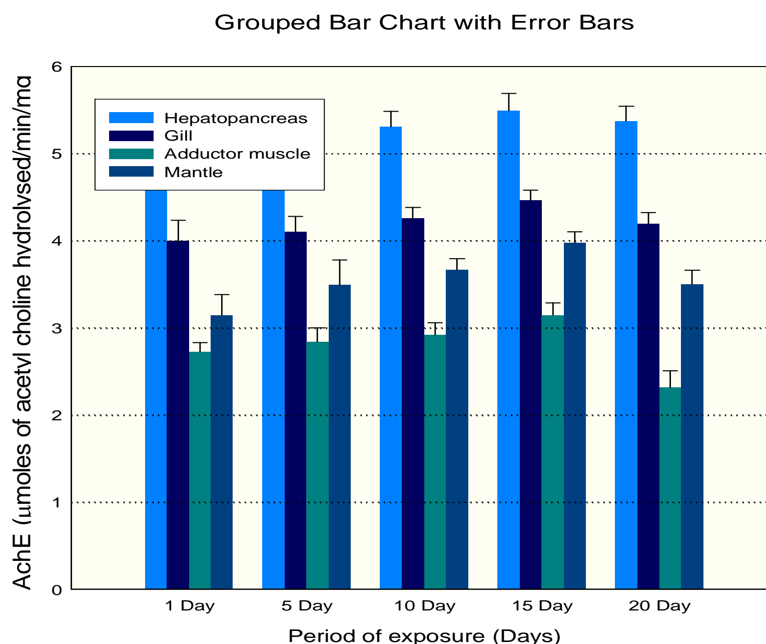


Figure 5.A7: AChE activity of different tissues of *P. viridis* exposed to 5 ppm of BHC WAF

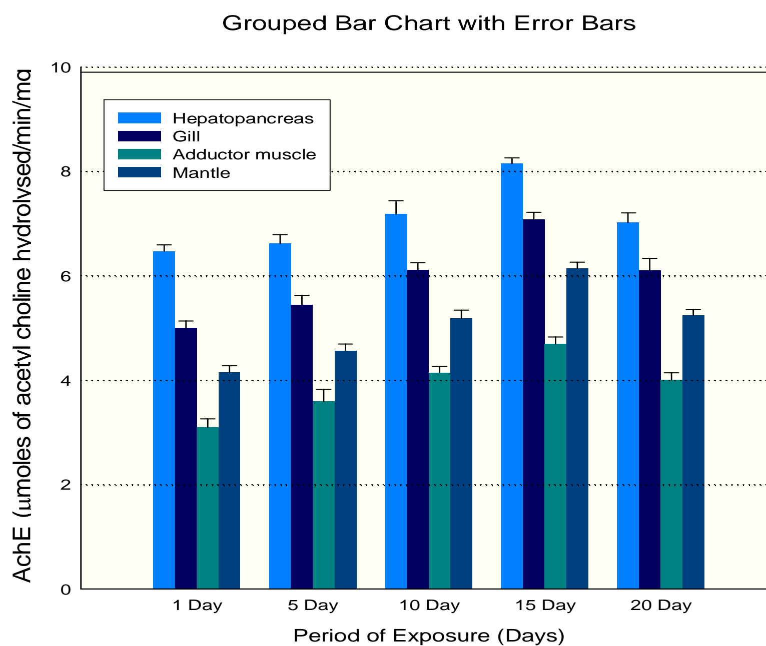


Figure 5.A8: AChE activity of different tissues of *P. viridis* exposed to 8 ppm of BHC WAF

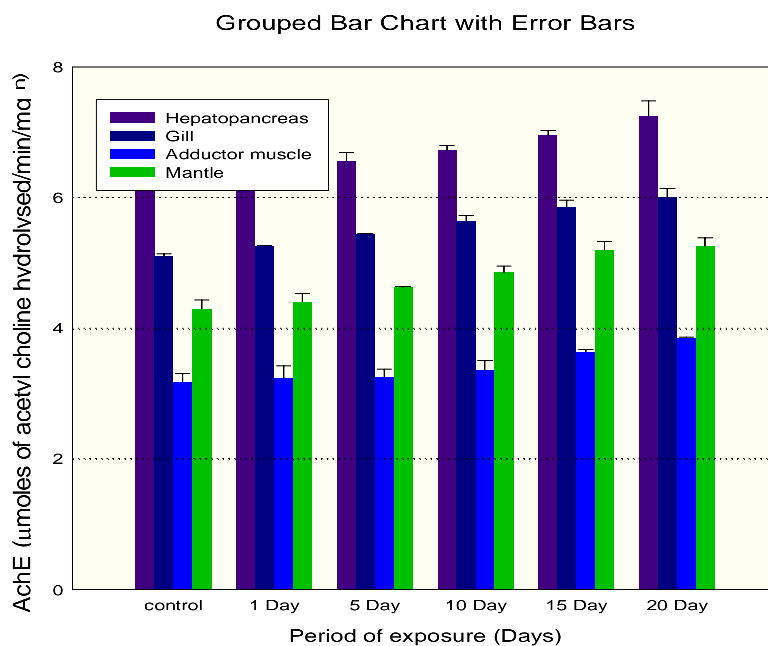


Figure 5.A9: AChE activity of different tissues of *P. indica* exposed to 0.04 ppm of LDO WAF

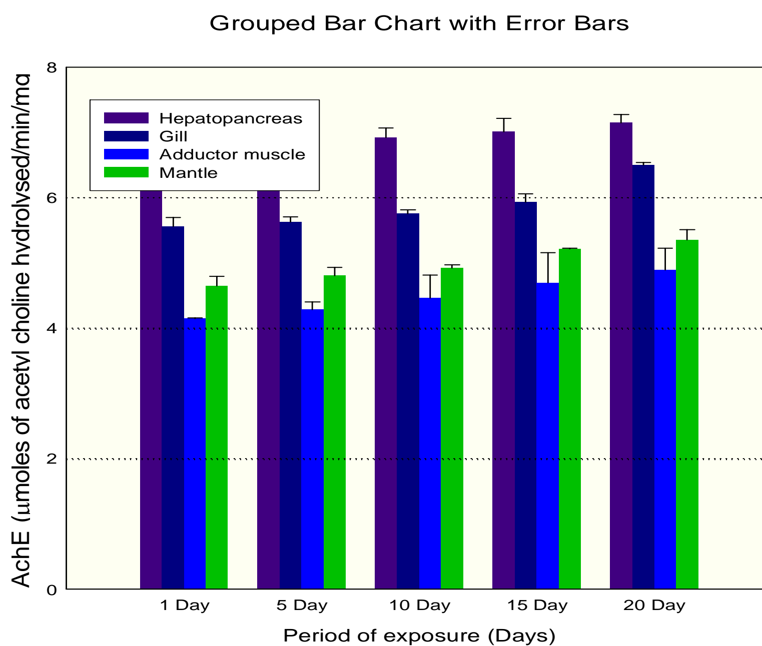


Figure 5.A10: AChE activity of different tissues of *P. indica* exposed to 0.4 ppm of LDO WAF

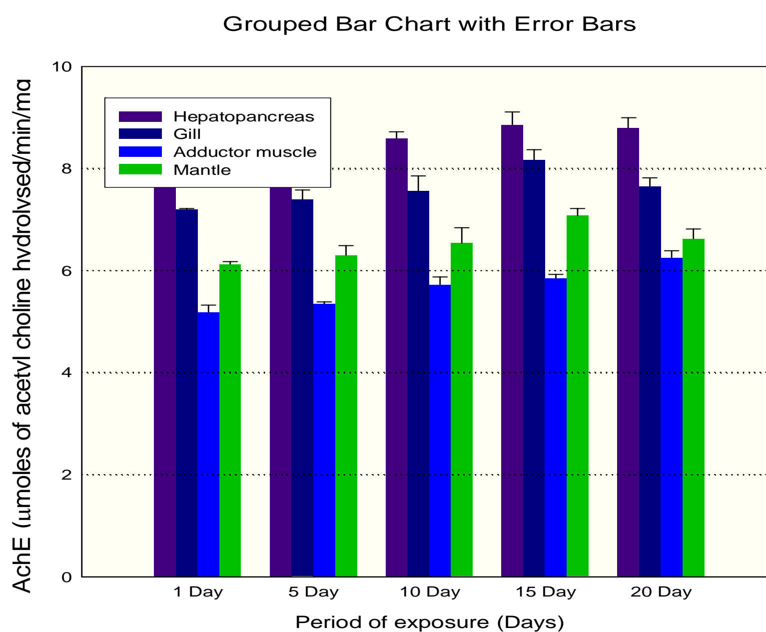


Figure 5.A11: AChE activity of different tissues of *P. indica* exposed to 1 ppm of LDO WAF

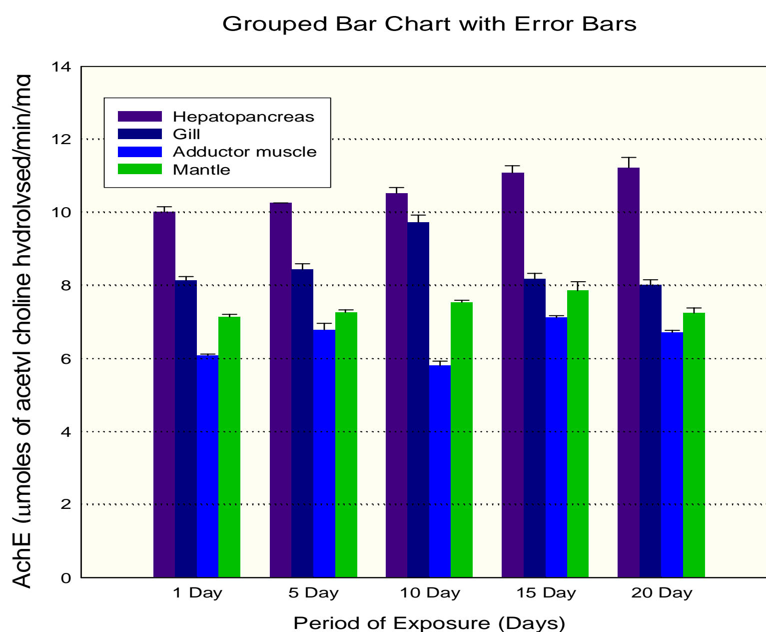


Figure 5.A12: AChE activity of different tissues of *P. indica* exposed to 3.5 ppm of LDO WAF

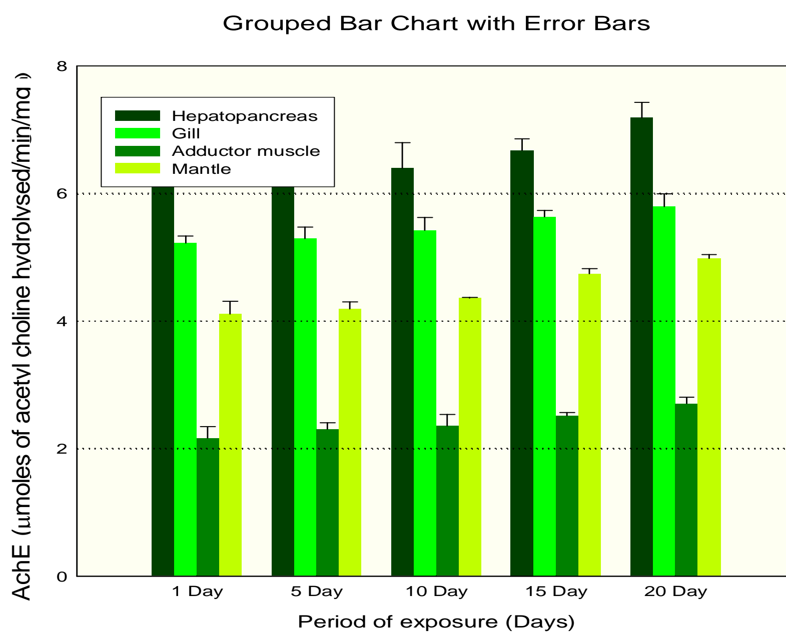


Figure 5.A13: AChE activity of different tissues of *P. viridis* exposed to 0..05 ppm of LDO WAF

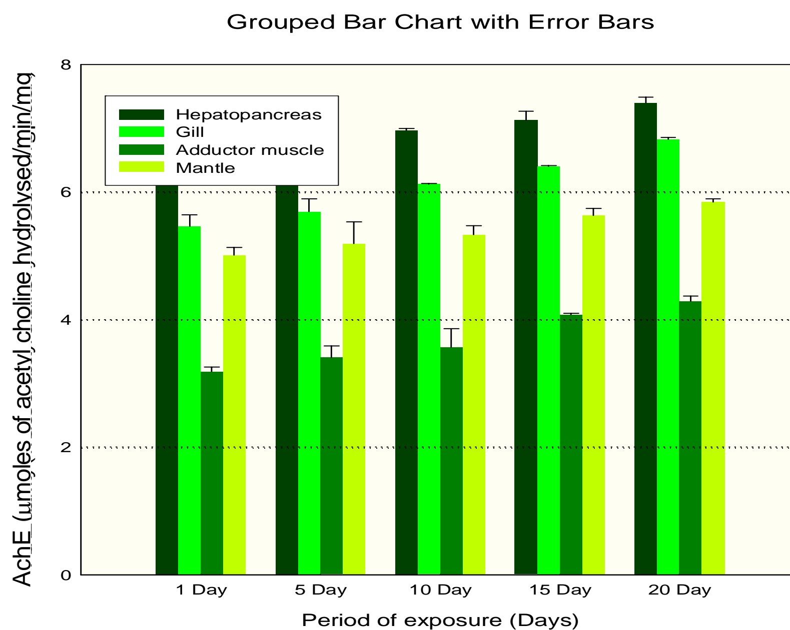


Figure 5.A14: AChE activity of different tissues of *P. viridis* exposed to 0.5 ppm of LDO WAF

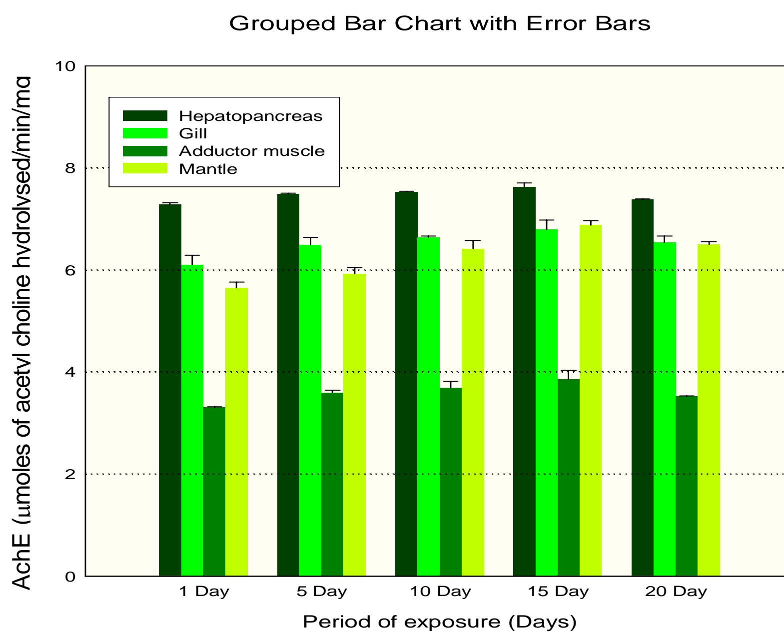


Figure 5.A15: AChE activity of different tissues of *P.viridis* exposed to 2 ppm of LDO WAF

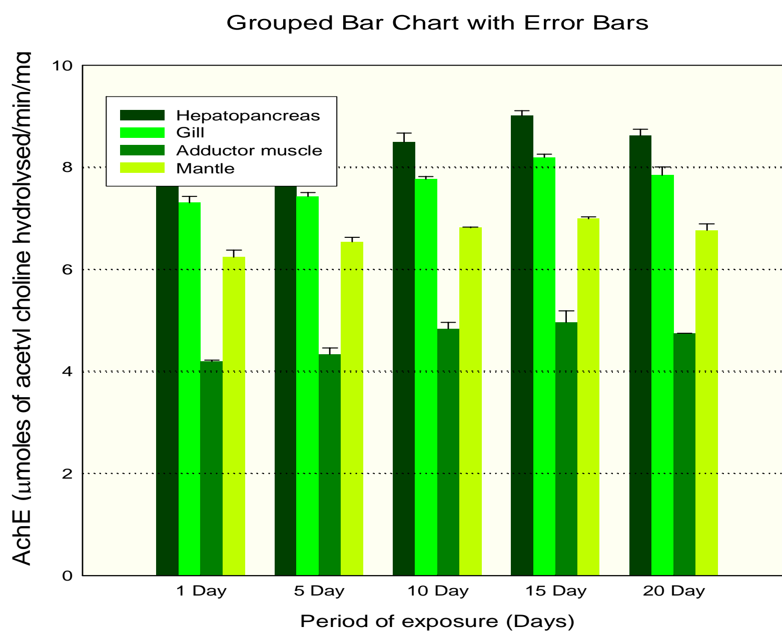


Figure 5.A16: AChE activity of different tissues of *P.viridis* exposed to 4.8 ppm of LDO WAF

5.A.4 Discussion

Cholinesterases are considered reliable markers for exposures to PAHs. Since the enzyme activity is based upon the oxidation of thiol groups from acetylthiocholine and Ellman's reagent (DTNB), metals tend to disrupt the assay. Enzyme values should also be normalized to length of the organism as recent studies by Rodriguez-Fuentes *et al.* (2007) indicated in flatfish collected near reference and oceanic wastewater outfall sites in Southern California. Site specific differences were observed in acetylcholinesterase activities in muscle but size-dependent differences were also observed and shown to correlate with activity.

There was, however, a sublethal effect, in that the same concentration resulted in a 90% inhibition of the AChE activity in the gill. Estimation of AChE activity in marine organisms (fish, Galgani *et al.*, 1992; mussels, Bocquene *et al.,* 1993; Narbonne *et al.*, 1993) has been made under sublethal conditions in field studies, and the results have been related to pollution gradients. Elevations in acetylcholine have been shown to affect the water pumping rate in the mussel (Jones and Richards, 1993).

Many field studies have demonstrated the interest of the measurement of Acetylcholinesterase (AChE) activity in invertebrates as exposure biomarker in coastal waters and rivers (Moulton, Fleming, and Purnell, 1996; Stien, Percic, Gnassia-Barelli, Romeo, and Lafaurie, 1998; Varela and Augspurger, 1996). AChE activity is inhibited in the presence of organophosphorus compounds and carbamates (Day and Scott, 1990; Holland, Coppage, and Butler, 1967; Weiss, 1965; Weiss and Gakstatter, 1964; Williams and Sova, 1966; Zinckl, Shea, Nakamoto, and Callman, 1987). Because of the relative short life of organophosphorus in the environment (Barron & Woodburn, 1995), the determination of the organophosphorus effect on the cholinesterases (ChEs) in non target organisms has been suggested as a tool for monitoring environmental contamination and organismal exposure to those compounds. In addition, heavy metals

have been reported to inhibit AChE activity in fish (Payne, *et al.*, Melvin, and Fancey, 1996), clams (Hamza-Chaffai, *et al.*, 1998) and mussels (Najimi *et al.*, 1997). Apart from its role in biomonitoring, AChE is a group of enzymes important in transmission of nervous impulses and is found in all animal phyla, including molluscs (Walker and Thompson, 1991). Apart from AChE a number of esterases as butyrylcholinesterase (BChE) and carboxylesterase (CbE) has been evaluated as biomarkers. According to Escartin and Porte (1997), there are tissue specific differences in esterases, since AChE is typically more abundant in gills while BChE and CbE are more abundant in digestive gland of *Mytilus galloprovincialis*. Furthermore, fish three-spined stickleback BChE showed higher sensitivity to organophosphorus insecticide compared to AChE and CbE (Wogram, *et al.*, 2001). It appears that mollusks present a higher diversity of cholinesterases than what is known for mammals (Edwards and Fisher, 1991).

Extracellular signals such as hormones, neurotransmitters and growth factors control their physiological effects on target cells by binding to specific receptors and thus activating a signaling cascade within the cell. The physiological responses to such extracellular signals are mediated through "second messengers" (Sutherland, *et al.*, 1968). It is reported that exogenous chemicals (endocrine disrupters) may also bind to a receptor and mimic or block the actions of natural hormones (Mendes, 2002). A pollutant may act as an endocrine disrupter and interferes with the signal transduction pathway.

In *Perna perna*, highest values were observed in the hepatopancreas and muscles and minimal in the gills, while in *M. edulis* the highest activity was reported in the gills (Bocquene, *et al.,* 1990). The role of the enzyme may be related to tissue distribution. AChE activity has been related to nerve impulse transmission in tissues with high neuromuscular activity (Najimi *et al.*, 1997; Walker and Thompson, 1991). Furthermore, the existence of cholinergic transmission in the peripheral nervous

system in molluscs has not so far been demonstrated (Heyer, *et al.*, 1973; Mercer and McGregor, 1982).

Acetylcholinesterase (AChE) is commonly located into different cell membranes of mussel tissues and its activity may be affected by a wide range of contaminants or their mixture (Bonacci *et al.,*2008; Jebali *et al.*, 2006; Vioque-Fernandez *et al.,* 2007).., Basu *et al.* (2009) showed that phenolic substances could impair fish reproduction via inhibition of neurotransmitter receptors and enzymes, such as AChE, while their interference with mitochondrial membranes could lead to membrane dysfunction and neuronal degenerative diseases (Martins *et al.*, 2008).

Elevated AChE content observed in the present study might be a response of PAHs toxicity. PAHs may alter the enzyme activity by binding to the functional group of proteins. PAHs can alter the AChE activity not only by inhibiting but also by stimulating the catalytic function of the enzyme (Jackin, 1974). Another possibility for AChE activation by PAHs could be related to de novo synthesis of the enzyme (Romani *et al.*, 2003). The increase in AChE activity may be due to interaction of PAHs with AChE receptor, and thereby affect its binding efficiency, leading to an increase in AChE synthesis, to decompose the higher levels of neurotransmitter. Dailianis *et al.,* (2003) reported increase in AChE activity in *Mytilus edulis* collected from polluted sites.

5.B Lysosomal stability

5.B.1 Introduction

Cellular biomarkers are used as a measures of the sublethal effects of pollutants that can indicate a progressive cell damage with physiological consequences .Thus measuring at the cellular level may provide a fast and sensitive indication of environmental pollution.

Lysosomes constitute a group of subcellular structures involved in intracellular digestion that compartmentalize and accumulate a wide range of pollutants (Viarengo and Canesi 1991; Hole *et al.,* 1993; Viarengo and Nott 1993). Thus pollution causes damage in these organelles, which can be measured by different means, including lysosomal stability tests (Lowe *et al.,* 1992, 1995; Regoli 1992; Lin and Steichen 1994; Krishnakumar *et al.* 1994; Hole *et al.,* 1995).

The measurement of lysosomal membrane stability is used as an integrative biomarker of cellular stress, as membrane integrity is affected by different pollutants (Moore 1985; Viarengo *et al.,* 1987; Mayer *et al.,* 1989; Lin and Steichen 1994). There is evidence of decreased lysosomal membrane stability in mussels exposed to pollutants under laboratory conditions and mussels collected from contaminated areas (Moore *et al.,* 1978; Widdows *et al.,* 1982; Ward 1990; Krishnakumar *et al.,* 1994).

The Neutral Red retention time assay (NRA)evaluates the lysosomal membrane integrity, which can be used as an indicator of exposure to xenobiotics (Moore, 1990; Lowe *et al*., 1995; Cheung *et al*., 1997; Zaroni *et al*., 2001). It involves exposing cells to a coloured dye, which is taken up by the lysosomes. Healthy cells retain the dye for more time than damaged cells, in which the dye rapidly leaks out into the cytoplasm.

The NRA can be used to measure alterations in lysosomal stability induced by various stress factors. In their stable form (no stress response), lysosomes will accumulate and retain the neutral red dye for an extended period of time. However, once destabilized (stress response) following a stressor, the lysosomes will coalesce to form larger lysosomal structures and the neutral red dye will leak into the cytosol of the cell through damaged membranes (Moore 1980, Lowe *et al*., 1995a). The rate of lysosomal changes following the addition of the neutral red dye is indicative of the stress response and can be directly related to the degree of stress being imposed on the mussels.

Particularly, various investigations have been addressed to study lysosomal membrane destabilization in hemocytes (Lowe *et al.,* 1995; Svendsen and Weeks 1995). In addition, digestive cells also posses a well-developed lysosomal system that is responsive to diverse pollutants (Moore, 1985, 1988).

The lysosomal system in the digestive cells has been identified as a target site for the toxic effects of many environmental xenobiotics (Moore, 1990; Cajaraville *et al.*, 1995a). Lysosomal responses to environmental stress fall into essentially three categories: reduced membrane stability, increased lysosomal size and changes in lysosomal contents (Marig´omez and Baybay-Villacorta, 2003).

The cellular composition of the digestive epithelium was examined as a marker of general condition of the digestive gland since it is known that pollutant exposure may induce alterations in cell-type ratios (basophilic cells become more abundant than digestive cells) (Cajaraville *et al.*, 1990; Marig´omez *et al.*, 1990).

Therefore, the critical issues involve, determining the organisms that should live and thrive in a habitat and identifying the effects of chronic stress on biotic health. In some cases, compensatory mechanisms may function to sequester, detoxify, or ameliorate the effects of stressors so exposures do not always translate into adverse effects. In other cases, individual stressors or combinations of stressors may cause chronic stress that can compromise basic physiological functions, including reproduction, so that long-term population dynamics and sustainability are endangered.

Hepatic tissues can be used most broadly and are also one of the most important sites for contaminant deposition and effects. The lysosomal assay is most readily used for hepatic tissues and blood cells.

The measurement of lysosomal membrane stability is used as an integrative biomarker of cellular stress, as membrane integrity is affected by different pollutants

(Moore 1985; Viarengo *et al.,* 1987; Mayer *et al.,* 1989; Lin and Steichen 1994). There is evidence of decreased lysosomal membrane stability in mussels exposed to pollutants under laboratory conditions and mussels collected from contaminated areas (Moore *et al.* 1978; Widdows *et al.,* 1982; Ward 1990; Krishnakumar *et al.,* 1994). Particularly, various investigations have been addressed to study lysosomal membrane destabilization in hemocytes (Lowe *et al.,* 1995; Svendsen and Weeks 1995).In addition, digestive cells also posses a well-developed lysosomal system that is responsive to diverse pollutants, including heavy metals (Moore 1985, 1988). Exposure to pollutants causes changes in size and number of lysosomes, alterations in membrane permeability and osmotic disruption, which altogether lead to vacuolization of digestive cells (Moore 1985, 1988; Cajaraville *et al.,* 1989; Lowe *et al.,* 1992; Hole *et al.,* 1995).

Our aim is to explore the utility of lysosomal responses as prognostic biomarkers for putative pathophysiology that will permit prediction of animal health status;

5.B.2 Materials and Methods

Lysosomal membrane destabilization was assessed by neutral red retention time according to Lowe *et al.*, (1995). Haemolymph was withdrawn from the posterior adductor muscle and macerated hepatopancreas incubated on a glass slide with a neutral red (NR) working solution. Granular haemocytes and digestive cells were microscopically examined at 15 min intervals, for up to 2h, to evaluate the time at which 50% of cells had leaked the dye previously trapped into lysosomes to the cytosol.

For each examination, 25 hemocytes from each mussel sample were examined and given a rating based on 1 of the 4 following characteristics: 1.No stress response = characterized by appearance of tiny pink dots, which are intact lysosomes containing neutral red dye; 2.moderately low stress response = increase in the size of lysosomes due to lysosomal membrane fusion; 3.moderately high stress response = lysosomes appear larger and more faint in color due to fusion of lysosomal membranes and

leakage of neutral red dye into the cytosol; 4.high stress response = cytosol is completely tinged pink due to leakage of neutral red dye out of the lysosomes, and only remnants of the lysosomal membranes may be still visible.

A mean NR retention time was calculated for each group, and the means were compared by analysis of variance (ANOVA) followed by a Tukey's multiple comparison (PASW 18 software). Also, the percent of organisms showing effects at each reading time was calculated, aiming to compare the response behaviour.

When higher than 50% of the 25 ceils exhibited high stress response at a particular time point, then the assay was stopped for that mussel and the previous examination time period recorded as the score for NRR.

5.B.3 Results

Haemocytes were observed under a light microscope at 2-min intervals and only the most abundant cell type, namely the smaller hyaline and agranular haemocytes with pseudopodia, were considered. The NRRT was calculated as the time at which 50% or more of the counted cells presented reddish cytosols after the leakage of the dye from lysosomes.There was no difference in the neutral red retention time between the control mussels, whereas the organisms exposed to BHC and LDO showed a significantly lower retention time. Mussels exposed to BHC and LDO WAF concentrations in the laboratory showed a significant reduction in neutral red retention time compared to controls.

Despite the absence of statistical differences, animals from controls showed a faster response than the organisms from exposed, which could be observed in the measurements made at 15 and 30 min exposure (Plate.5.B1).

The results of the neutral red retention assay following exposure to BHC and LDO WAF in both *P.viridis* and *P.indica* demonstrate a significant reduction in lysosomal

retention capacity (Table 5.B2-5.B3) in both the blood cells (Plate. 5.B1) and isolated digestive cells (Plate 5.B2 and Plate 5.B3) as compared to their controls. NR dye can only be retained in healthy cell lysosomes (Plate 5.B2a), but leads to lysosomal swelling (Plate 5.B2b) and rupture (Plate 5.B2c), and subsequent leakage of the NR dye will occur in un-healthy cells (Plate 5.B2d).

Furthermore, there was a significant reduction in the retention time in both cell types from the mussels exposed to the BHC and as compared to the control. Digestive cell lysosomal membrane stability (latency) was significantly reduced in mussels exposed to LDO as compared to the control ($P < 0.001$) .Significant differences were found in digestive cells of mussels exposed to sublethal concentrations of BHC and LDO WAF in laboratory experiments.

The maximum NRR times were as high as 120 or even 180 min *et al.*, (1995). The lower NRR values obtained by the present study may be attributed to the different experimental procedures used, at least compared with that of Fernley *et al.*, (2000) where mussels were allowed to 24–36 h acclimatization prior to the NRR application. In the present study, the NRR value was taken immediately after the mussels was transferred to the laboratory. It is also possible that the low NRR times of the present study constitute a characteristic of the indigenous mussel populations, or even reflect persisting pollution levels, as similar NRR values were reported in other field studies conducted by (Koukouzika and Dimitriadis,1992).The results of the present study indicated that the NRR assay might constitute a good marker of lysosomal damage, as it is precise, sensitive to pollution gradient and minimally affected by natural factors' variation. In addition, the results of previous studies concerning LMS evaluation of the digestive gland (Domouhtsidou *et al.*, 2002), and the results of Kohler, Deismemann, and Lauritzen (1992), Lowe *et al.*, (1992) and Lowe, Fossato *et al.*, (1995) further enhance the use of lysosomal damage evaluation as a good biomonitoring tool.

Table 5.B1: One way analysis of variance (ANOVA) of neutral red retention times of blood and digestive cell lysosomes from mussels exposed to BHC and LDO WAF in *Perna viridis*.

	Control	**0.1 ppm**	**1 ppm**	**5 ppm**	**8 ppm**
Blood cell NRR statistics					
Perna viridis BHC WAF	P<0.001	P<0.01	P<0.04	P<0.02	P<0.03
Digestive cell NRR statistics					
Perna viridis BHC WAF	P<0.01	P<0.04	P<0.001	P<0.04	P<0.01
	Control	0.05 ppm	0.5 ppm	2 ppm	4.8 ppm
Blood cell NRR statistics					
Perna viridis LDO WAF	P<0.001	P<0.02	P<0.04	P<0.01	P<0.01
Digestive cell NRR statistics					
Perna viridis LDO WAF	P<0.001	P<0.001	P<0.04	P<0.01	P<0.01

Table 5.B2: One way analysis of variance (ANOVA) of neutral red retention times of blood and digestive cell lysosomes from mussels exposed to BHC and LDO WAF in *Perna indica*

	Control	**0.1 ppm**	**0.6 ppm**	**2 ppm**	**5 ppm**
Blood cell NRR statistics					
Perna indica BHC WAF	P<0.001	P<0.001	P<0.03	P<0.02	P<0.01
Digestive cell NRR statistics					
Perna indica BHC WAF	P<0.001	P<0.001	P<0.04	P<0.03	P<0.01
	Control	0.04 ppm	0.4 ppm	1 ppm	3.5 ppm
Blood cell NRR statistics					
Perna indica LDO WAF	P<0.001	P<0.04	P<0.04	P<0.03	P<0.01
Digestive cell NRR statistics					
Perna indica LDO WAF	P<0.001	P<0.03	P<0.02	P<0.03	P<0.01

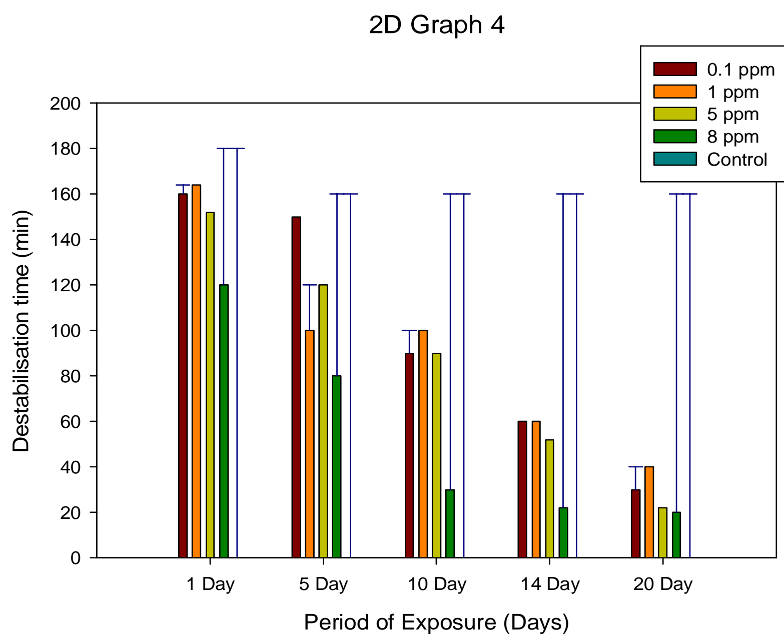


Figure 5.B1: Lysosomal membrane labilization in digestive cells of *P. viridis* exposed to BHC

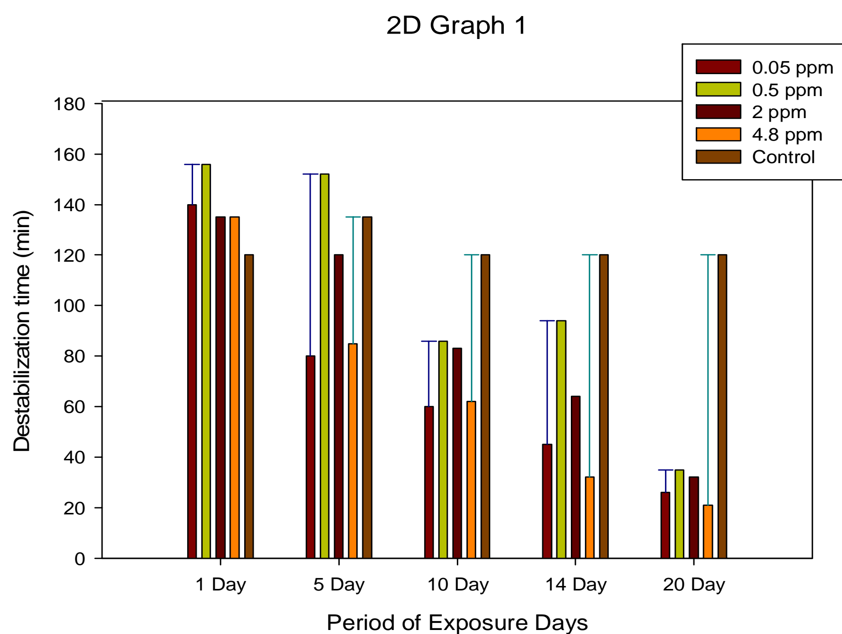


Figure 5.B2: Lysosomal membrane labilization in digestive cells of *P. viridis* exposed to LDO

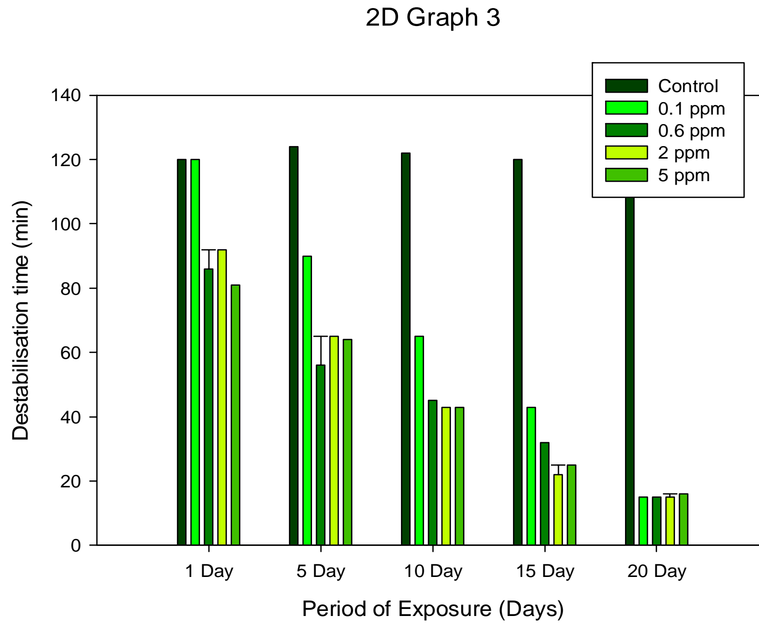


Figure 5.B3: Lysosomal membrane labilization in digestive cells of *P. indica* exposed to BHC

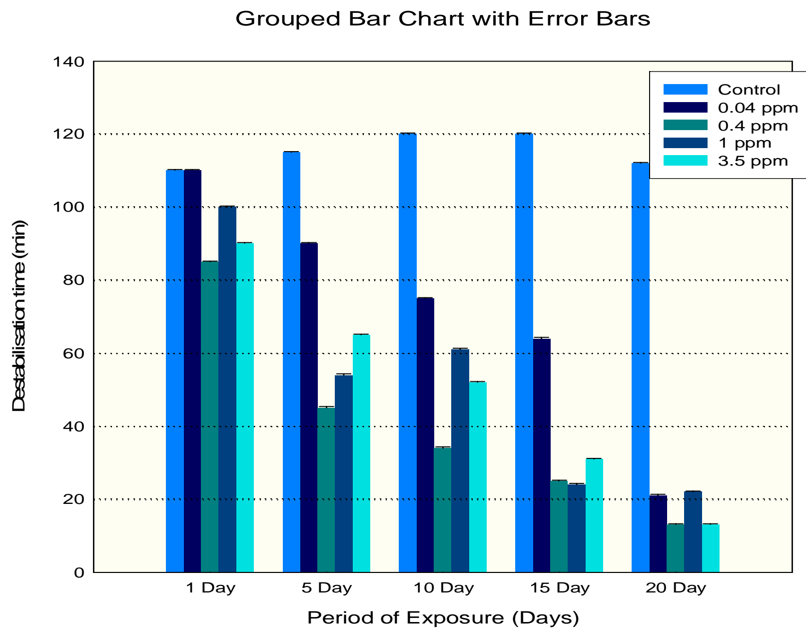


Figure 5.B4: Lysosomal membrane labilization in digestive cells of *P. indica* exposed to

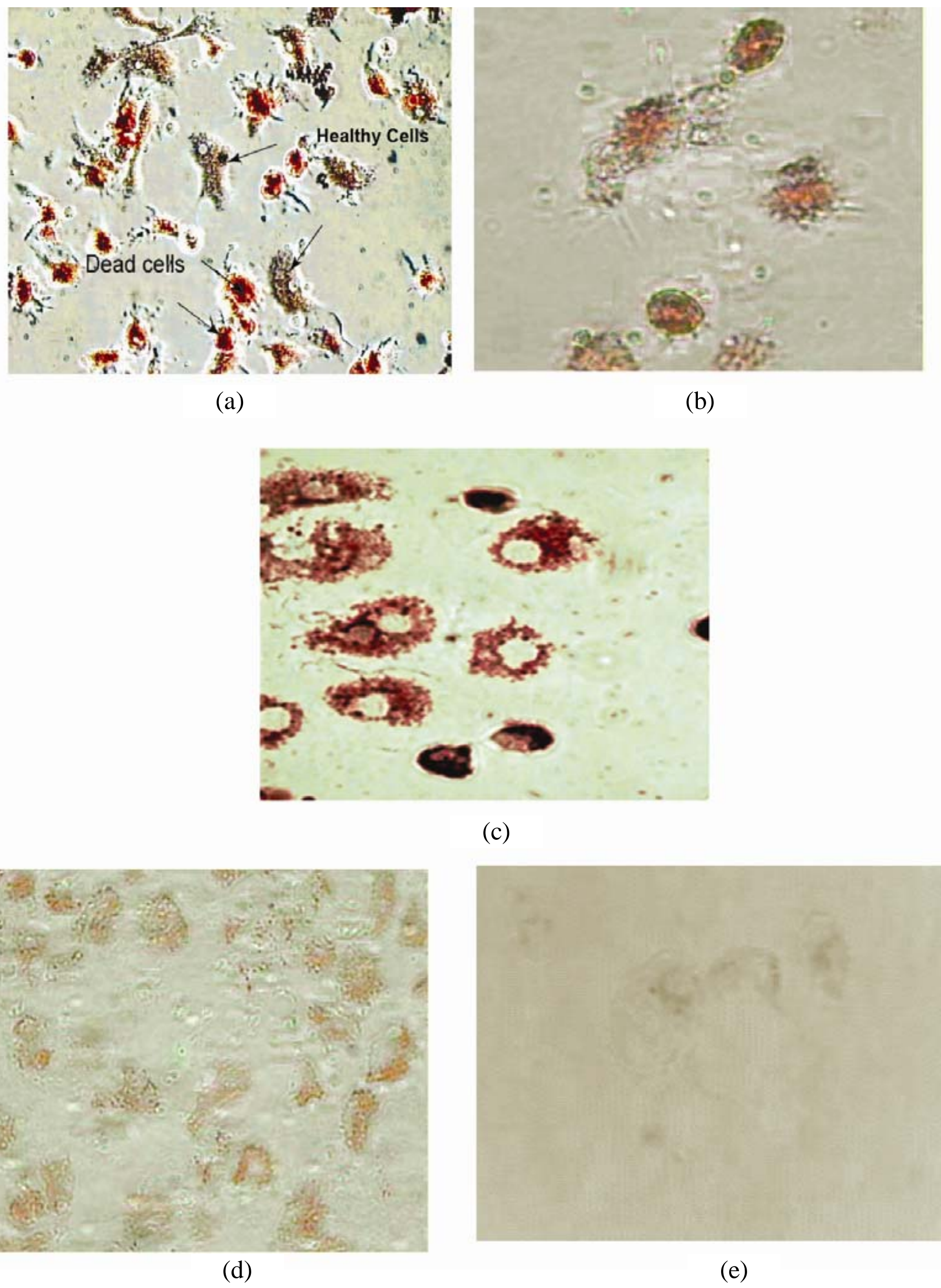


Plate 5.B1: Photomicrograph showing NRRT in Heamolymph cells in *Perna viridis* (a) control (b-e) destabilized stages

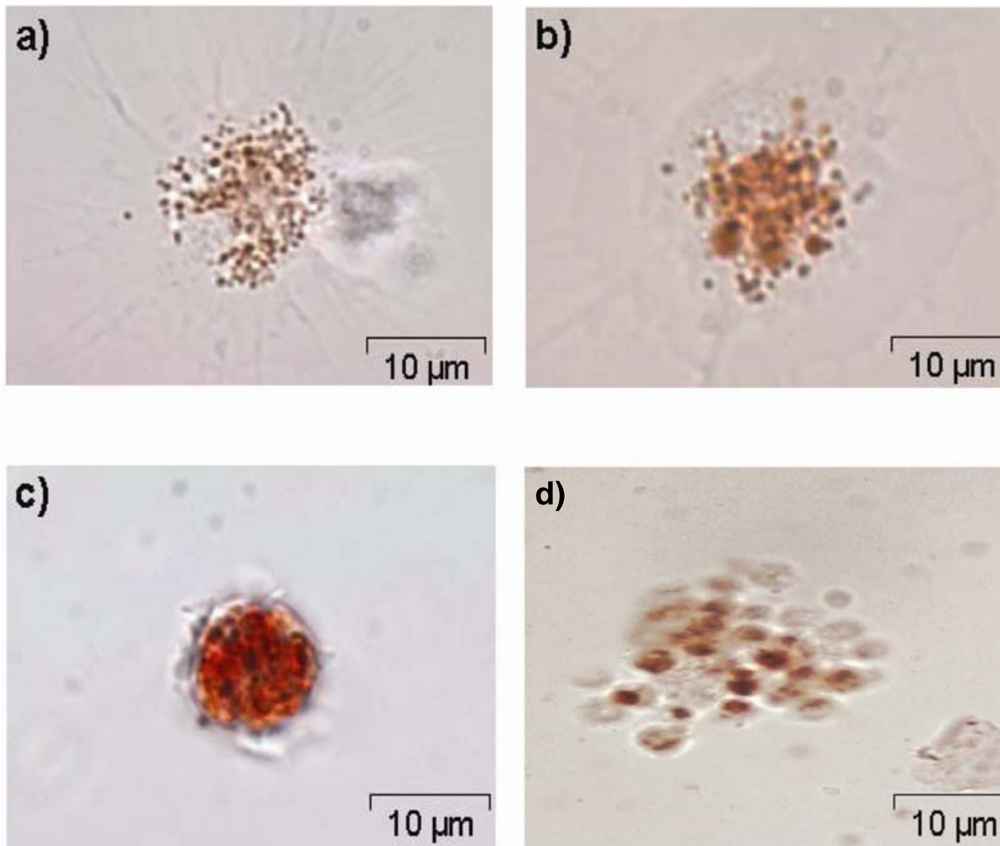


Plate 5.B2: Microscopic images of four states of lysosomes (the dark dots inside a cell) affected Neutral red dye in *Perna indica.*

a) Robust lysosomes

b) Lysosomal swelling

c) Lysosomal rupture

d) Leakage of neutral red dye

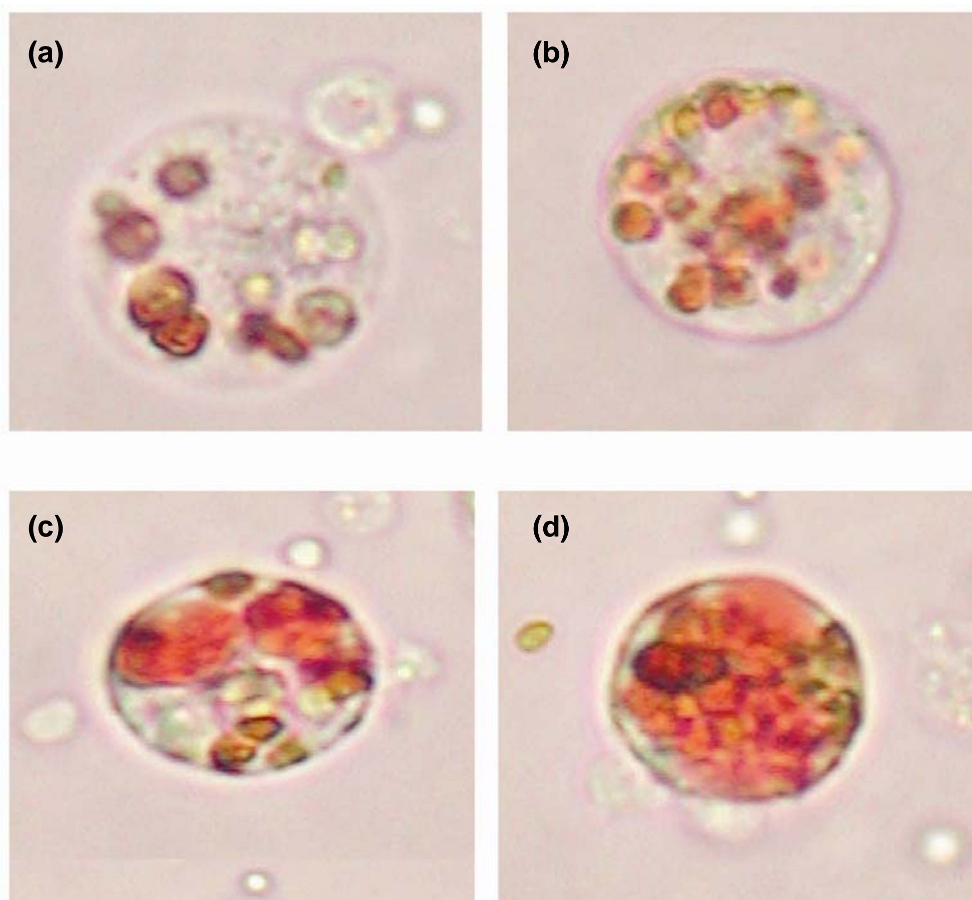


Plate 5.B3: Microscopic images of *Perna viridis* hepatopancreas cell scored as dye present in the lysosome (a-b) Stable condition.

Microscopic images of *Perna viridis* hepatopancreas cells scored as dye present in the cytosol (c-d) Destabilised.

5.B.4 Discussion

For comparisons of the health conditions between exposed organisms, the evaluation of different levels of organisation may provide a more comprehensive understanding of the actual status of the studied organisms.

Lysosomal stability has been defined as a very sensitive index of cellular condition (Moore 1978, 1985; Widdows *et al.*,1982; Viarengo *et al.,* 1987).

It has been reported that alterations in mussel hemocytes have consequences in the immune defense mechanisms (Lowe *et al.,* 1995). Lysosomal membrane destabilization provokes a release of hydrolitic enzymes, which can cause damage to cytosolic components due to proteolysis and lysis of organelles (Moore 1985; Cajaraville *et al.,* 1989). Because lysosomal membranes may not recover integrity in the short term, this alteration could have negative effects on reproduction and growth in the long term (Moore *et al.,* 1978; Lowe *et al.,* 1995).

It is worth to emphasize that the used technique to determine lysosomal stability (neutral red retention assay) possesses several advantages: low cost, short measuring time, and small sample size (only a tiny volume of blood is required) and, therefore, the same individual mussel can also be used to measure other biological responses.

Changes in size and number of lysosomes in the digestive cells in mussels have also been used as an indicator of environmental stress (Etxeberria *et al.,* 1995). The enlargement of lysosomes could be due to alterations in fusion events in the lysosomal-vacuolar system of these cells and has been related to the lysosomal membrane destabilization in the presence of pollutants (Moore 1985, 1988; Lowe *et al.,* 1992; Hole *et al.,*1995).

At the cellular level, the lysosomal system has been identified as the target for the effects of many readily accumulated contaminants (Moore, 1990). This is due to

their ability to accumulate a diverse range of toxic metals and organic chemicals which can result in enhanced toxicity and cell injury (Lowe and Pipe, 1994). Adverse changes in the lysosomal system are significant since they can contribute directly to pathology (Hawkins, 1980) and have been related to adverse alterations in scope for growth in marine mussels (Widdows *et al.,* 1982). Lysosomal integrity has been utilized in a number of field studies as a biomarker for the consequences of environmental contamination (Regoli, 1992; Krishnakumar *et al.,* 1994; Lowe *et al.*, 1995a).

The major function of lysosomes in bivalves is food degradation in the digestive diverticula cells. Lysosomes contain hydrolytic enzymes which are responsible for autophagy (breaking down substances within a cell) and heterophagy (breaking down substances invaded in a cell) (Holtzman, 1976). As these hydrolytic enzymes are capable of degrading cellular constituents, they are predominantly sequestered in an inactive form inside lysosomes enclosed by lysosomal membranes in order to prevent free access to the cytosol. Another function of lysosomes is storage of both metals and organic chemicals in order to reduce their toxicities to other sensitive cytosolic components. However, over-accumulation of these chemicals can destabilize the lysosomal membranes (Moore, 1985). Subsequently, lysosomal membranes become more permeable to the hydrolytic enzymes, which then freely pass into cytosol, leading to abnormal autophagy (Lowe *et al.*, 1992, 1995; Nicholson, 1999a; Au, 2004; Nicholson and Lam, 2005). Lysosomal damage resulting from excess intra-lysosomal storage, i.e., lysosomal integrity, shows a good dose- response relationship to a wide range of pollutants and pollutant-induced stress (Au, 2004 ; Nicholson and Lam, 2005).

The neutral red retention technique has been shown to be a rapid and sensitive test for determining the lysosomal stability of indigenous invertebrate populations (Lowe *et al.,* 1995a; Wedderburn *et al.,* 1998; Cheung *et al.,* 1998) and to be relatively robust against environmental factors, such as salinity and temperature (Ringwood *et al.*, 1998). The neutral red technique measures the retention of neutral red, a weak

base dye, within the lysosomal compartment. The dye is added to isolated live blood cells and permeates into the lysosome in its unprotonated form where it becomes trapped by protonation (DeDuve *et al.,* 1974) inducing perturbations such as swelling and enhanced autophagy (Robbins *et al.*, 1964). Sub-cellular retention of the neutral red dye is measured at timed intervals using light microscopy. Given the susceptibility of the lysosomal system to xenobiotic insult the exposure to the neutral red probe will represent an additional challenge. Thus, cells from animals exposed to environmental contaminants will exhibit reduced retention times for the neutral red probe (Lowe *et al.,* 1995b; Wedderburn *et al.,* 1998; Cheung *et al.,*1998). Conversely, animals in control will be able to maintain lysosomal integrity against the challenge of the neutral red for longer periods of time.

Lysosomal damage has been shown to be a prognostic biomarker for pathology and reduced fitness (Hawkins, 1980; Moore, 1990; Lowe *et al.*, 1992; Depledge *et al.,* 1993)

The morpho-functional alteration of the lysosomes is a well-known consequence of chemical pollution, and lysosomal membrane destabilisation was first reported as a precocious biomarker of stress in marine mussels (Moore, 1976, 1982). Lysosomes have an important role in the cell compartmentation of heavy metals and organic xenobiotics, being also subject to the toxicity of these substances (George *et al.*, 1976; Lowe and Moore, 1979;Winston and Di Giulio, 1991).

Biomarkers include a variety of measures of specific molecular, cellular and physiological responses of key species to contaminant exposure (Depledge, 1994, 1999; Depledge *et al.*, 1993; Moore, *et al.,* 2004). A response is generally indicative of either contaminant exposure or compromised physiological fitness. The challenge is to integrate individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation in health of a particular type of sentinel organism.

Responses of the lysosomal-vacuolar system may provide a solution to the question of prognostic biomarkers, since injurious lysosomal reactions frequently precede cell and tissue pathology. Lysosomal perturbations have been widely used as early indicators of adverse effect to various factors, including pollutant exposure

Lysosomes are highly conserved multi-functional cellular organelles present in almost all cells of eukaryotic organisms from yeast to humans. Their function in the cellular economy includes the degradation of redundant or damaged organelles (e.g., mitochondria and endoplasmic reticulum) and longer lived proteins as part of autophagic cellular turnover (Klionsky and Emr, 2000). Lysosomes are also involved in the digestion of materials ingested by endocytosis and phagocytosis (i.e., intracellular digestion).

According to our results, the digestive gland AChE could serve as potent biomarkers of organochlorinated and PAHs compounds. Our suggestion is also enhanced by the significant positive correlation of the AChE activity in the digestive gland with the values of the NRR assay, which as it has already been discussed could constitute a potent marker of environmental stress.

Molecular and cellular biomarkers are very sensitive "early warning" tools for biological effect measurements in environmental quality assessment, and a battery of them is recommended for the most appropriate use as biomonitoring tools (Ring-wood *et al.*, 1999; UNEP, 1997). In the present study, the validity of performing the core biomarkers of the NRR assay and of the AChE inhibition (especially in the haemolymph and the digestive gland) is supported, firstly by their ability to respond to different pollution levels and secondly by the significant linear correlation among them.

However, MN responses need more research in order their use as stress indices to be validated. In addition, the first results on cAMP levels, whose concentrations correlated to both, NRR and AChE introduce this signal transduction molecule as a promising biomarker, although further laboratory and field validation are needed in order to be proposed for pollution monitoring.

Chapter 6

FIELD STUDIES ON TOXICITY EMPLOYING TRANSPLANTS

Contents

6.1 Introduction
6.2 Review of Literature
6.3 Materials and Methods
6.4 Result
6.5 Discussion and Conclusion

6.1 Introduction

In reality, most aquatic systems experience some level of chronic and/or sublethal exposure from environmental pollutants which impact these systems through point source or non-point source pathways (Schlenk, 1996). While impacts on aquatic systems from point-source pathways are relatively straightforward to assess using standard toxicological and ecotoxicological approaches, effects from non-point sources are much more difficult to assess and quantify primarily due to multiple stressor effects.

Classical toxicology has been one of the primary approaches by which the effects of environmental pollutants on aquatic organisms have been assessed. A variety of standardized laboratory toxicology studies have been conducted where contaminant dose, for example, has been related to responses of individual organisms particularly at the biochemical or physiological levels. These types of controlled lab studies, however, provide limited information about how organisms, short of mortality or other survival endpoints, respond to environmental stressors in their natural environment. The experimental conditions of laboratory studies seldom accurately reflect natural conditions and they lack ecological realism (NRCC, 1985; Lagadic *et al.,*

1994). In addition to these laboratory toxicology studies, efforts to assess and evaluate the effects of environmental stressors on organism health have involved examination of the relationships between contaminant concentrations and specific biological responses, usually at only one or two levels of organization (e.g., Huggett *et al.*, 1992; McCarthy and Shugart, 1990).

Ecotoxicological studies have also utilized correlation statistics or even semiquantitative procedures to try and establish relationships between specific types or groups of contaminants and various biological responses (Schlenk *et al.*, 1996; Adams *et al.*, 1999). Although statistical correlations may suggest cause and effect, they cannot be used, however, to establish definitive causality between a stressor (e.g., contaminant) and specific biological responses.Because of the problems and limitations of laboratory toxicology studies and most other methods which have been used in attempts to evaluate and quantify the effects of environmental stressors on the health of aquatic systems, better field bioassessment approaches are needed which provide an integrated framework for addressing cumulative and/or synergistic environmental impacts on the health of aquatic systems. One such method, the bioindicators approach, has proven successful in assessing and evaluating the effects of contaminants and other types of environmental stressors on the health and integrity of key components of aquatic systems. The bioindicator approach is based on using sentinel or ecologically important organisms and specific responses of these organisms as integrators and sensitive response indicators of past and existing environmental conditions (Adams, 1990; Adams *et al.*, 1999). Both rapidly-responding exposure biomarkers such as bimolecular and biochemical responses (Huggett *et al.*, 1992), and slower-response but ecologically relevant bioindicators such as population and community responses are included in this bioindicator assessment methodology.

The primary advantages of this integrated bioindicators approach, therefore, is that it can provide (1) early warning signals of environmental damage, (2) assessment

of the integrated effects of a variety of environmental stressors on the health of organisms, populations, and communities, (3) sentinels of potential hazards to human health based on the responses of fish, shellfish, and others to environmental stressors, (4) evaluation of the effectiveness of remediation efforts in clean up of contaminated areas, and (5) scientifically sound information for addressing ecological and possibly human health risk issues at contaminated sites. Within this context, the primary objective of this presentation is to demonstrate, using two brief examples, how bioindicators can be utilized in field situations to address environmental issues related to water quality.

Biomonitoring of marine pollution using the common mussel as a bioindicator has received special attention in the past few years. There are many advantages of using *in situ* bioassays, i.e. to transfer organisms to different sites of interest, as the results give direct information on marine environmental quality that would be impossible to obtain through chemical monitoring alone or laboratory assays. Biological impact of PHC was studied using the bivalves *Perna viridis* collected from the Tangassery regions of Kollam (Plate 6.1). Seeds of 20 mm average size were used in the experiment. The number of specimens for use depends on the kind of measurement to be done, but in this experiment 150 test animals per site were used.

Amounts of bioaccumulated contaminants are closely linked to the mussel's life cycle, particularly the individual's age and sexual maturity. Characteristics of the immersion site, such as salinity and food availability, affect pollutant bioavailability and speciation of the pollutant, as well as the metabolism and tissue growth of the mussel, in which the pollutant is diluted (Cossa, 1989).Although the concentrations measured in the tissue are a function of bioavailable pollutant levels, the bioaccumulation factor depends on mussel growth in relation to the primary food production, or trophic capacity, of the environment.

The Cochin backwater area has not only had much heavy industry including oil refineries and also jetties for commercial and oil tanker ships. There is heavy mixing of water between the Cochin estuary and the Arabian Sea takes place during tides.

Field validation of results is a useful approach for ultimately assessing environmental impacts, and also for determining confidence in predicting impacts from laboratory studies.

The results of the present study are expected to provide useful information for biomonitoring application, regarding, in particular, the differential profitability of transplanted mussels with respect to specific biomarker responses.

6.2 Review of Literature

Mussels and other filter feeding molluscs are widely used in laboratory and field experiments as sensitive biomarkers of chemical contamination (Moore *et al.*,1978; Moore,1985; Viarengo *et al.*, 1987; Lowe,1988; Viarengo and Canesi,1991; Krishnakumar *et al.*, 1994; Lowe *et al.*,1995; Ringwood *et al.*, 1998). Sublethal effects of contaminants on marine organisms can also be determined using such approaches, and one advantage of transplanting bivalves for this purpose is that it provides a combination of the experimental control of laboratory bioassays with the environmental realism of field monitoring (Salazar and Salazar 1997). The premise for transplanting marine organisms from clean environments to those containing contaminants, is that the individuals have not previously had the opportunity to regulate their physiology in order to withstand contaminant stress. This enables the estimation of initial stress response (lethal and sublethal), which may be correlated with levels of measurable contaminants.

Owing to their ability to accumulate various xenobiotics and toxic compounds and due to their extensive distribution along the coastal marine environment, mussels

represent appropriate tools and useful sentinel organisms for biomonitoring the presence and perhaps the effects of different chemical contaminants.

The use of bivalve molluscs as indicators of xenobiotics or biological stress in aquatic environments has gained widespread acceptance (Goldberg *et al*., 1978; Phillips, 1980). Most biomonitoring studies have employed bivalves native to the site of interest, but some studies have transplanted indicator organisms along a suspected pollution gradient (Bayne *et al*., 1979; Martin, *et al*., 1984). Transplanting organisms has some advantages. Transplants can also be taken from a single population, thus reducing genetic variability and improving the homogeneity of response among test environments. Questions remain, however, about how closely transplanted organisms follow the responses to contaminants of organisms native to an area.

The interest in using these organisms relies on the fact that they are permanent inhabitants of specific sites and rather resistant to local pollution; they are also filter-feeders and have only a limited metabolic ability. Further, contaminants present in the water column, which may have not been recorded in the sediments, can accumulate in bivalve tissues to levels several orders of magnitude higher than those found in the surrounding seawater (Neff, 2002).

Transplanted animals used to compare levels of contamination at several locations must be selected from the same population (Ritz *et al.,* 1982; Widdows *et al.,* 1981).

The compounds selected for the mesocosm exposures were selected on their ecological relevance. Environmental pollution related to petroleum exploitation and transport in the aquatic environment is a general problem worldwide.

A mixture of alkyl phenols (APs) and PAHs was designed to simulate the exposure of marine organisms to produced water from offshore oil installations.

Produced water, a by-product from oil production, is a highly complex mixture of water and trace amount of oil. PAHs and APs are some of the compounds present and aromatic compounds give the most important contribution to toxicity (Harvey *et al.,* 1999; Aas *et al*., 2000),

Among the numerous ecotoxicological biomarkers proposed in the last three decades, those based on responses at the molecular and cellular level represent the earliest signals of environmental disturbance and are commonly used for biomonitoring (Bayne *et al.,* 1985; Depledge, 1994; Lowe *et al.,* 1995). The translocation of sentinel species, mainly mussels, from a reference site to the study areas has been demonstrated as a useful strategy for the assessment of water quality in coastal and estuarine environments, either through bioaccumulation or biomarkers analysis (Regoli and Orlando, 1994; Regoli, 2000; Da Ros *et al.,* 2002; Regoli *et al*., 2004). Caged mussels facilitate the investigation of areas where native specimens are absent, and/or the reduction of the influence of genetic differences and adaptive phenomena, which can attenuate the capacity of biomonitoring to discriminate different levels of environmental disturbance (Regoli and Principato, 1995).

A consolidated review of the information on the ecology of the Cochin estuary was provided recently (Menon and Menon, 2000).

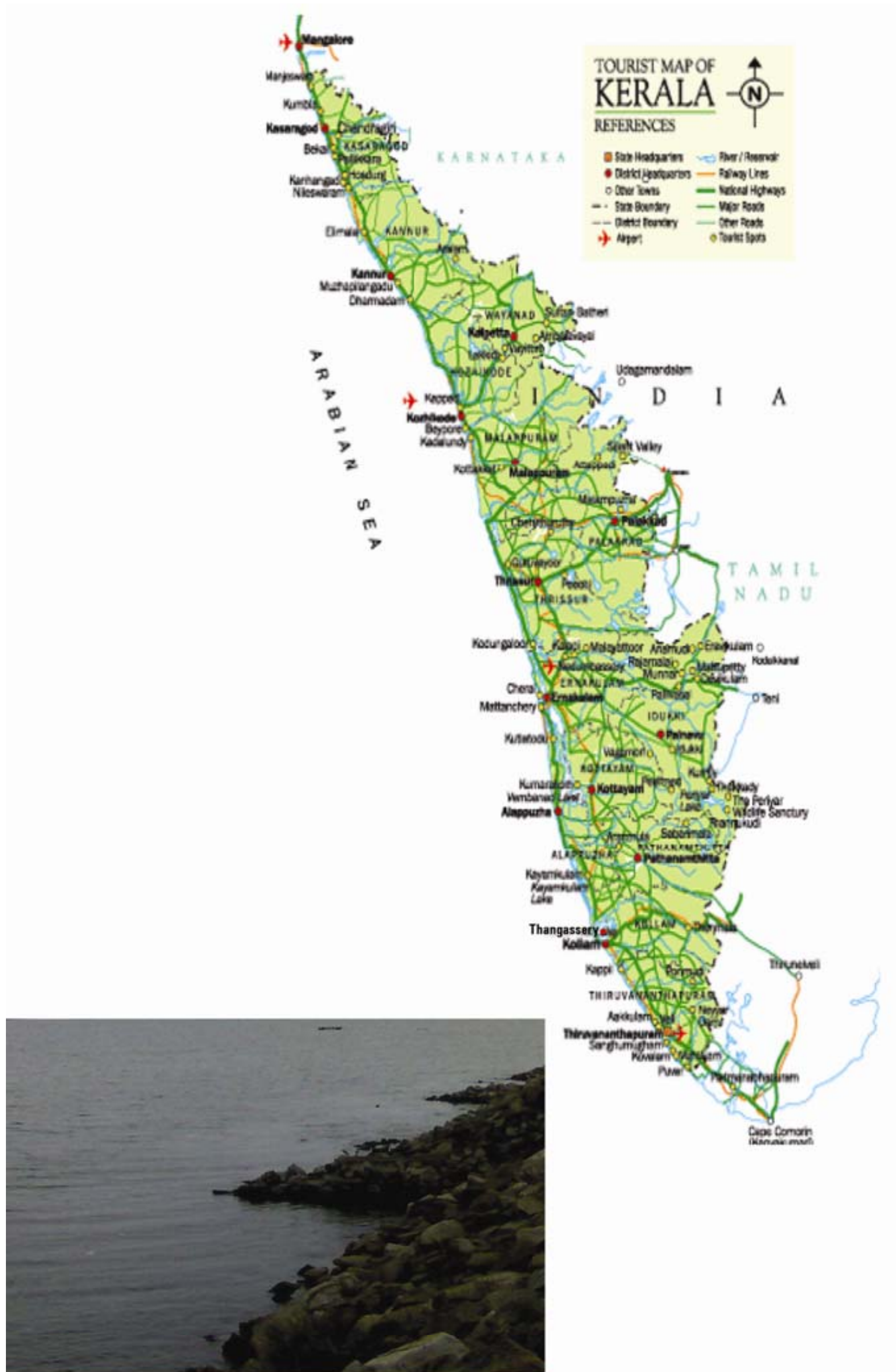


Plate 6.1 Mussels Collecting Site

6.3 Stations investigated

The Five Stations investigated during this study were selected to represent five significantly different ecological habitats in the Cochin Harbour (Plate 6.2). Cochin backwater, situated in the norlhward extensions of the Vembanad Lake, having an area of 300 km^2, is the largest estuarine water body on the southwest coast of India.

Station 1: **Manassery (Reference station)** [9^{0}53"N and 76°16"Ej

An open sea site situated in a rather unpolluted, less turbid location. There is a thriving mangrove near this area and the depth of the station is 2 m.

Station 2: **Ernakulam Channel -** [9°57'81"N and 76°16'93"E]

This station is nearly 4.5 km away from the bar mouth. The pier is used to load oil into barges and occasionally, oil pollution is bound to take place during these operations. Dredging in the channels release a cloud of detritus and silt, this raises the turbidity of the area. Foreign and inland ships frequent this berth Depth of the station is about 5 m.

Station 3: **Mattancherry Channel** [9°58'04"N and 76°15'57"E]

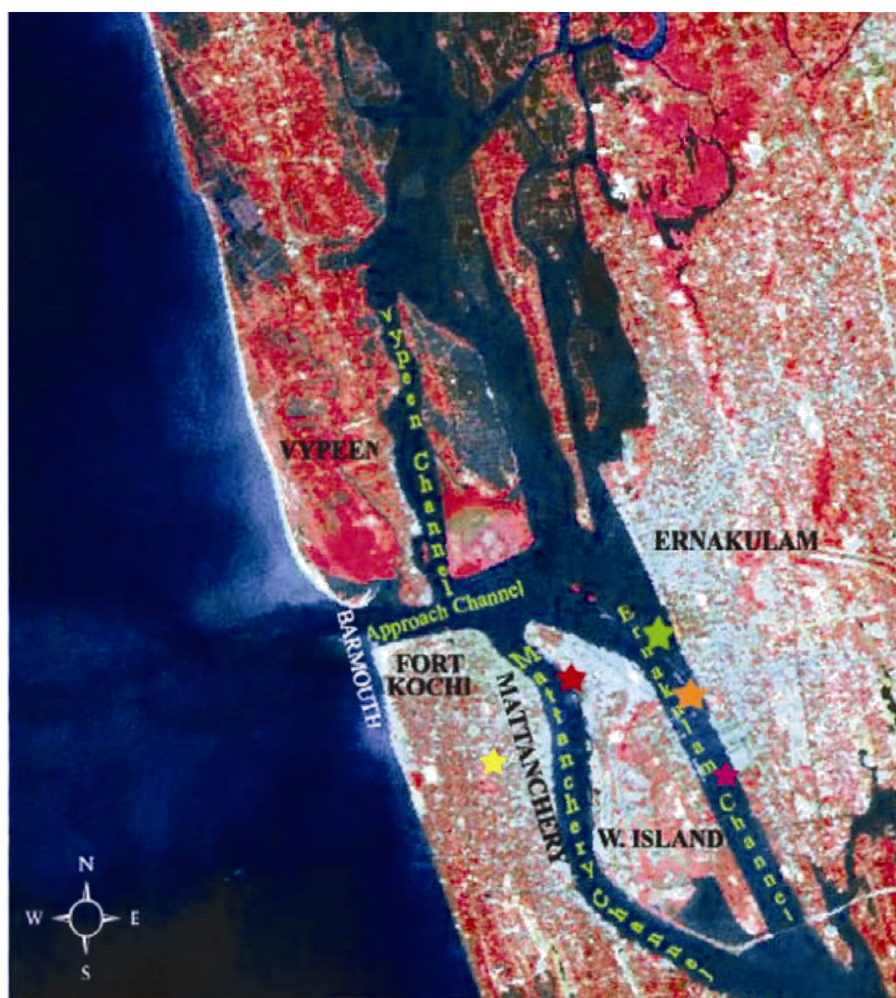
This station is situated in the Mattancherry Channel on the western side of the Willington Island nearly 3 km away from the barmouth. The water at this site is turbid due to dredging activities and is polluted as the cargo and passenger ships regularly anchor at this wharf. The depth of the station is 5 m.

Station 4: **South Oil Tanker Berth** [9°58'04"N and 76°16'83"E]

This station is also situated in the vicinity of oil transportation and shipping infrastructure.

Station 5: **North Oil Tanker Berth** [9°58'04"N and 76°16'73"E]

This station is located on the eastern side of the Ernakulam Channel, which is nearly 4 km away from bar mouth. This berth is used for transferring crude oil and petroleum to Kochi Refinery and other oil companies. Foreign and inland ships frequent this berth. Siltation is high due to dredging operations in the channel. Depth of the station is 5 m.



STATIONS

- Manassery
- Ernakulam Channel
- Mattanchery
- South Oil Tanker Berth
- North Oil Tanker Berth

Plate 6.2 Satellite Imagery of Cochin Estuary showing the mussel exposed stations

6.4 Materials and Methods

Exposure Apparatus

In this study, the type of exposure apparatus was a polyvinyl chloride (PVC) frame supporting fifteen cylindrical plastic mesh bags commonly used for mussel breeding (Plate 6.3) as described by Salazar and Salazar (1997). The frame made of ~ 2cm diameter PVC tubing, had dimensions of ~ 100 cm by ~ 80 cm. The mussels were held in place individually by tying off sections of the mesh bag with plastic tie wraps. The mussels got

attached firmly to the mesh bag by means of freshly secreted byssus threads. The cages were laid vertical on the substrate and secured with anchors in 5m of water.

Hydrographic Conditions

During the course of experiment, 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 and stored in pre cleaned polyethylene bottles for analysis.

Water quality parameters such as pH was analysed immediately after collection, dissolved oxygen (DO) was measured using Winkler method. Temperature was measured to the nearest 0.1^{0}C. Salinity was measured by salinometer. Nutrients such as nitrate, nitrite, ammonia, inorganic phosphate and reactive silicate were measured as per procedures described by Grasshoff *et al.,* (1983). Petroleum hydrocarbons in seawater were extracted in double distilled hexane and quantified using Shimadzu RF-1501 spectrofluorometer, at excitation-emission wavelengths of 310 and 360nm.
Sediment samples were collected by van Veen grab. Triplicate sub samples of approximately 100g were taken carefully from the surface layer of the sediment collected. Organisms and shell debris were removed and stored frozen at –20^{0}C prior to analysis. Thawed samples were dried under vacuum at 40^{0}C. Then, 0.5g of sediment was 20ml of methanol and 0.75g of potassium hydroxide for 2h. Products

were then extracted three times with 25 ml of hexane. PAHs in hexane were estimated by comparison of the fluorescence at 360 nm (excitation at 310 nm) with that of hexane solutions of chrysene (IOC,1982). The organic carbon in the sediment was determined using the method of Walkley and Black (1990).

The mussels were sampled from each site. Shell length was measured, and after shell opening and gross examination, the body was removed and weighed. Biota were treated similarly to sediments. PAH concentrations were calculated from the equivalent chrysene fluorescence (Donkin and Evans, 1984).

The mussels were then fixed for histological examination in Bouins fixative for 24h, and kept in 10% formalin until processing. The exposed mussels were then subject to extensive histopathologic and cytological observations to look for early indication of cytologic and physiologic alterations in the tissue and organs.

Plate 6.3 Exposure apparatus

6.5 Results

Hydrological parameters of five different stations located in the Cochin estuary during the study period (Table.6.1) and PAHs concentrations in surface water, subsurface water and sediment were given in (Table 6.2 to 6.4). Caging had no dramatic impact on PAHs concentrations in tissues of *P.viridis*.

Phosphate shows a concentration range between 2.888 to 8.850 µg/l in surface water and 1.832 to 6.850 µg/l in column water.

Both the Nitrite and Nitrate values are low both in the surface and column waters of all stations. Considerably low values of ammonia and silicate were noted in the study period in all the stations.

Surface water showed maximum variance over the seasons followed by subsurface waters. Within stations also the pattern was the same with least variance noticed in PHC values in sediments.

South oil Tanker Berth stations appear to be most polluted showing consistently higher values for the sediment. Tissue load of PHC of *P.viridis* showed a positive correlation with the pollutional status of the environment. Thus, animals exposed at Manassery, the reference site, harboured minimum PHC in their tissues. Animals at the North oil tanker berth accumulated the maximum quantity of oil in their tissues. The variance between stations and variation of PHC concentration within station over time were found to be significant ($P<0.001$).

Histopathology in Manassery station of the Cochin estuary

The frequency of general indices of lesions was derived for more than 650 individuals of the mussels *P.viridis* exposed in the five stations located in the Cochin estuary from May 2005 to 2007.

Typical lesions measured in tissues of *P.viridis* from different areas of the Cochin estuary are shown in Plate 6.4. Highlighting for *P. viridis* infiltrations leucocites

at level of gills, mantle, digestive glands, and ovary as well as alterations in epithelial and ciliar structures of gills and hepatopancreas. In *P.viridis* an increase of mucous secretion was observed at level of gills as well as infiltration leucocytes in the mantle. In the digestive apparatus it has been detected an apparent ciliar loss and sloughing of the cells epithelial decamation; without any significant alterations in the gonads.Digestive glands of control mussels are shown in Plate 6.4a.

Histopathology of mussels from other stations 2 to 5

The results of the lesions measured in *P.viridis* from four selected areas such as Ernakulam Wharf, Mattanchery Wharf, South oil Tanker Berth, and North oil tanker Berth were identified in the digestive gland of the exposed mussels: tubular dilation or atrophy (TDA), macrophage aggregates (DMA), tubular cell necrosis (TBN), and inflammation (DINF).

The number of digestive gland lesions observed was not significantly different between male and female mussels. There was no indication that Apicomplexan diseases observed in intestine were responsible for the increased number of lesions in the digestive gland. Lesions in the exposed site mussels were significantly more than in control animals. Comparing with the control mussels, lesions increased significantly in mussels exposed in the three most contaminated stations. (Plate 6.5-6.8).

More concrete evidence of contaminant-related pathological changes in cell structure has been obtained from investigations of the hepatopancreas of *Perna viridis*. This has involved pollutant induced alterations in the epithelial cells of the digestive tubules, often leading to epithelial thinning or apparent atrophy.

Table.6.1. Hydrological parameters of five different stations located in the Cochin estuary during the study period

Parameter	Stations and locations				
	North oil Terminal	South oil Terminal	Ernakulam Wharf	Mattancherry Wharf	Manassery
Water Temperature (^{0}C)	28	29	33	32	28
Salinity (ppt)	28	27	30	32	30
DO (mg/l)	4.3	5.53	5.15	4.97	4.81
pH	7.9	7.6	8.2	8.3	7.2
Nitrite (µg/l)	0.903	0.845	0.966	0.518	0.804
Nitrate (µg/l)	9.234	8.844	11.03	18.38	6.24
Ammonia (µg/l)	0.018	0.013	0.019	0.008	0.013
Phosphate (µg/l)	3.488	3.675	8.850	4.275	2.888
Silicate (µg/l)	0.52	0.312	0.627	0.451	0.322
Column water					
Nitrite (µg/l)	0.226	0.468	0.776	0.651	0.221
Nitrate (µg/l)	16.94	11.68	24.21	21.03	6.313
Ammonia (µg/l)	0.019	0.018	0.014	0.018	0.008
Phosphate (µg/l)	2.100	3.641	5.971	6.850	1.832
Silicate (µg/l)	0.364	0.421	0.381	0.322	0.348

Table. 6.2 Petroleum hydrocarbon concentrations (µg/l chrysene units) in surface waters of various stations located in the cochin estuary.

Months	PHC concentration (µg/l)				
	Stations and locations				
	North oil Terminal	South oil Terminal	Ernakulam Wharf	Mattancherry Wharf	Manassery
May	20.82	30.42	32.24	16.15	2.25
Jun	10.42	16.15	30.15	10.12	2.85
Jul	15.04	18.42	30.26	12.23	2.42
Aug	35.64	30.15	20.18	16.23	5.12
Sep	42.49	40.26	35.54	20.72	4.73
Oct	54.21	61.88	57.58	34.16	4.52
Nov	60.3	59.55	45.63	48.12	5.46
Dec	22.42	35.28	28.51	32.15	3.41
Jan	164.16	112.54	87.85	50.46	6.32
Feb	254.76	169.13	117.1	80.21	6.54
Mar	189.12	212.46	184.25	69.15	3.42
Apr	152.28	172.25	168.36	68.2	4.53
May	20.04	20.25	32.16	30.14	3.51
Jun	15.16	10.2	17.7	28.18	1.26
Jul	10.15	11.12	20.22	22.12	2.25
Aug	20.18	22.46	21.44	20.72	2.72

Surface Waters

Source of Variation	DF	SS	MS	F	P
Rows	15	135617.291	9041.153	7.988	<0.001
Columns	4	45573.189	11393.297	10.066	<0.001
Residual	60	67910.451	1131.841		
Total	79	249100.931	3153.176		

Table. 6.3. Petroleum hydrocarbon concentrations (μg/l chrysene units) in subsurface waters of various stations located in the cochin estuary.

Months	PHC concentration (μg/l) Stations and locations				
	North oil Terminal	South oil Terminal	Ernakulam Wharf	Mattancherry Wharf	Manassery
May	22.42	16.53	21.24	16.17	3.18
Jun	20.16	18.48	18.18	15.34	2.45
Jul	20.22	16.32	23.44	12.14	1.35
Aug	38.41	30.25	20.01	11.23	2.42
Sep	35.26	25.31	48.34	9.45	3.88
Oct	42.18	40.33	15.33	18.42	4.54
Nov	50.20	26.13	28.34	16.33	4.18
Dec	44.12	50.45	22.46	18.69	3.21
Jan	120.42	143.21	88.44	48.35	4.18
Feb	250.54	114.23	82.38	60.10	3.25
Mar	188.22	89.62	104.36	69.34	4.18
Apr	111.43	76.54	78.43	50.32	4.68
May	80.42	60.37	63.14	40.38	2.11
Jun	20.41	35.42	40.31	11.5	2.49
Jul	18.24	20.01	25.11	12.2	2.31
Aug	25.35	10.05	20.28	18.42	1.58

Subsurface Waters

Source of Variation	DF	SS	MS	F	P
Rows	15	66964.844	4464.323	6.048	<0.001
columns	4	38110.713	9527.678	12.908	<0.001
Residual	60	44288.354	738.139		
Total	79	149363.911	1890.682		

Table. 6.4: Petroleum hydrocarbon concentrations (μg/g chrysene units) in the sediments of various stations located in the cochin estuary.

Months	**PHC concentration (μg/g)**				
	Stations and locations				
	North oil Terminal	**South oil Terminal**	**Ernakulam Wharf**	**Mattancherry Wharf**	**Manassery**
May	18.31	16.44	22.15	10.11	0.49
Jun	10.42	10.35	48.16	18.12	0.33
Jul	11.16	10.88	32.15	12.55	0.59
Aug	8.38	10.49	40.16	9.33	0.98
Sep	12.31	12.54	18.12	8.56	0.25
Oct	11.48	13.28	15.35	9.55	0.99
Nov	10.45	9.35	16.49	10.35	0.86
Dec	8.32	8.11	22.54	16.58	1.52
Jan	8.48	15.34	30.19	15.32	3.5
Feb	25.38	28.59	56.11	16.42	4.52
Mar	18.15	29.52	29.48	16.38	1.22
Apr	16.34	18.43	33.49	10.44	1.57
May	16.45	15.31	18.35	12.38	0.91
Jun	4.59	4.99	20.34	14.39	0.43
Jul	18.33	7.33	18.66	20.59	0.22
Aug	16.21	6.89	20.31	8.37	0.49

Sediments

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Rows	15	1379.478	91.965	2.650	<0.001
Columns	4	5900.658	1475.164	42.510	<0.001
Residual	60	2082.096	34.702		
Total	79	9362.232	118.509		

Lysosomal membrane stability

Digestive cells from animals exposed to environmental contaminants has exhibited reduced retention times for the neutral red dye. Conversely, animals in relatively clean sites has the ability to maintain lysosomal integrity against the challenge of the neutral red for longer periods of time. Retention time were in the range of 5-25 min. The deployed animals from the Manassery site were shown to have greater retention time than the other stations, indicating greater lysosomal stability (Figure 6.1).

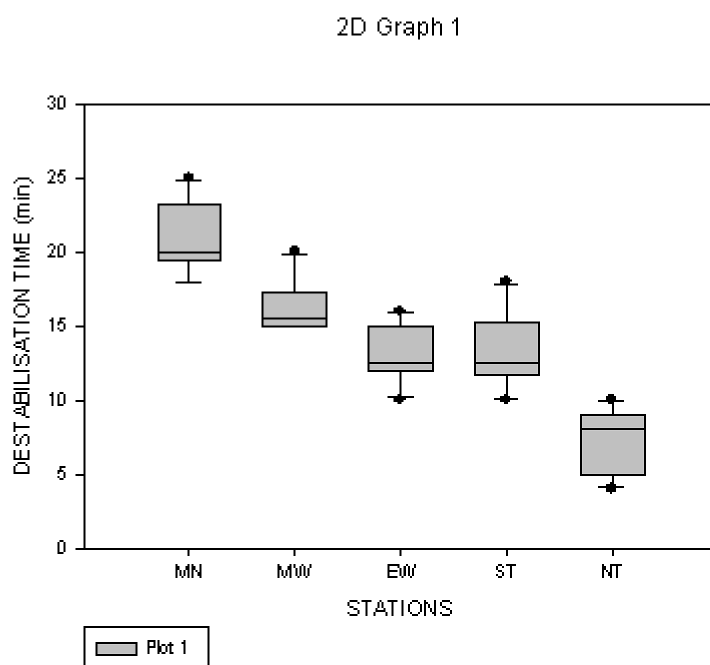


Figure 6.1: In situ studies: Lysosomal Box – whiskers plots of lysosomal membrane stability in the digestive gland *Perna viridis* at five different locations in Cochin estuary. MN-Manassery, EW-Ernakulam wharf, MW-Mattanchery Wharf, ST- South oil Terminal, NT – North oil Terminal Median (line), 25% and 75% quantiles (boxes), 10% and 90% quantiles (bars) and outliners (dots)

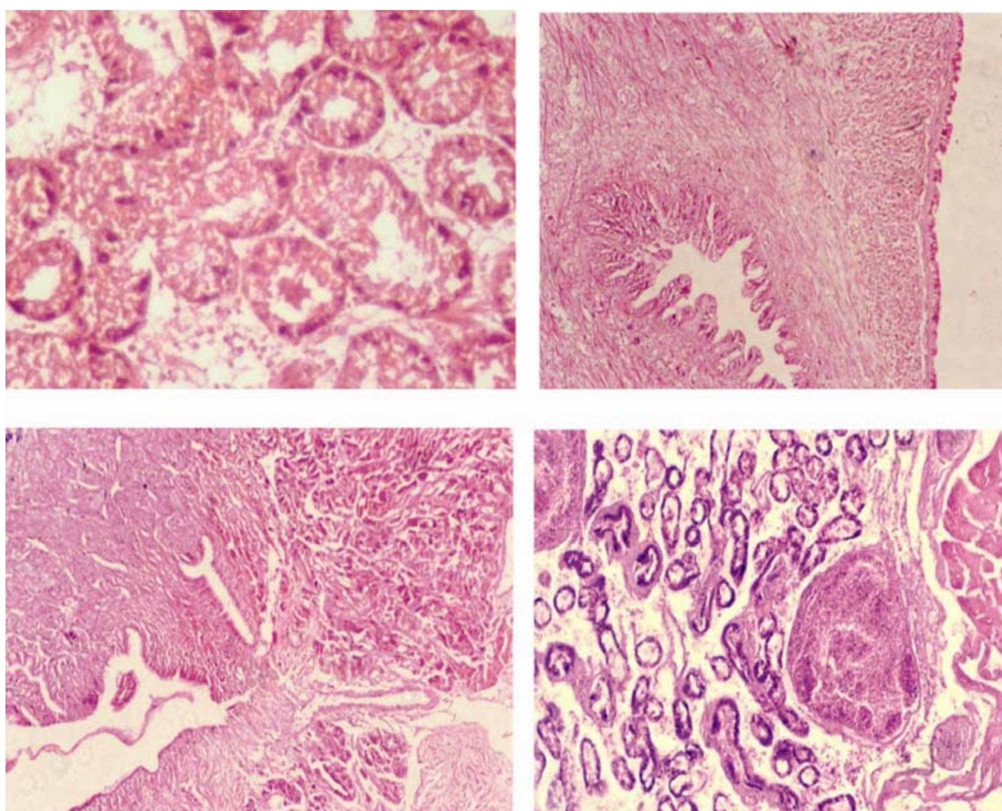


Plate 6.4: In situ studies: Light micrographs of sections through the digestive gland of *P.viridis* transplanted to Station 1 Manassery (reference) to station 5. a) Typical appearance of digestive tubules of mussels from Station 1 X 200 b) Mantle margin of *P. viridis* , showing decreased number of vacuolated connective tissue cells (VC) and detached epithelial cell layers X 100 c) Mantle margin of *P. viridis* showing vacuolation X 100 d) atrophic tubules in mussels from Station 4 X 200

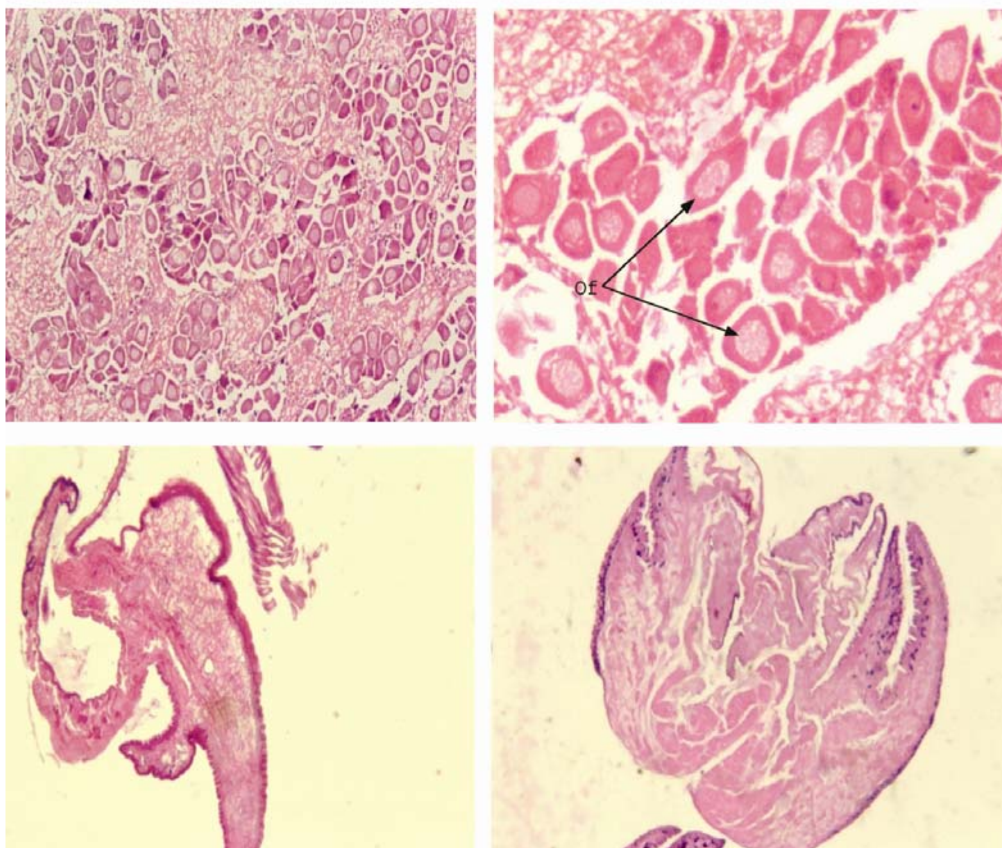


Plate 6.5: In situ studies: a) developing stage of female gonad from reference station. X 100 b)Female gonad lesions in *P. viridis* exposed to Ernakulam Wharf sediments with wide spread occurrence of athretic oocytes.Infiltration by haemocytes is also observed. Each mature oocyte contains a germinal vesicle (GV) nucleolus (N), vitelline layer (VL) amd yolk granules (YG) X 200 c) Transverse section through the mantle margin of control *P. viridis* X 200 d) Distorted manle margin in *P.viridis* from station 2 X 200

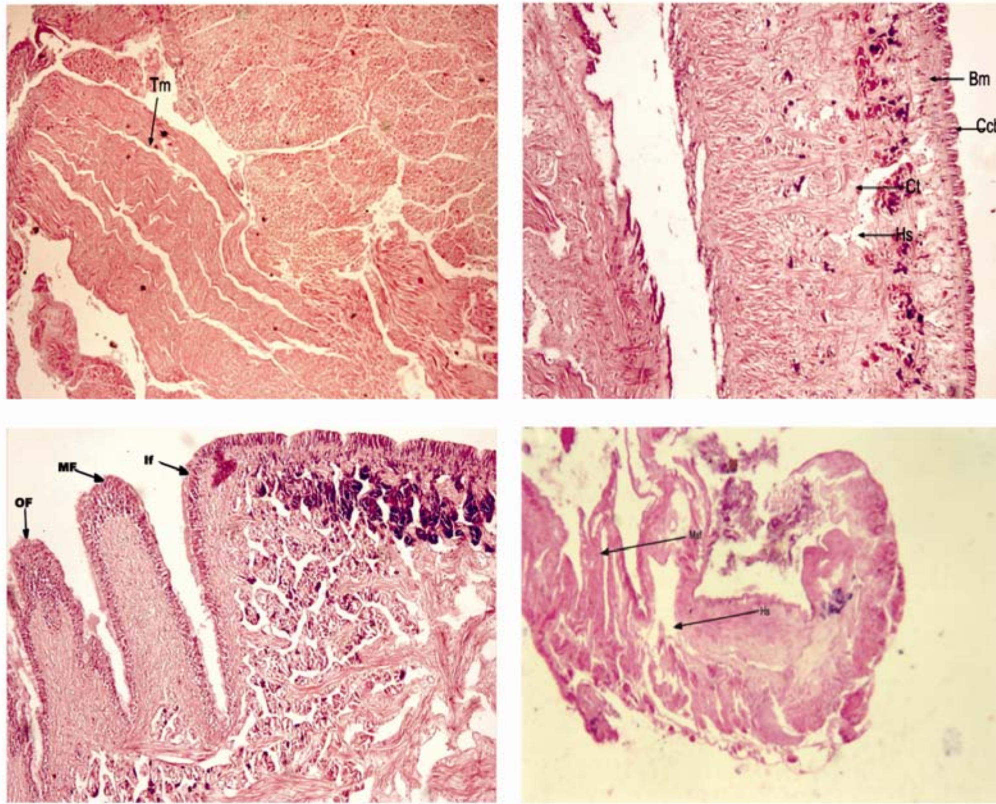


Plate 6.6 : In situ studies: a-d) showing cellular damages in mantle of *P. viridis,* (inner mantle fold (IF), middle mantle fold (MF), outer mantle fold (OF and vacuolated cell (VC) X 200

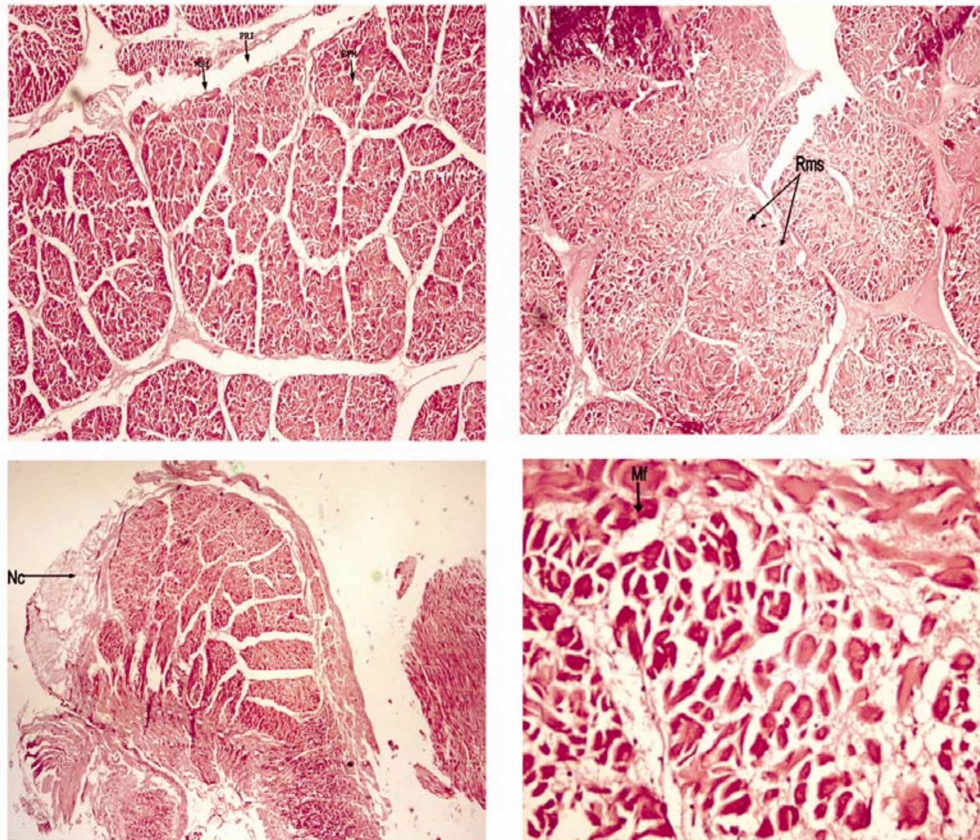


Plate 6.7: In situ studies: a-d) showing cellular damages in adductor muscle of *P. viridis X* 200

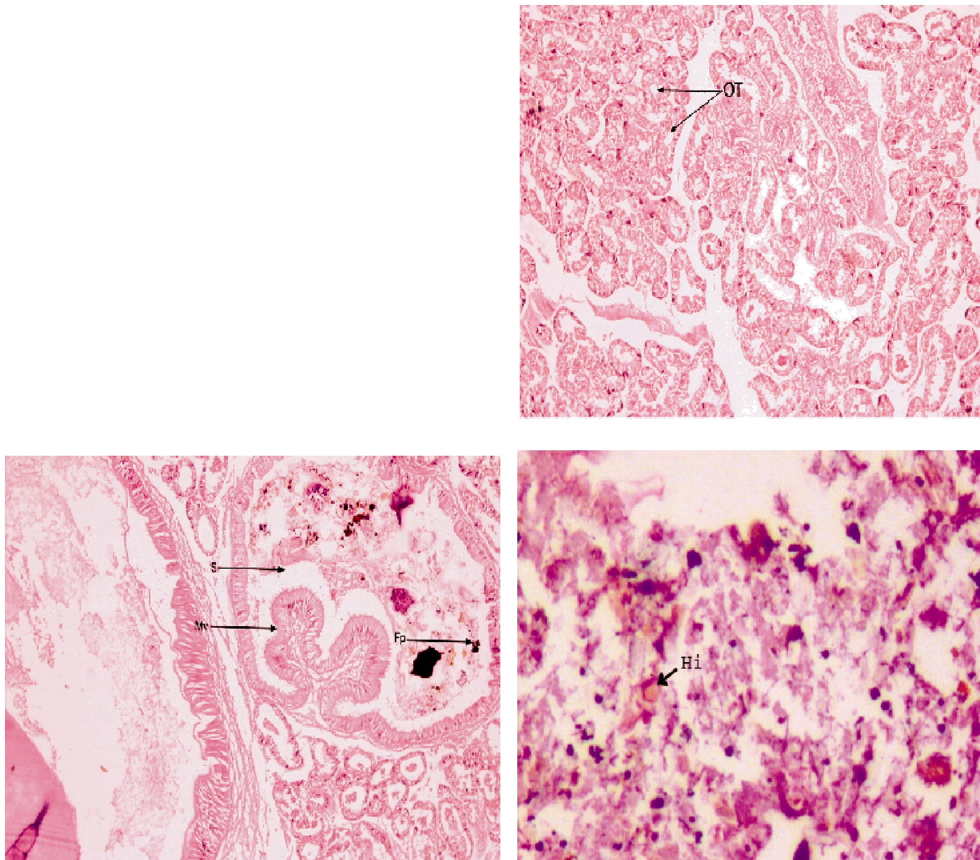


Plate 6.8: In situ studies: a-d) showing cellular damages in digestive diverticula of *P. viridis* X 200

6.6 Discussion and Conclusion

An understanding of the petroleum hydrocarbon metabolism in the environment is fundamental to the assessment of its effects on the biota. Monitoring the total concentrations, and chronological variations in PHC quantities provide baseline information for assessing and predicting the health of the ecosystem.

The Ernakulam channel is around 5 km in length with a width of around 250-500 m. The Mattanchery channel has a length of 3 km and a width of around 170-250 m. Depth of the two channels vary between 1.5 m and 6.0 m in most parts except the dredged channels, which are 10-13 m deep. The sediments of these channels are a mixture of clay and silt and vary based on the season (Menon *et al.,* 2000).

The transplantation of mussels from control to impacted area is commonly used for the environmental quality evaluation in coastal environments and is receiving growing interest for ecotoxicological investigations (Regoli and Orlando, 1994; Regoli, 2000; Da Ros *et al.*, 2002; Regoli *et al.,* 2004). In the present study, a set of cellular biomarkers and PAHs bioaccumulation have been investigated, in transplanted specimens, in order to evaluate the sensitivity of the approach and their profitability for monitoring estuarine environments.

In this study, we used a suite of histopathologic biomarkers and a semiquantitative scoring system to assess the chronic effects of contaminated area on *P. viridis*. A screening evaluation was conducted on major organs and tissues of mussels to identify lesions that can be used as indicators of biologic effects. Based on the results of this evaluation, the digestive gland and gonads were selected for more detailed observation. Studies using both histopathology and biomarkers to assess chronic effects of sediment contaminants on *P. viridis* are rare.

The seasonal cycle in Indian sub-continent is mainly dependent upon the monsoonal cycle. This monsoonal cycle has some pronounced effect on the physico-chemical parameters of aquatic medium, particularly on salinity and to some extent on pH in the Cochin estuary.

The salinity gradient of this area is greatly influenced by the seawater intrusion and the emptying of rivers. Saline water intrudes the estuary through the bar mouth area in the approach channel during the daily tidal rhythm. Major rivers, such as the Periyar and the Moovattupuzha empty either into the Vembanad Lake or into the backwaters. Another factor influencing the salinity of this area is the yearly rainfall. Maximum rainfall is recorded in this region during the southwest monsoon, which bursts along the southwest coast during late May or early June. Some precipitation occurs during the northeast monsoon and also during the period October - December. The rainfall, during the two monsoons and the consequent drainage, influence the salinity of the backwaters from June to December, and in July and August the surface water of Cochin backwaters may almost be fresh. This fluctuating salinity conditions bring about variations in the availability of food, variation in the substrate characteristics and also other changes in the physical characteris tics of the water body. The salinity gradient in Cochin backwater supports diverse species of flora and fauna according to their tolerance for saline environment (Menon *et al.,* 2000).

The decrease in salinity and pH in monsoon period is due to massive rainfall that causes a considerable increase in freshwater runoff from the upper stretch of the estuary. The biochemistry of bivalve molluscs is known to vary seasonally in response to both environmental parameters like temperature, oxygen levels, salinity etc and to the physiological status of the animals, depending on the food availability and on the gametogenic cycle (Widdows, 1978; Livingstone, 1981).

The sediments serve as long term sink for petroleum hydrocarbons and the table 6.4 shows that Ernakulam Wharf stations station recorded the highest value indicating that the station is heavily oiled. Chapman *et al.*,(1987) have the opinion that sedimentary concentrations of 4-10 µg/g of polycyclic aromatic hydrocarbons can cause measurable biological changes which, in turn, may affect the physic-chemical characteristics of the overlying water column.

The higher concentrations of phosphate can be attributed to domestic sewage discharged through rivers. This high content of phosphate helps to degrade the oil, as phosphate increases the rate of hydrocarbon oxidation (Zobell, 1962).

Commonly harbor waters are characterised by high inorganic nutrients attributed to sewage and industrial waste discharges. (Raman and Prakash 1989).Nitrate values are influenced by oil that hindered the activity of bacterial decomposition of planktonic detritus, thereby preventing nitrifying bacterial activity (Segar and Hariharan, 1989).
The lower values of nitrate may be attributed to presence of bacteria that can utilize nitrate as a source of oxygen for oxidation of excess hydrocarbons due to the oil spill. During the period of DO depletion due to oxidation of oil residue by bacteria, substantial removal of nitrate is common, because nitrate partially serves as an oxygen donor in the aerobic conditions of the coastal waters to degrade oil. (Zobell, 1962).
Cochin estuary experiences continuous oil input associated with shipping. Fishing vessel operations, transportation, urban run-off, accidental spills during tanker operations etc.

Menon and Menon (1998) are of the opinion that natural causes that control the distribution of petroleum hydrocarbons in the surface and subsurface waters of the estuary are the estuarine circulation, oscillatory nature of tidal currents and dissolved and suspended load of waste.

Agriculture, urban settlement and industrial activities around the world have contributed to the widespread contamination of global marine ecosystems with organochlorine compounds, petroleum products and heavy metals. All of these types of pollutants are conservative and many are essential permanent additions to the environment. They are also often highly toxic to biota. (Clark, 1992).

The fate of petroleum hydrocarbons once they enter the marine environment is similar to that of many organic pollutants. The bulk of the petroleum initially introduced into the water column rapidly becomes associated with hydrophobic organic matter and suspended particulates, the volatile compounds then evaporate and the non-volatiles are deposited into the sediment (Capone and Bauer,1992). The component of petroleum left, the emulsion or mousse, is not likely to dissolve, adsorb, evaporate or be rapidly biologically degraded and will eventually sink to the bottom and settle in the sediment. While the lighter fractions are suspended in the water column. Once the PAHs have settled in the sediment, filter feeders and benthic organisms are affected with the bioaccumulation of toxic compounds in their tissues, genetic mutation and cell atrophy often occurring. (Peters *et al.,* 1997).

The interest in using organisms to monitor pollution relies on the fact that fugacious contamination may often not be recorded in the sediment while bivalve may accumulate and therefore 'record' the contamintants behavaviour.When an organism is exposed to organic contaminants, the concentration of these xenobiotics in its tissue varies until it reaches a steady-state level. This apparent constant concentration results from a balance between uptake and depuration.

Molluscs took up petroleum hydrocarbons somewhat more slowly, but accumulated them in their tissues to considerably higher concentrations and released them more slowly than did crustaceans (Anderson, 1973; Neff and Anderson, 1974).

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

The digestive gland of molluscs has been known as a target organ for contaminant effects because this organ plays a major role in contaminant uptake and metabolism of inorganic and organic chemicals in the organisms. Digestive gland lesions including tubular dilation, cell vacuolation, and tissue inflammation induced by exposure to contaminated sediments were reported in mussels, *Perna viridis* Tripp *et al.*, (1984) reported severe inflammation and moderate atrophy of primary ducts and diverticula in the digestive gland of an euryhaline species of bivalve mollusc, the Asian clam (*Potamocorbula amurensis*). Working in a controlled laboratory environment, they also observed similar lesions in *P. viridis.*. Bell and Gosline (1997) described vacuolation of digestive cells and fragmentation-phase tubules in *Macoma carlottensis*. Tubular dilation of digestive gland in this study is similar to those observed in the Asian clam by Tripp *et al.* (1994). No vacuolation of digestive cells and fragmentation-phase tubules were detected in the reference site.

Pathological alteration of mussel tissue, as a result of pollutant exposure, has been reported in a number of studies (Lowe *et al.,*1981 ;Moore *et al.,* 1987 ; Lowe.,1988). Increased number of granulocytes were observed in the gonad and digestive gland in mussels from the impacted sites. Granulocytes are phagocytic in nature (Carball *et al.*, 1997); their increased presence in mussels may be indicative of greater catabolism of damaged tissue. Increased vacuolation was observed at the impacted sites. Vacuolation of bivalve digestive gland has been previously demonstrated after pollutant exposure (Sarasquete *et al.*, 1992 ; kela and Bowen.,1995).Bivalve epithelial thinning after pollutant exposure has been shown in a

number of studies (Lowe *et al.*,1981 ;Moore *et al.*, 1987). This suggests a possible reduction in the ability of these mussels to digest food material adequately.

The low dye retention times, indicating poor membrane integrity, exhibited by lysosomes from mussels deployed from the industrialized sites is consistent with the findings of other studies on lysosomal integrity in chronically exposed mussel populations (Lowe *et al.*,1995 ; Cheung *et al.*,1998). Lysosomal system plays an important role in maintaining homeostasis.Lysosomal damage has been shown to be a prognostic biomarker for pathology and reduced fitness (Moore, 1990 ; Lowe *et al.*,1992 ; Depledge *et al.*,1993).

Many xenobiotics induce alterations in the bounding membrane of the lysosomes leading to destabilization (Moore *et al.*, 1980, Lowe *et al.*, 1995). The digestive cell atrophy or thinning results a significant increase in lysosomal volume in mussels (Lowe *et al.*, 1981). This increase in lysosomal volume involves the formation of pathologically enlarged or 'giant' lysosomes and this alteration is associated with destabilization or increased permeability resulting in reduced latency of lysosomal enzymes (Moore *et al.*, 1995). Release of degradative hydrolytic enzymes from the lysosomal compartment into the cytosol may result from destabilization of the lysosomal membrane (Moore *et al.*, 1978), and such destabilization may also involve increased lysosomal fusion with other intracellular vacuoles leading to the formation of pathologically enlarged lysosomes. The consequences of these lysosomal changes would be increased autolytic activity leading to atrophy of the digestive cells.

Bivalve molluscs are appropriate sentinel species in the aquatic environment as they are sedentary and live in sediment or at the interface of water and sediment. They filter large amounts of water to cope with nutritional and respiratory needs and are able to bioaccumulate environmental micropollutants. Since several years, bivalve

transplantation is also used in order to assess water quality (Romeo *et al.*, 2003; Camus *et al.,* 2004).

The use of species transplanted from unpolluted to study areas was found to offer a valuable means for detecting disturbances of natural environments. The results of such transplant studies do not always preclude adaptation or compensation mechanisms that may occur in native species in the long term. Yet this kind of study represents a useful tool for environmental biomonitoring and ensuring environmental safety. The impact of human activities on marine environment is an ongoing and increasing problem. Various potentially dangerous chemicals like polyaromatic hydrocarbons (PAHs)., polychlorinated biphenyls(PCBs)., heavy metals etc. are continually entering the coastal waters. These pollutants are being taken up by resident animals, which ultimately endangers their very existence.

Oil pollution in the Cochin estuary arises from local exploration, exploitation, refining and routine handling of petroleum at ports. Contamination of oil could originate also from heavy maritime transport of crude and refined oil through the region, as a result of the discharge of ballast water from tankers. The analysis of hydrocarbons in the water column can provide insights into short-term variations in pollutant discharges, and their transport mechanisms and fate.

Detection of sub-lethal effects of such pollutants on marine and estuarine organisms at population and community levels of biological organization often becomes problematic. This has led to suggestions that changes in various physiological and biochemical parameters at the individual species level of sentinel organisms may be useful for identifying and delineating the impact of pollutants, thereby, developing the concept of biomarkers(Depledge and Fossi, 1994).The results of this study carried out in the Cochin estuary revealed clear biomarker signals in green

mussels. The study showed that, the biomarker responses could reflect both acute exposure as well as chronic exposure to pollutants.

Salazar and Salazar (1997) suggested that the use of caged bivalves to estimate chemical and biological effects represents a cost-effective approach that could reduce the uncertainties associated with more traditional approaches.

Overall, the results of the study on *P. viridis* showed that digestive cell and germ cell necroses and inflammations in both digestive gland and female gonad. The observed responses of these histopathologic biomarkers were a likely indication of chronic biologic effects induced by exposure to a mixture of inorganic and organic contaminants in the sediments. In addition to showing early indication of environmental stress on the health of the test organisms, they can also be used to localize the target tissues and organs of sediment contaminants in the affected individuals.

The utility of biological indicators as tools to monitor environmental pollution has been demonstrated in many studies. However, the results of this study and others indicate that the response to an environmental contaminant is population-specific and may be greatly influenced by the populations histories of exposure. Given the ease with which these generally non destructive techniques can be implemented, their application to long-term monitoring programmes, particularly with reference to the remediation of impacted areas, is promising.

Chapter 7

CONCLUSIONS

The present study on bioaccumulation and physiological responses of *Perna spp*. to varying concentrations of the water accommodated fraction of petroleum hydrocarbons has revealed that the secretion of the byssus thread is phenomenally affected by the presence of hydrocarbon. The disruption of secretion was due to the reduced or inactivity of the foot of the mussel and negative impact on the performance of the adhesive gland. This is amply testified by the presence of twisted byssus threads and distorted adhesive discs of the byssus complex. Corrugation appearing in the discs and inward bending of the byssus discs were clear indications of structural malfunction of this important organ of the mussel which ensures both attachment and control filtration efficiency of the gills.

Hepatopancreas was the most affected internal organ of the animals. The route of entry is assumed to be through oil adsorbed food particles and probably direct entry through the cells of the gills involved in respiration. Entry through oil coated mucous globules also cannot be ruled out.

Performance of respiratory processes was influenced by the presence of oil in the media. It is possible that heavy secretion of mucous accompanied by the rapid decrease in the ciliary activity have affected the quantum of water that was circulated for the absorption of oxygen.

Histopathology which reveals the damages caused on the structure and function of important organelles is a very useful tool to assess the pollutional effects of oil on the sensitive tissues of bivalves. Both the gills and digestive tubules were heavily damaged due to prolonged exposure to very low concentrations of PAH in the culture media. Lesions and neoplasia were the most recognizable damages of the gill and gastric tubules. There was a gradation in these insults as a function of concentration and duration of exposure. Substantial wasting which was recorded as severe intracellular damage resulted in the death of the experimental animals. Another conspicuous indication of toxicity at intracellular level was the occurrence of heterolysosomes in the digestive cells. These contained inclusions of medium density of variegated shapes. Active excretion of cytoplasmic granule into the cell cavity was often indicated explaining the effects of PAH on the general health and rate functions of digestive cells. Another remarkable conclusion is the resilience of *P.indica* and *P.viridis* regaining normalcy on exposure to healthy field conditions for a period of 20 days after being subjected to pollutional stress for 35 days in the laboratory.

Electron microscopic studies of pedal disc, mantle, adductor muscle, gill and hepatopancreas are essential to explain the declension in the rate functions of these cardinal tissues and behavior of the mussels subjected to exposure to low levels of toxicants. Various indications in cellular damages can be adopted as very useful indication of cell damage. Fusion of cell lamina, disarray of cytoplasmic contents, total degradation of nuclei and rampant occurrence damaged golgi apparatus at intracellular levels are a few possible indicators that could be employed to assess the rate of cellular, intra and subcellular damages. Distorted endoplasmic reticulum, presence of dense sub cellular particles in the cells of adductor muscle probably explains for the reduced rapidity in the closure of the shells when the intoxicated mussels were subjected to mechanical stimuli in the laboratory.It is also concluded that the species of *Perna* tested do accumulate PAH in their soft tissues. It is also suggested that

prolonged exposure to low levels of PAH in natural conditions would lead to recognizable morphological changes of the gills.

Oxidative stress studies of cells proved to be a very useful tool to explain the effects of PAH on bivalves. Since the antioxidant systems protect the cells from the deleterious effects of oxyradical generation, a range of antioxidant defence mechanisms could be delineated. The most conspicuous mechanism was the lysosomal membrane labilization of digestive cells, haemolymph cells. Lysosomal swelling and rupture also indicate lysosomal instability resultant of oxidative stress.

Exposure studies conducted employing intoxicated mussels to field conditions helped in understanding the chronic biological effects that could be induced by prolonged exposure to an array of toxicants. Rehabilitation of toxicant exposed laboratory test animals to natural conditions indicated the resilience of these bivalves to correct the damages by effective depurative processes.

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