HEAVY METAL TOXICITY IN BIVALVE - HISTOLOGICAL AND HISTOCHEMICAL ENQUIRY

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1993

To My Beloved Sons . . .

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CERTIFICATE

This is to certify that the thesis entitled ***"Heavy metal toxicity on bivalve - Histological and histochemical enquiry"****, is an authentic record of the research work carried out by* ***Ms. Sreekala Pillai P.****, under my scientific supervision and guidance in the School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the Cochin University of Science and Technology and that no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.*

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December 1993

DECLARATION

I hereby declare that this thesis entitled ***"Heavy Metal Toxicity On A Bivalve - Histological and Histochemical Enquiry"****, is a genuine record of the research work done by me under the scientific supervision of Dr. N. Ravindranatha Menon, Director, School of Marine Sciences, Cochin University of Science and Technology, Kochi and that this has not previously formed the basis of the award of any degree, diploma or associateship in any University.*

Kochi - 16

December 1993

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PREFACE

A quantification of the toxic effects in marine animals is an important aspect of biological monitoring of pollution effects. There are various methods which are adopted to understand and explain the toxic effects in marine organisms. Examining morphological changes and histochemical alterations are among the important ready reckoners adopted by researchers. Marine bivalves are excellent experimental material that could be conveniently used for biological assessment of functional and structural modifications and alterations brought about by chronic or episodal exposure to toxicants of anthropogenic origin. This has resulted in an exhaustive utilisation of intertidal and subtidal bivalves, as experimental animals to delineate pollution effects.

Perna viridis is a very common intertidal and sub-tidal bivalve occupying the entire stretch of South West coast of India. This animal has proved to be an excellent material which can get adapted to laboratory conditions without drastic changes in the basic physiological characteristics (Hawkins et al., 1986). Therefore, Perna viridis was exposed to copper and mercury individually and in combination under laboratory conditions for varying periods. The effects of exposure was analysed employing histopathological and histochemical techniques, the former to understand alterations and damages and the latter to identify

functional variations based on histochemistry. Since it was necessary to correlate the quantity of heavy metals entering the body with histological and histochemical variation, the body burden of animals by the metals was estimated using Atomic Absorption Spectophotometer. The results obtained are presented in this thesis which embodies the findings and explanation of heavy metal toxicity.

GENERAL INTRODUCTION

Investigations on toxic effects of heavy metal to marine invertebrates is a field of study which has gained momentum in recent years. There has been a spectacular increase in basic and applied research connected with metallic contamination in the sea. Metals like iron, copper , zinc, cobalt, manganese , molybdenum, chromium, nickel, vanadium, arsenic and tin are biologically essential. However, many of these essential metals are toxic and in this respect can be considered along with non-essential elements like silver, cadmium and lead. Binding at centers where metals are normally not bound on living systems can lead to toxicity. Thus mercury can bind at certain ligands under conditions where none of the essential metals can bind. Some metals could substitute essential elements at the active sites of enzymes. Replacement of groups, precipitation and formation of insoluble metal phosphates, alteration of membrane permeability etc. are some other biochemical aspects of an organ system which could be influenced by heavy metals.

Marine bivalves have been used as very useful experimental organisms to assess the toxic effects of heavy metals. These animals are capable of tolerating variable conditions provided in the laboratories. The capability of bivalves to function normally under laboratory conditions have made them potential experimental animals to assess rate functions, structural

modifications and accumulations of heavy metal insult. On exposure to contaminant sea water, bivalves are known to take up heavy metals. The absorption of these heavy metals can occur through food or by pinocytosis of gill epithelia (Coombs and George 1978). However, it is not clear whether the same processes are involved in the absorption of heavy metals by bivalves. Storage and ultimate removal of heavy metals which are toxic could be via kidney and digestive glands (Janssens and Scholz, 1979). Opinions differ regarding mode of entry of heavy metals into the body of bivalves. Evidences show that heavy metals could enter the body either through food or water. Therefore, the tissues that could be involved in the process are the digestive glands, mantle and the gill. It is not clear whether there is relative importance of these media in mode of uptake process. Since it is known that sea water is the main source of supply of heavy metals to marine bivalves, it is obvious that water is a prime source of heavy metals to bivalves. Therefore, the experiments were performed using sea water as the major medium to supply heavy metals to experimental animals, namely Perna viridis. It is known that for most metals the concentration in the mussel will come to reflect that of the environment. This implies that sea water could be the main source. The exact relationship between heavy metal concentration in water and the tissue load achieved after a defined period of time is not probably understood.

Histological and histochemical investigations also throw

light on toxic effects of heavy metals. The sequential reactions of a marine animal exposed to heavy metal contaminated sea water should necessarily commence from histochemical alterations culminating in mortality through a series of physiological, anatomical and behavioural steps.

Therefore, to identify organismic effects it is sufficient to look into only one or a few of the above series of chain reactions. It is known that manifestation of toxic effects is better seen at cellular and subcellular levels. This is mainly because of the fact that the pollutant employed affects the basic functions of the experimental animals and that these effects are best seen at cellular and subcellular levels. Identification of such changes can be facilitated by histological and histochemical enquiry. Physical presence of heavy metals in the tissue of an intoxicated organism, can be identified only by extraction and estimation of heavy metals by spectrophotometry.

In the present investigation, Perna viridis, one of the commonest of the mytillids, encountered in intertidal and subtidal realms of the South West Coast of India was identified as the experimental animals. Representative of this species inhabiting the coastal waters of Cochin was sampled many times stretching for a span of three years and was used as experimental materials to analyse the effect of copper and mercury on histology, histochemistry and rate of uptake of metals.

During this study at a few instances the animals were maintained in laboratory for considerable length of time to

faciliate utilising individuals from the same cohort to reduce inter individual variations in reactions. The information gathered on histology, histochemistry and accumulation are presented in this work.

CHAPTER III

HISTOPATHOLOGY

III . 1 INTRODUCTION

Marine bivalves have proved to be an excellent material for histological and histopathological assay. This quality of the material has helped in utilising marine bivalves as materials to assess the effects of toxicants, mainly heavy metals and polycyclic aromatic hydrocarbons. The efficacy of histology to delineate the effects of toxicants at tissue levels probably became evident after Goldberg (1975b) developed the idea of "Mussel Watch" . The mussel watch strategy is based on the concept that bivalves are capable of accumulating reasonably higher concentration of heavy metals when they are variable in the environment. When the cause and defects of heavy metal pollution are analysed experimentally, various morphological, physiological and biochemical assays become useful methodologies to understand the degree of effect that can be quantified chemically and qualified structurally. Therefore, structural changes represented by way of alterations, modifications and damage, should be looked into, to employ these as basic tools to explain heavy metal toxicity. Bivalves are exposed for a considerable period of time, lasting for a few days to weeks in toxicants with a view to allowing the animal to manifest the toxic effects in cardinal tissues involved in performing functions which are responsible for tolerance, adaptation and successful existence in a dynamic environment like the tropical

coastal waters. The present investigation envisaged a histopathological assay of two important tissues namely, the gills, which are copiously bathed by external medium resulting in considerable water tissue contact and the gastric diverticula, an internal organ which gets the impact of heavy metal toxicity via water or food. Structural changes as envisaged in the present investigation could be the result of either contact or absorption. The former, being the main stay for gill lamellar damages and the latter, the hepatopancreas. In this chapter therefore, a histopathological enquiry has been conducted and presented with the help of photomicrographs, visual assessment expressed in terms of intensity of damage of numerous sections of these two tissues prepared from animals sacrificed after varying periods of exposure to heavy metal intoxicated culture media.

III . 2 REVIEW OF LITERATURE

Heavy metals form one among the innumerable contaminants added to our marine ecosystem every day mainly due to anthropogenic activities or otherwise. Their contributions in making life hazardous to all organisms especially the sessile filter feeding bivalves, widely inhabiting the coastal areas, are well documented by workers in India and abroad.

As a group, heavy metals form a dangerous group of potentially hazardous pollutants , particularly in estuaries and nearshore waters (Bryan,1984). Murphy and Spiegel(1983) while

defining heavy metals, states that they are a group of metals having specific gravity greater than 4 or 5 , located from atomic number 22-34 and 40 - 52 on the periodic table (as well as the lanthanide and actinide series) and having specific biological response.

Toxicity and biological effects of heavy metals have been reviewed by Akberali and Truemann (1985). The toxic effects of copper and mercury are extensively studied on marine organisms, especially the sedentary filter feeding bivalves which are considered as sentinel organisms in biomonitoring of environmental pollution (Goldberg et al., 1978 ; Davies and Pierre,1978). The chemistry of copper in sea water, sources and levels of environmental copper, toxic concentrations , accumulations, storage and excretion of copper along with its behavioural, physiological and metabolic effects are summarised by Davenport and Redpath (1984). Though copper is listed in the grey list of contaminants released in the environment, it plays a very essential role in molluscs. Its function is three fold 1)It forms a part of the respiratory pigment. 2)It is present in the cytochrome system of electron transfer. 3)Finally it forms an essential part of the enzyme system involved in removing the toxic effects of aerobic metabolism (Simkiss et al.,1982). International Copper Research Association (1982) gives us a detailed review of acute and sublethal toxicity of copper .

The biological significance of mercury is unknown. On the other hand, its toxicological effect on the marine biota and its

significant role as a potential hazard to mankind have been emphasized by many workers. (D'itri and D'itri, 1977;Taylor, 1979; Bryan, 1984; TOI, 1986). In fact the Oslo and Paris Commission has included it in the black list of contaminants and its release into the marine biota has completely been banned.

Man's increasing awareness of the impact of pollutants, which has become a threat to the stability of our biosphere, has made many a scientists to devise efficient diagnostic biomarkers to warn him of the perturbations in organisms exposed to pollutants at a very early stage. One such approach is the application of cytochemical methods which probes the alterations at the molecular and biochemical levels. This would be of advantage in that these alterations, though unnoticed at the early stage, could be detected and deleted or controlled at the onset (Moore, 1991). Sedentary filter feeding bivalves, especially mussels are often used to assess the quantity of heavy metals in aquatic environment (Bryan, 1976). The capacity of the bivalves to accumulate trace metals and other toxicants has led to the selection of this group as an important bio indicator for the reason that they satisfy the basic requisites as proposed by Butler et al.(1971); Phillips (1976a,b). The adverse effects of pollutant accumulation in coastal waters are often reflected as minute alterations in the structure, biochemistry and physiology of the organism inhabiting that area. In fact, though these changes are commonly classified under different categories, they are all interrelated.

The prominent organs which are often prone to the effect of pollutant accumulation are the gills and digestive glands. The former organ is always in close proximity with the aquatic environment and the latter, the chief sites of intracellular digestion and detoxification. As the present study involves cytochemical methods to evaluate alterations caused by selected trace metal impact, an understanding into the basic structure is considered a must.

A comprehensive knowledge of the structure and function of the digestive glands and gills of bivalve molluscs can be obtained from the extensive literature available on them (Atkins, 1937; Owen 1955, 1956). The preliminary concept of the structure and function of digestive diverticula of bivalves was derived from the earlier works of Yonge (1926), which stated that the cells lining the digestive divertucula to be made of a single type of cell. This was constantly being replaced by single type of darkly staining undifferentiated pyramidal shaped cells, present in the crypts of tubules. These cells were believed to be concerned with intracellular digestion. Contrary to these findings, numerous reports that in some species of bivalves, the digestive cells performed secretory function were put forth. (Mansour, 1946; Mansour and Zaki, 1946; Owen, 1956; Reid, 1965). Owen (1955, 1956) reported that the darkly stained cells of many bivalves are flagellated and appear to undergo a secretory cycle. This idea held true in the case of cells in the digestive diverticula of gastropods.

Based on these findings, it was confirmed that the digestive cells of most bivalves were mainly concerned with functions of absorption, digestion and secretion. This view was supported by the works of Sumner (1966b), which is perhaps the only published account of electron microscope study of the digestive tubules of bivalves at that time.

In the case of most bivalves studied, it was found that the blind ending tubules communicate with the stomach by means of partially ciliated ducts and non ciliated secondary ducts. (Owen, 1955). These digestive tubules were noted to be made of two distinct types of cells. 1) Digestive cells containing numerous membrane bounded vesicles, 2) Basophilic darkly stained cells. (Owen,1972a ; 1973). However, in Cardium edule , Owen (1970) reports the epithelium lining of digestive tubule to be composed of three cell types 1) mature digestive cells 2) mature secretory cells 3) immature flagellated cells. The same configuration of cells were noted in some other bivalves like Anodonta anatina (Sumner, 1966a), Mytilus edulis (Platt, 1971), Mya arenaria (Pal,1971; 1972) and in some other fresh water lamellibranchs. Studies by Lowe and Clarke (1989) on the structural alterations of the digestive epithelium exposed to contaminants also noted changes in only two different types of cells. In short, all these reports confirm that the basic structure of the digestive tubules of most bivalves is more or less the same in appearance.

Fundamentally, the digestive diverticula consists of blindly ending tubules which are linked with the stomach by a system of partly ciliated ducts. Under the light microscope the epithelium of the tubules are of two cell types. 1) acidophilic columnar and vacuolated 2) pyramidal and basophilic.

In Mytilus edulis the basophilic cells are seen to be provided with an extensive granular endoplasmic recticulum, free ribosomes, active golgi body and granules at cell apex and tubule epithelium indicating its possible role in protein synthesis. Though the exact function of basophilic cells still remains a subject of controversy, many workers have come to the conclusion that it can either be enzyme secreting or immature digestive cells.

Thompson et al. (1974) while examining the structure of basophilic cells in some bivalves stated it to be of two forms. The same observations were reported in Anodonta anatina (Sumner, 1966b). Another important finding put forth by Thompson et al. (1974) while examining the fine structure of degenerating tubule of starved Mytilus edulis was that the number of flagellated and non flagellated cells varied. At the same time, under suitable conditions, or when subjected to less severe stress, the normal mature digestive cells were more observed. Owen (1970) too, was of the same opinion that the flagellated cells in Cardium edule represented immature cells which later gave rise to mature digestive cell. The second type of cells namely the acidophilic cells, commonly referred to as the digestive cells, are the cells

mainly concerned with digestion. They are cuboidal or columnar in shape. Describing their structural details, Thompson et al. (1974) stated about the presence of numerous cytoplasmic vesicles, and microvilli projecting from the cell apex into the lumen of the tubule. A large nucleus is also prominent. It is possible that particulate material from the lumen is taken at the base of microvilli by pinocytosis and pinocytotic vacuoles so formed fuse to form heterophagosomes. (Owen, 1972a). An important characteristic feature of digestive cells is that it is made up of various types of microvesicles and macrovesicles. Structural and functional differentiation on micro and macrovesicles have been studied by numerous workers. (Owen, 1970; Platt, 1971; Thompson et al.,1974). However, Pipe and Moore (1985) have identified three types of macrovesicles as hetrophagosomes, hetrolysosomes and residual bodies.

Recent research conclude that digestive cells in Mytilus are multifunctional (Moore, 1991). They are found to be analogous to vertebrate liver cells as they are found to be important storage sites of glycogen and lipids. Apart from storage, these cells have turned out to be the major sites of physiological processes like detoxification and removal of toxicants entering the system (Moore, 1985; Moore et al., 1987).

Gills are yet another organ of much importance in bivalves in that they are structures which next to the mantle are always in close proximity with the aquatic environment.

The complicated ciliary mechanisms in the gills, make bivalves the most efficient filter feeders.

Most of the anatomical works on structure of gill dates back to the late nineteenth and early twentieth century, at a time when photomicrography, modern histological techniques and electron microscopy were not so well developed (Kellogg, 1892; Janssens, 1893; Ridewood, 1903; Drew,1906; Setna, 1930). The work of Atkins (1937a, 1937b, 1938a, 1938b, 1938c) was mainly confined to ciliary mechanism and gives us a beautiful illustration of how movement of water retention, sorting and transport of particulate matter takes place in ctenidia.

In recent years studies of bivalve gill structure have focused more on details of ciliature (Moore, 1971; Owen, 1974; Owen and Mc Crae,1976). Reedmiller and Greenberg (1982), gave a detailed account of ultrastructure of ciliated junctions between gill filaments. Nelson (1960) while working on gill anatomy in Ostrea edulis, had tried to make an attempt in discussing the macro and microanatomical details of gills, but attention was once again directed to ciliary tracts. Yet the gross morphological structure of gill in Mytilidae have not been well defined .

A general description of organisation, general anatomy

and surface microanatomy of gill of giant scallop has been done by Benninger, Le Pennec and Salaün (1988). This description coincides with that given by Drew (1906). Another important work reflecting the anatomical structure and function of gill was done on boring eulamellibranch, Pholas dactylus (Knight, 1984; Knight and Knight, 1986). Apart from these, the late discoveries of endosymbiotic bacteria inhabiting the gills of the bivalves occupying the hydrothermal vent, (LePennec and Hilly, 1984; Fiala Medioni and Metivier, 1986; Fiala Medioni et al.,1986), has renewed interest in workers in the study of functional anatomy of gills, especially of ones belonging to littoral species.

The classical picture we have of the function of gills is that of respiration (via gas exchange across the surface of gill) and suspension feeding (Jorgen sen, 1966). Studies on a variety of gills of littoral and deep sea bivalves prove an alternate trophic function of gills. This is mediated by either direct uptake of dissolved or particulate organic matter (Henry et al.,1981; Manahan et al., 1982; Wright et al.,1984) or by symbiotic relationship with chemotrophic bacteria.(Felbeck et al., 1981; Henry et al.,1981; Dando et al., 1985; Spiro et al.,1986).

While studying the ultrastructure of the gill of two hydrothermal vent mytilids, namely Bathymodiolus thermophilus and Calyptogena magnifica, Fiala Medioni et al.(1986) reports the gills to be rich sites of symbiotic bacteria. The bivalves

therefore gain an additional advantage of nutrition through this association because these bacteria are able to fix carbon-di-oxide by utilisation of sulphides.

The basic organisation of gill in mussels is that it is composed of an ascending and descending lamellae. Each lamellae is composed of filaments which are joined by ciliary interfilamentar junctions. These lamellae are interconnected by interlamellar blood vessels. Ciliated and nonciliated cells are found in the epithelium of branchial vein which runs through the filament. Chitinous rods are seen along with the muscles (abofrontal and frontal) which help to preserve the integrity of the structure .

The arrangement of cilia follow a definite pattern (Sunila, 1986). 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 called latero frontal cell and the cilia associated with it is called laterofrontal cilia. A row of lateral cells bear lateral cilia. Interfilamentar junction is present. At the abofrontal end can be seen abofrontal cells which bear abofrontal cilia. It is also noted that beneath the ciliated abofrontal cells mucous glands are present (Lucas, 1931; Satir and Gilula, 1970; Aiello and Sleigh, 1972; Paparo, 1972). A comparative study of the basic structure of the gills and digestive tubules to that of pollutant dosed tissues helps a lot to assess the

extent of cytological perturbrations in the concerned tissue. Earliest detectable changes of primary events are associated with particular type of subcellular organelle such as lysosomes, endoplasmic reticulum and mitochondria. (Moore, 1985). Many ways in which the structure or function of organelles and cells can be disturbed by toxic contaminants are elucidated by Slater (1978) which includes (1) depletion or stimulation of metabolites or co-enzymes, which produce morphologically evident lesions in cells (2) Inhibition or stimulation of enzymes which results in damage to cellular functions (3) Activation of a xenobiotic to a more toxic molecular species (4) Membrane disturbances resulting in damage to cell injury.

Pathological disturbances in organisms due to organic and inorganic pollutants have been widely documented. (Hawkins, 1980; Moore and Clarke, 1982; Moore, 1985; 1988; Lowe, 1988;).Some egs. cited are the occurrence of neoplastic lesions in fishes and non-neoplastic abnormalities in crabs. (Malins et al.,1984), hepatopancreatic epithelial reduction in bivalve molluscs by a variety of contaminants, (Lowe et al., 1981; Couch, 1984), lysosomal disruption in response to copper and phenanthrene (Pickwell and Steinert 1984; Moore, et al., 1984).

Abnormal cellular conditions like proliferative neoplastic cells in Mytilus edulis on exposure to polynuclear aromatic hydrocarbons were observed (Lowe and Moore, 1978). Though reports state that blood of bivalve molluscs transport metals from organs that are in immediate contact with surrounding

water (egs. gill, mantle, digestive gland) to deeper lying tissues (kidney) where detoxification and accumulation may occur, there has also been instances of occurrence of abnormal occlusion of haemolymph sinuses by large number of haemocytes or blood cells termed granulocytomas (Lowe and Moore, 1979).

The digestive diverticula in some invertebrates showed the following pathological alterations namely, atrophy of cells, reduction in height of tubular epithelium, tubular dilation, necrosis and desquamation of tubular epithelium. when exposed to cadmium chloride (Establier et al., 1978b). Auffret (1988) describes severe degenerative changes in the epithelium and digestive glands of Mytilus edulis when exposed to high concentrations of a mixture of copper and diesel oil. Lesions of similiar type have been reported in mussels exposed to sub lethal stress (Gonzales and Yevich, 1976). Oysters from contaminated estuaries presented atrophic epithelium, sloughing of cells and necrosis. (Couch, 1985). Martin (1971) showed histopatholigical changes in digestive tubules and gills of fresh water clam C. fluminer exposed to copper.

Phagocytosis of sperm in follicles and gill lesions (hyperplasia of epithelia, metaplastic interfilamentar junctions and a chronic inflammatory reaction) along with occurrence of inflammatory reactions, ulcers, haemorrhages of the digestive tract and kidney lesions were observed in M. edulis sampled from polluted areas in the Baltic Sea (Sunila, 1988).

The degree of pathological alterations varied with the pollutants. It was found that exposure to cadmium, copper, lead and silver evoked an inflammatory reaction in gills of Mytilus edulis, while cobalt, iron or dieldrein had no effect. On the other hand it was found that organic pollutants caused loosening of intercellular connections in the epithelia and shrinkage of cells and inflammatory reactions.

The connections between cilia and microvilli are seen to break following uncoupling of the gill filaments due to copper and cadmium exposure. (Sunilla & Lindstroem, 1985). Short term exposures to cadmium and copper indicated swelling of endothelial cells and detachment of abofrontal cells and dilation of brachial veins (Sunilla, 1986). Pathological alterations observed in Perna indica when exposed to 10 ppb of mercury for twenty one days were sloughing off of lateral and frontal cilia and infiltration of blood cells in gill filaments. The digestive cells showed degeneration of cells. Twenty one days exposure to 10 ppb copper in Perna indica led to thorough damage of gills with complete destruction of cells and bulging of lumen and gill filaments.

On the other hand combined toxicity of copper and mercury resulted in the distortion of gill filaments with no cellular damage to distal cilia and total degeneration of digestive tubules. (Philip, 1990).

III.3 Materials and Methods

This part of the investigation involved collection of test animals from their natural habitat, transportation to laboratory, acclimatization to laboratory conditions, exposure of a fixed number of animals to known concentrations for fixed time and finally preparing a series of permanent histological slides of selected tissues of the exposed animals to get an insight into the histopathological alterations caused. Finally using suitable statistical tests evidence of enlargement of cells as a consequence of pollution, repair of cells during exposure of toxicant exposed animals, to raw sea water, was confirmed.

III. 3 . 1 Test animals

Perna viridis

This commercially important edible bivalve was collected from an unpolluted area near to the sea wall near Narakkal, Cochin, during November - December for this study. They were then brought to the laboratory in polythene bags filled with sea water taken from the site.

III .3 .2 LABORATORY CONDITIONING OF TEST ANIMALS

In the laboratory the animals were cleaned and acclimatised for two days in sea water collected from the site of occurence. Animals of uniform shell length (30-40 mm) were

taken for the experiment. Specimens from the same population was used for a single set of experiments. Contamination by pseudo-faeces and metabolites was checked by daily renewal of water .

III .3 .3 TOXICANTS AND CONCENTRATIONS EMPLOYED

III .3 . 3 .a Copper

Analar grade of copper sulphate (M.W. 249.68) was the source of copper. The salt was dissolved in distilled water and added to achieve the required concentration.

III . 3 . 3 .b Mercury

Standard solutions of mercury were prepared using analar grade mercuric chloride (M.W. 271.50) in glass distilled water and stored in amber coloured bottles . Since mercury solutions are not stable for long periods , they were prepared afresh for each set of experiments and added to make up the required concentrations.

Ten animals each were taken from the samples and introduced into ten litre tubs containing filtered sea water,to which the following concentration of metals inthe form of their salt solutions were added.

III . 3 . 3 . 4. Concentrations used :-

Individual

Copper- 10ppb ; 20ppb

Mercury-20ppb ; 50ppb

Combinations

10ppbCu 20ppb Hg ; 10ppbCu 50ppb Hg

20ppbCu 20ppb Hg ; 20ppbCu 50ppb Hg

All experiments were carried out in duplicate. For each experiments control was maintained. Prior to the experiment the animals were fed with algae (S. salina). This process was repeated , every day for either seven days or thirty days. The animals exposed for a week were then exposed to raw sea water for depuration.

For routine histological studies, under light microscopy, the digestive tubules and the gills were dissected from animals exposed to various concentrations after the fixed time and immediately fixed in the Bouin's fixative. Following dehydration, they were embedded in paraffin wax and serial sections of 5 to 8 µmm. thick were cut and stained with Triple Mallory Stain.

III . 3 .5 **Chemical composition of the fixative used**

Bouin's Fluid

Picric Acid (saturated aqueous solution)-75ml

Formalin (40%formaldehyde solution) -25ml

Glacial Acetic Acid -5ml

The foregoing chart was used to making paraffin wax blocks used for histological studies.

1) Washed overnight in running water.
2) The tissues were treated with saturated solution of lithium carbonate in 70 % alcohol to remove yellow colour of picric acid.
3) After softening , the tissues were treated were washed in 70% alcohol and transferred to 90% alcohol for 2h.
4) Transferred to 95% alcohol for 1h.
5) Transferred to absolute alcohol (2 changes) for 1 hr. each.
6) Placed the tissue in 1:1 mixture of absolute alcohol and methyl benzoate until tissues become transparent.
7) Cleared in methyl benzoate until tissues become transparent.
8) Placed tissues in benzene for 15 mins.
9) The tissues were then transferred to benzene, saturated with paraffin wax of melting point 58-60^{o}C for 6 hrs.
10) Infiltered the tissues in 2-3 changes of molten paraffin wax of m.p. 60-62^{o}C
11) Embedded in paraffin wax of m.p. 60-62^{o}C .
12) The blocks were trimmed and sections at 5to8µ mm were taken for histological studies.

III .3 . 6. Staining techniques followed with Triple Mallory Stain

Solution -1 (Mallory-1)

Acid Fuschin - 1gm

Distilled water- 100ml

Phosphotungstic Acid

Phosphotungstic Acid-1gm

Distilled water-100ml

Solution -2 (Mallory 2)

Aniline Blue-0.5gm

Orange G -2gm

Distilled water-100ml

Procedure

1) Deparaffinise and hydrate slides to water. If mercuric chloride is absent from fixative mordant in saturated aqueous mercuric chloride, plus 5% glacial ascetic acid for 10 mins.
2) Wash treat with Lugol's Iodineand Sodium thiosulphate.
3) Wash and rinse in distilled water
4) Stain in Mallory 1 (15) secs.
5) Rinse in distilled water (10) secs
6) Treat in phosphomolybdic acid (1-5) mins.

7) Stain in Mallory-2 (2mins).

8) Rinse in distilled water.

9) Differentiate aniline blue in 90% alcohol.

10) Dehydrate in absolute alcohol.

11) Clear in xylene.

12) Mount in D.P.X.

III . 3 .7.STATISTICAL ANALYSIS EMPLOYED

To confirm the evidence of enlargement of cells consequent on the application of various pollutants statistical analysis was carried out using the following methodology.

III . 3 .7.a WILCOXON SIGNED RANK TEST

The experiment conducted consisted in taking count of number of cells within $1mm^2$ in eight different fields. Since the area considered in all was the same, the decrease in number of cells could be considered as evidence of enlargement.

The statistical test used in this connection is the **WILCOXON SIGNED RANK TEST** for paired observations. Since the two samples under comparison could not be viewed as independent the hypothesis considered is that there is no difference in number of cells before and after pollution. The alternative considered is that average number of cells is lesser under the influence of pollution. We compare the two readings at individual fields to see whether there is evidence of decrease after implementation of pollution.

The test statistic is the sum of the rank of negative differences. If the hypothesis of no differences in the number of cells is true we expect the sum of the ranks of positive and negative to be roughly equal. On the other hand if the alternative is true we would expect one of those ranks to be large and the other to be small. Thus we take the sum of negative ranks T^- and reject the hypothesis of equal averages if the observed value of T^- is less than or equal to 5 at 5 % level of significance.

Result of analysis is presented in tables 2, 3, 10 & 11.

III.3.7.b. **RANK CORRELATION**

While analysing the histological status of cells which had undergone degeneration due to the effect of pollutants a method of rank correlation was employed. The statistical problem is to test whether there is an increase in degree of degeneration due to the effect of pollutants. Since it is unlikely to observe an increase in degeneration in the absence of pollutant we have to test positive correlation between the detailed ranks. Thus the hypothesis considered are (1) there is no tendency for pollutant dosed sample to change the status of degeneration against the alternative that (2) there is some tendency to increase positive correlation.

We calculate **SPEARMAN'S CORRELATION COEFFICIENT**

$$r = 1 - (6\Sigma d^2 / n(n^2-1))$$

where n = number of observation in sample

d = difference in rank

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

The conclusions are presented in tables 5 & 13.

III .3 .7.c **PAGE STATISTIC**

In analysis the average effect under exposure and depuration in comparison with control multiple comparisons are involved. The problem here is to test whether there is a steady enlargement of cells during control, depuration, exposure. Therefore, we formulate the hypothesis as there is no difference in the average number of cells per square millimeter area under three different stages. The alternate hypothesis is that the number of cells under exposure is lesser than number of cells under depuration which inturn is lesser than number of cells under control (E<D<C). The test is carried out using **PAGE STATISTIC.**

$$L = \sum_{i=1}^{3} iR_i$$

where R_1, R_2, R_3 are the rank sums in control, depuration and exposure respectively. The hypothesis of no difference between the three states will be rejected if at 5% $L \geq 104$.

Conclusions are presented in Table 8 & 9.

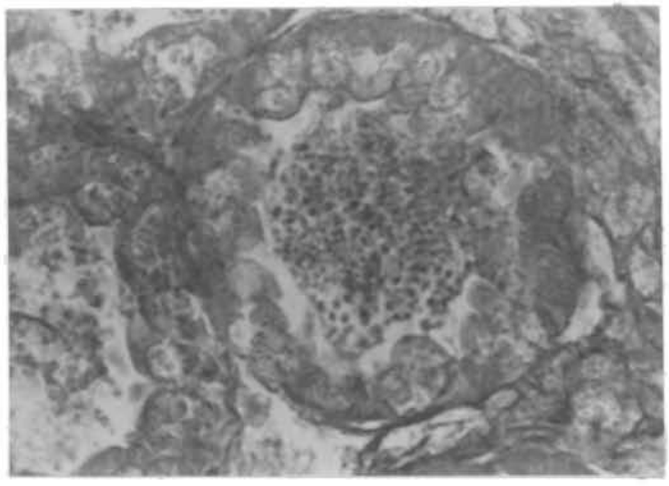
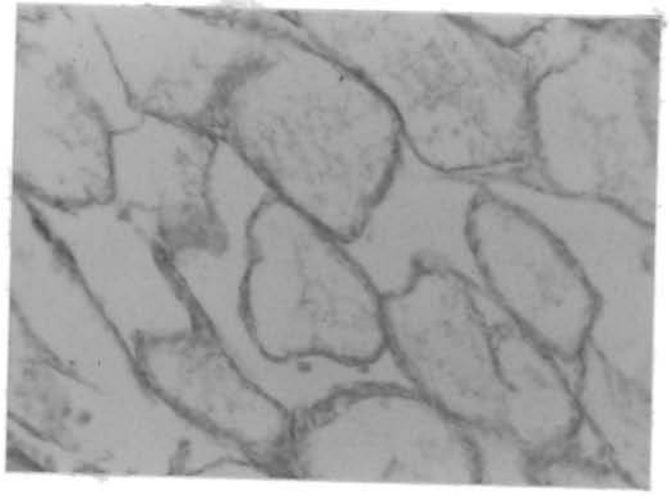


Fig. 1 *C.S. of digestive tubule of P. viridis maintained under controlled conditions for 4 weeks x 400.*

Fig. 2 *C.S. of digestive tubules of P. viridis exposed to 20 ppb of mercury for 4 weeks indicating severe destruction leading to loss of the basic nature of cells x 200.*



III . 4 . RESULTS

Histological sections of control and stressed individuals of P.viridis exposed to mercury for a period of one month are presented in Figs.1 & 2

III .4 .1 Histopathology

III . 4 . 1 .1 Perna viridis subjected to 4 weeks exposure

III .4 .1. 1 a DIGESTIVE TUBULES

The digestive diverticula of Perna viridis consists of numerous digestive tubules which communicate with the lumen of the stomach through partially ciliated main ducts and non ciliated secondary ducts. Shape of the ducts could be circular or oval. The normal structure of the tubule as evidenced from the photomicrographs show a sheath of collagen fibers covered with smooth muscle fibers forming a meshwork. This is the normal structure of digestive tubule of majority of bivalves. The digestive cells which occupy the internal lining of tubules are large and are characterised by the presence of irregulalarly shaped empty vesicles. The basophilic cells may occur in distinct groups. The lumen of the tubules are provided with internal edentations , the cells occupying this area provided with long cilia probably

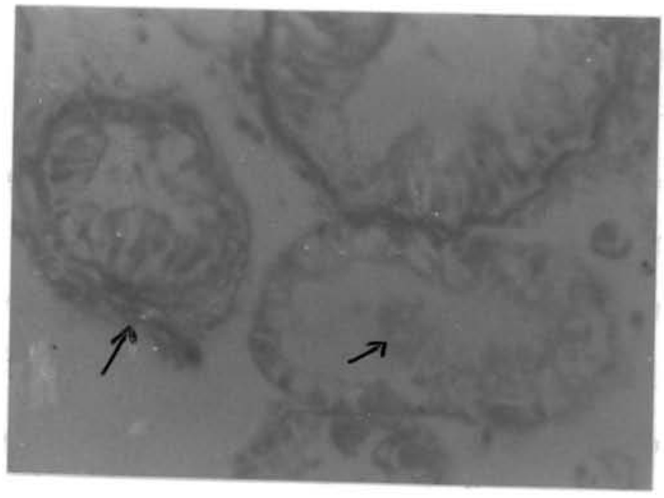


Fig. 3 *C.S. of Digestive tubules of P. viridis exposed to 50 ppb of mercury for 4 weeks showing dislodged cells and disintegrated collagenous layer x 400.*

helping in quick passage of foods. These edentations are known as crypts with normally two types of basophilic cells of which only one is ciliated .

A comparison of this structure with that of digestive tubules of those animals exposed to 20 ppb of mercury for one month shows the extent of damage caused. The tubules have virtually lost the basic characteristic and are so bulged and enlarged . The collagenous layer has disintegrated exposing the muscular layer. The cells have been found to be dislodged and in some cases remain distributed in the lumen. It is likely that sections which contained organelles of blue colourations are collagenous layer that are destroyed and remain distributed in the intertubular spaces. The examination of animals before sacrificing them for preparation of sections showed that they have become very weak and the mantle cavity was nearly filled with mucus reducing the quantity of water that normally gets circulated. Another conspicious feature was the reduction in number of tubules in unit area of gastric diverticula of the individuals maintained for thirty days in media containing 20 ppb mercury (Fig.2)

Those animals exposed to 50ppb mercury (Fig.3) also possessed gastric tubules with partial or complete damage reflected in form of disruption of collagenous layer , disarray of the muscle layer , dislodging of cells and partial or complete disintegration of both basophilic or acidophilic cells.

P.viridis was maintained in culture media containing two

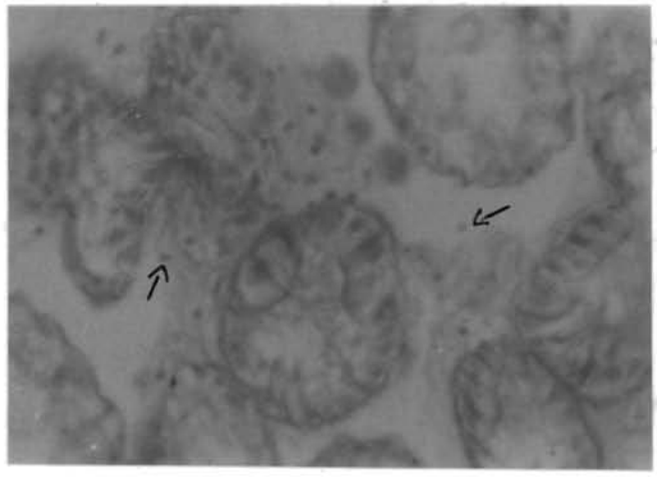
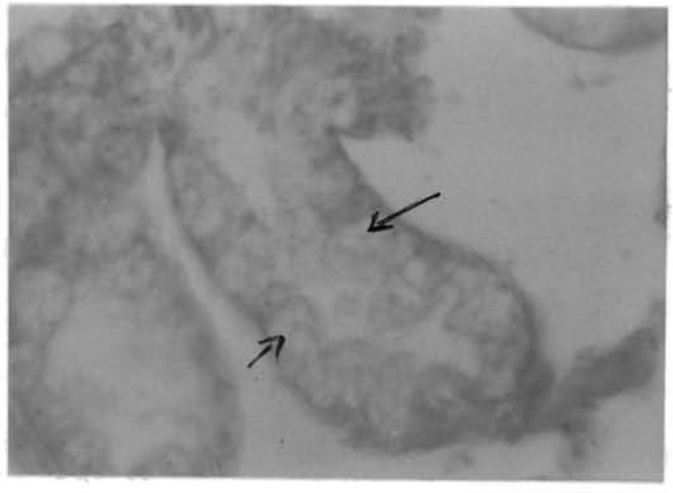


Fig. 4 C.S. of Digestive tubules of *P. viridis* exposed to 20 ppb copper for 4 weeks showing heavily vacuolated cells and wandering baemocyles x 400.

Fig. 5 C.S of Digestive tubules of *P. viridis* exposed to a combination of 10 ppb copper and 50 ppb mercury for 4 weeks showing heavily vacuolated cells x 400.



levels of dissolved copper containing 10ppb and 20ppb. The damages to the tubules were not as drastic as happened in the case of mercury ; although variations in nature of damage was noticed. Noticeable feature was the presence of wandering haemocytes in the lumen of gastric diverticula. Tubular damage involved disruption and dislocation of collagenous layer , loss of identity of tubules and heavy vacuolation of the two types of cells (Fig.4).

The animals were subjected to combined exposure of copper and mercury for thirty days . The damage assessed by way of histological examination of digestive tubules showed that basically the presence of copper sulphate and mercuric chloride resulted in tubular as well as cellular damage. It looked as if the damage was more manifested in the case of animals that encountered 10 ppb copper and 20 ppb mercury in the media. Heavy vacuolation was resulted in the case of cells of tubules of animals exposed to 10ppb copper and 50ppb mercury. (Fig. 5)

Gastric tubules of animals exposed to 20ppb copper and 50ppb mercury contained enlarged vacuolated cells to the extent of obliterating the lumen.

III.4.1.1.b - GILLS

The basic structure of gill of mussel shows a descending and ascending lamellae. The lamellae are formed of jointed filaments at interfilamentar junctions by means of cilia.

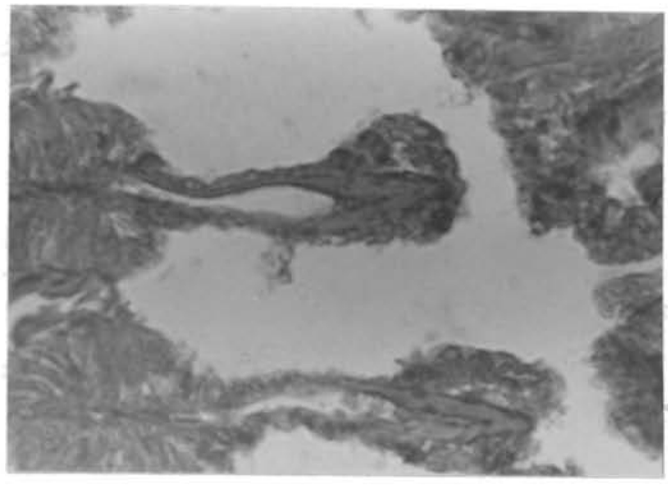
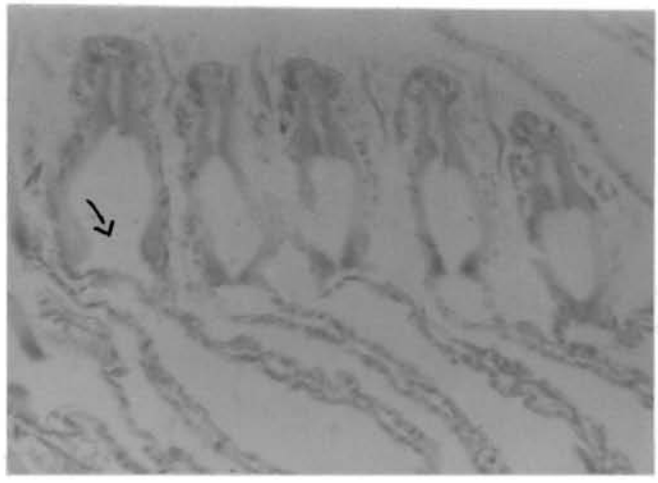


Fig. 6 *C.S. of gills of P. viridis maintained under normal conditions for 4 weeks x 400.*

Fig. 7 *C.S. of gills of P. viridis exposed to 50 ppb of mercury for 4 weeks showing dilated brachial veins x 400.*



Ascending and descending lamellae are connected by interlamellar blood vessels . The blood vessels have well defined epithelium formed of ciliated or non ciliated cells . A chitinous rod supports the whole soft tissue . The frontal cilia are connected to the frontal cells . Interfrontal cells and post laterofrontal cells are unciliated . Mainly the abofrontal cells and lateral cells carry the cilia.

As in the case of the study of the gastric diverticula , gills of such animals which were maintained in various concentrations of copper, mercury and copper and mercury combinations were examined histologically to assess the extent of damage . The basic structure of the gill as evidenced in (Fig. 6) was maintained only in the control animals .

Those animals which encountered 20 and 50 ppb mercury in the surrounding media for one month had partially or totally damaged gill filaments . The damage could be denudation of cilia or even chitinous rods . Dilation of brachial vein and rupture of blood vessels could also be noticed.(Fig. 7)

In the case of animals exposed to 10ppb copper (Fig.8) a noticeable feature of the filamentar tip was the crowding of cells carrying the cilia . Whereas animals exposed to 20ppb copper had gills at different stages of total damage . The range of cellular damage is comparable to that observed in case of mercury exposed animals.

A combination of copper and mercury obviously resulted in

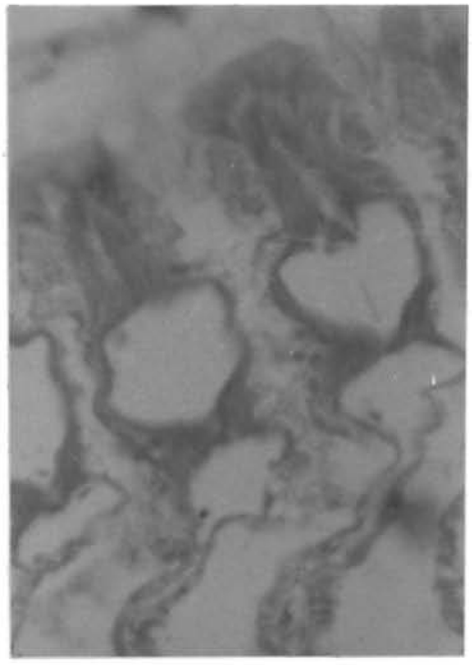
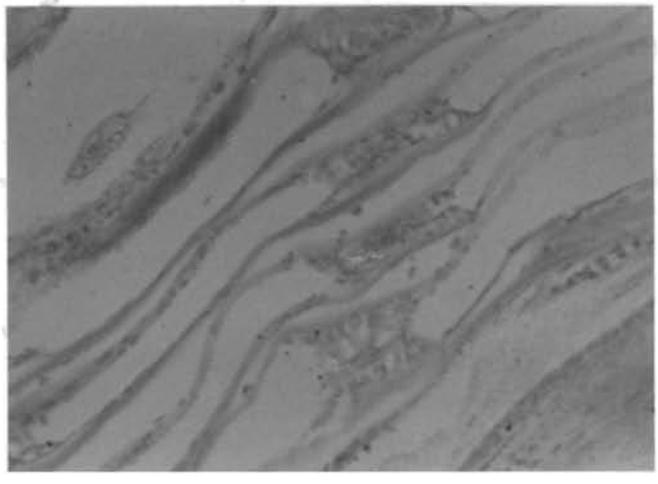


Fig. 8 *C.S. of gills of P. viridis exposed to 10 ppb of copper for 4 weeks showing crowding of ciliated cells at the filamentar tip x 400.*

Fig. 9 ***C.S. of gills of P. viridis exposed to a combination of 10 ppb copper and 20 ppb mercury for 4 weeks showing enlarged gill filaments.***



various degree of damage . A very clear cut difference was observed in the gills of those animals that lived for one month in media which contained 10ppb copper and 20ppb mercury . Here the gill filaments were found to attain incomparable length resulting in nearly the gills filling the mantle cavity. Fig. 9 shows the elongated gill filaments with totally disrupted structure . Major portions of gill filaments were denuded and only at points the lateral and abofrontal cilia were noticed . Curiously enough the extent of damage of gill filaments exposed to 20ppb copper and 50ppb mercury was minimal even after a month's exposure. Gill filaments were found to maintain a near minimal a near normal structure The damage being only in the reduction of the intralamellar space , which otherwise had a characteristic lumen.

III.4.1.2 Perna viridis subjected to one week exposure followed by one week depuration.

In a series of experiments involving histological enquiry into cellular damage of digestive tubules and gills of animals exposed to concentrations of mercury and copper for one week and subsequent transfer to clean sea water for another week form the subject matter.

III.4.1.2.a Digestive tubules

Fig. 10 a, is section of gastric diverticula of P.viridis exposed for one week to 20ppb mercury. It is clear from the

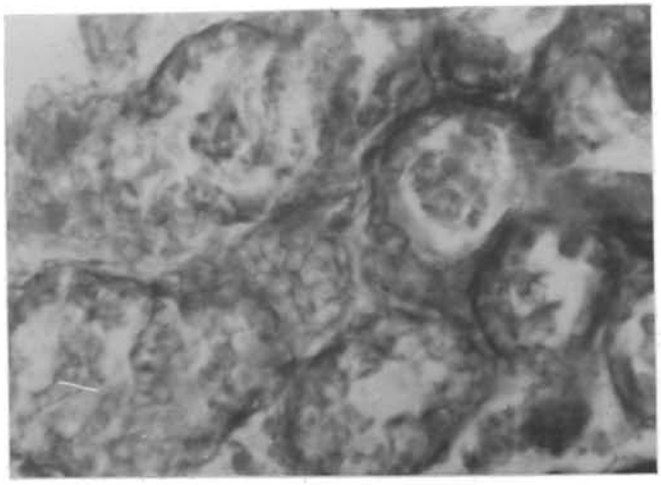
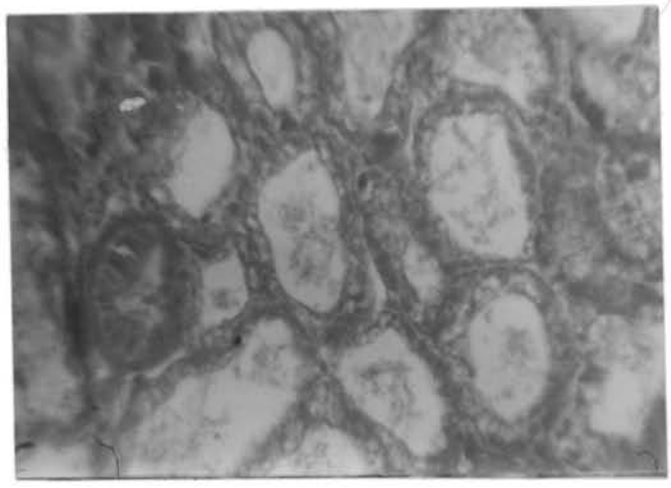


Fig 10 (a) *C.S. of Digestive tubules of P. viridis exposed to 20ppb Hg for 1 week showing presence of sloughed off cells x 400.*

Fig 10 (b) *C.S. of Digestive tubules of P. viridis which was exposed to 20 ppb Hg for 1 week and subsequently exposed to raw sea water for 1 week showing intact cells x 400.*



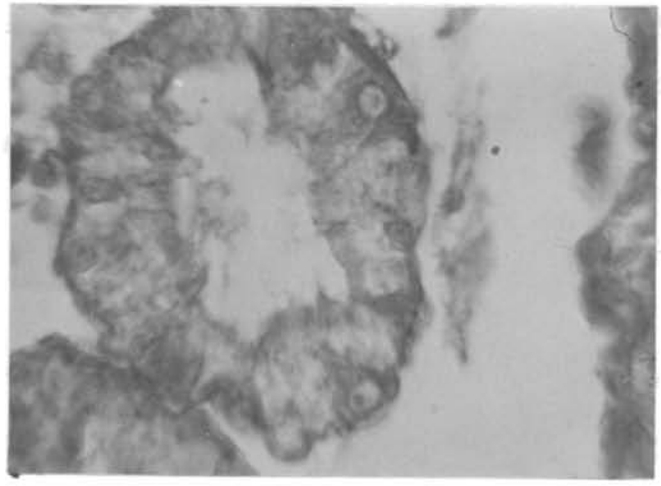


Fig. 11 *C.S. of Digestive tubule of P. viridis which was exposed to 50 ppb mercury for 1 week and subsequently exposed to raw sea water showing normal lumes and intact cells x 400.*

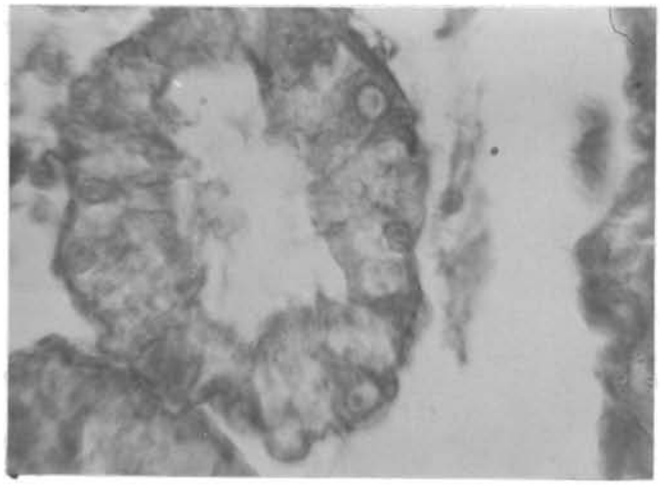


Fig. 11 *C.S. of Digestive tubule of P. viridis which was exposed to 50 ppb mercury for 1 week and subsequently exposed to raw sea water showing normal lumes and intact cells x 400.*

figure that cells of the tubules were sporadically sloughed off and remains within the lumen. These cells could undergo degradation and get cast off. It is not clear whether this sporadic sloughing off is the result of the shock reaction of the animal on account of heavy metal toxicity.

Fig. 10 b, is the section of digestive tubules of P.viridis allowed to depurate mercury after exposure to this for seven days. It is clear that the cellular organisation of digestive tubule is intact and lumen contains food particles (S. salina), in various stages of digestion . However heavy vacuolation was noticed. The outer collagenous layer also did not show any disruption, indicating that the damages that would have happened due to exposure have been repaired.

Contrary to the nature of damage underwent in the gastric tubules , in the case of animals exposed to 50 ppb mercury, prolific vacuolisation and obliteration of lumen were the nature of damage that had happened to the gastric tubules. Here also, the external lining mainly the collagenous layer showed shrinkage which looked to be a compensatory reaction to sloughing off cells. It is also evident that in general there was reduction in number of basophilic cells . As a rule , Basophilic cells are less in number and it is known that these cells are actively involved in secretory process and sometimes even in digestion. The rapidity with which basophilic cells regain normalcy either by regeneration or by repair is amply evidenced from the section of digestive tubules of animals allowed to depurate.(Fig.11)

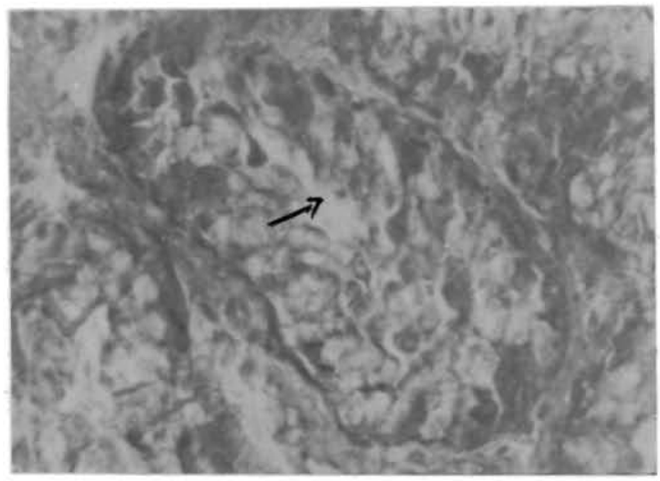
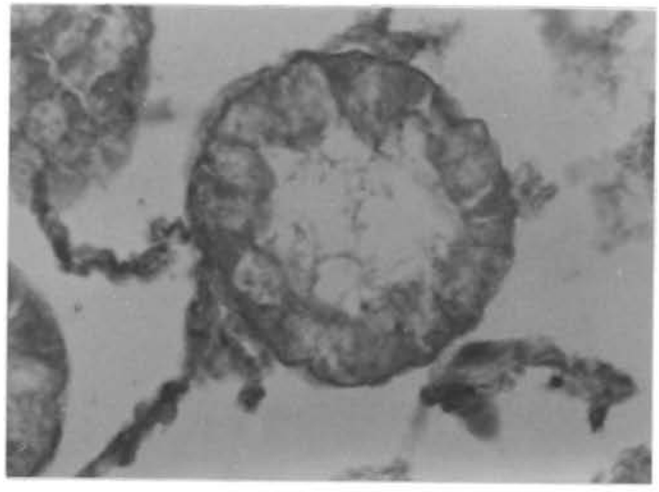


Fig 12 (a) *C.S. of Digestive tubules of P. viridis exposed to 10 ppb Cu. for 1 week showing obliterated lumen and enlarged acidophilic cells.*

Fig 12 (b) *C.S. of Digestive tubule which was exposed to 10 ppb copper for 1 week and subsequently exposed to raw sea water for 1* ***week showing normal epithelical cells and lumen.***



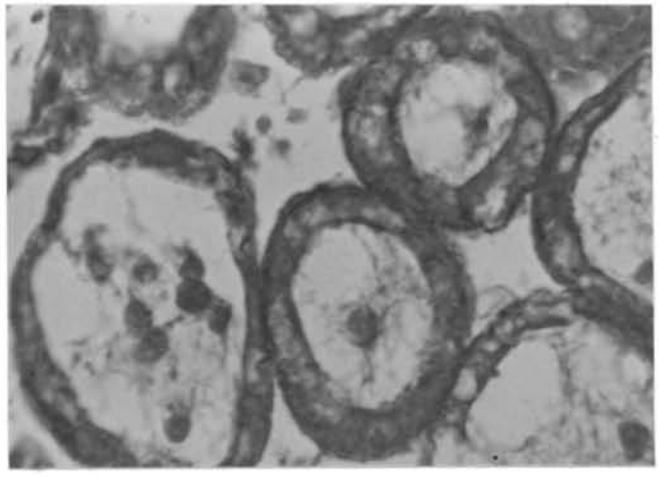


Fig. 13 *C.S. of Digestive tubules of P. viridis exposed to a combination of 10 ppb Cu and 20 ppb Hg for a week, showing enlarged lumen, wandering haemocytes and flattened epithelial cells x 400.*

Further the heavy vacuolation was reduced and obliterated . The lumen has regained the normal structure. This could have happened by reduction in size of the possibly enlarged acidophilic cells or regaining rigidity by different cellular layers of digestive tubule. In the case of digestive tubule of P.viridis exposed to medium containing 10 ppb copper (Fig. 12 a & b) obliteration of the lumen by enlargement of acidophilic cell was a reaction . Exposure to raw sea water could help in reversing the reaction.

In the case of those animals which was exposed to 20 ppb copper the extent of damage was more. Obviously it is to be believed that the intensity of damage is associated with increase in concentration of copper within the area .

III.4.2.b.METAL COMBINATIONS

Fig. 13 shows the damage caused to the gastric tubules of animals exposed to a combination of 10 copper and 20ppb mercury. The most noticeable feature was the loss of acidophilic cells which has resulted in the enlargement of lumen and digestive tubule is represented by collagenous layer , flattened basophilic cells and shrunken acidophilic cells. When the animals were allowed to depurate for a week in raw sea water there was regeneration of cells although the lumen was obliterated by prolification of acidophilic cells for reasons incomprehensible. In the case of those animals exposed to 10ppb

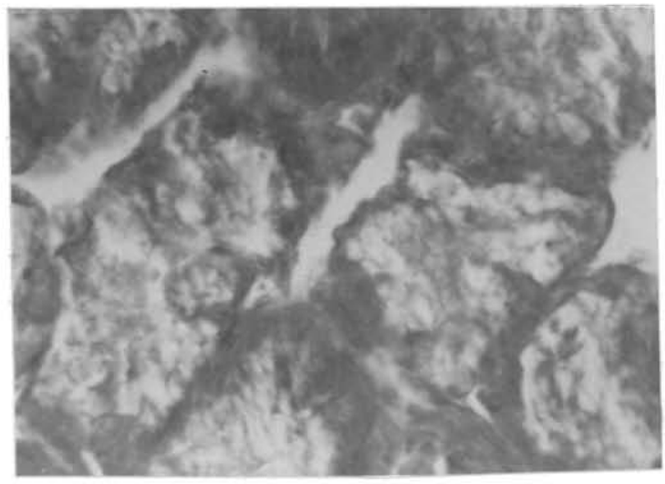
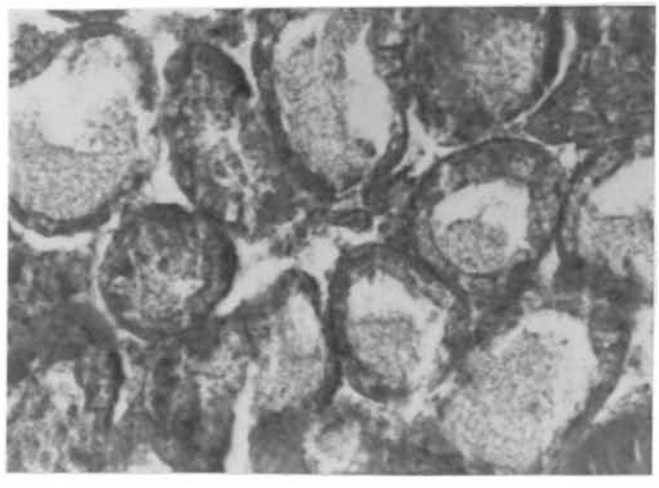


Fig 14 (a) *C.S. of Digestive tubules of P. viridis exposed to a combination of 20 ppb copper and 50 ppb mercury showing sloughed off epithelial cells x 400.*

Fig 14 (b) ***C.S. of Digestive tubules of P. viridis which was exposed to a combination of 20 ppb copper and 50 ppb mercury for a week and subsequently exposed to raw sea water for a week showing intact collagenous layer and normal lumen with food particles.***



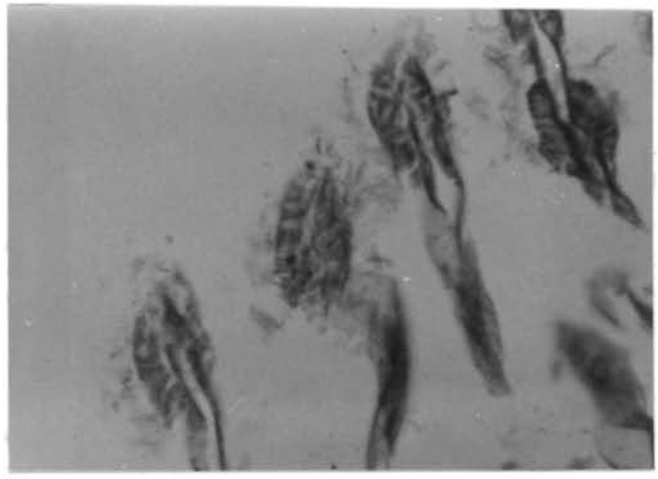


Fig 15 (a) *C.S. of gills of P. viridis exposed to 20 ppb mercury for a week showing damaged chitinous rods and cilia x 400.*

copper and 50ppb mercury the nature of damage of gastric tubules was totally different . Here the lumen was obliterated , the acidophilic cells were highly vacuolated and connective tissue showed thinning , mainly of the outer collagenous layer . Exposure to raw sea water did not substantially result in regaining normalcy in the structure of gastric tubules . High vacuolation was maintained even after exposure to raw sea water . Lumen was copiously supplied with dislodged cells . Heavy vacuolation of acidophilic cells was reaction of animals to exposure. As a consequence of depuration the cells were found to contain vacuoles and only the basophilic cells maintained certain degree of normalcy. It is clear that damage was drastic . A series of photographs (14 a & b) show that the effect of exposure of P.viridis to 20ppb copper and 50 ppb mercury , gastric tubules were found distorted with acidophilic and basophilic cells sloughed off. Connective tissue also showed shrinkage . The digestive tubules of depurated animals maintained a structure very similiar to that of animals exposed to lower concentrations of copper and mercury. The collagenous layer was found intact and lumen contained food particles.

III.4.1.2.b GILLS

Fig. 15a, is a section of gill filament of P. viridis exposed to 20ppb Hg. . It is clear from the section that chitinous rods and the frontal and abofrontal cilia have been damaged . The contiguity of the cell filaments effected by the cilia is lost resulting in filaments remaining as independent

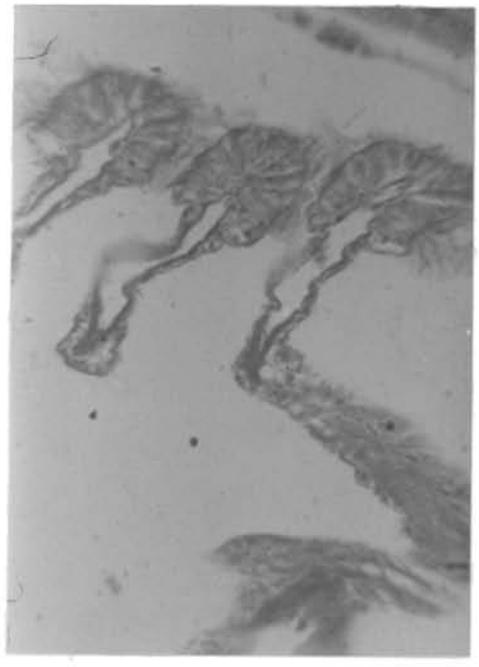
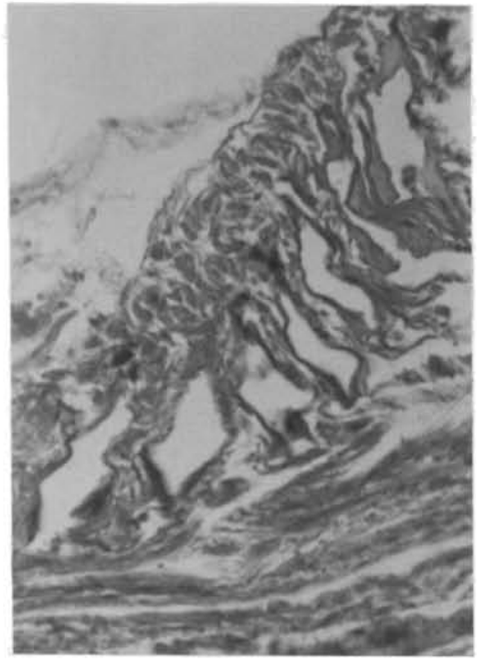


Fig 15 (b) *C.S. of gills of P. viridis which was exposed to 20 ppb mercury for a week and subesequently exposed to raw sea water for a week showing intact gill filaments x 400.*

Fig. 16 ***C. S. of gills of P. viridis exposed to 20 ppb copper for a week showing damaged chitinous rods.***



entities, which would have resulted in rendering gills inefficient for the circulation and filtration of water.

Fig. 15 b, is that of the gill of animal allowed to depurate mercury for seven days after exposure to 20 ppb mercury for seven days The gill filaments have intact structure indicating quick repair of damaged gill lamina . It is possible that exposure to clean medium for some more days would have resulted in the complete repair of damaged gill lamina.

In the case of gills of animals exposed to 50 ppb mercury, dilation of proximal region of gill filament and sloughing off of cilia of abofrontal cells were the main reaction . Curiously enough the extent of damage was limited when compared with that of Perna exposed to 20 ppb mercury . In the case of depurated animals the filamentar organisation was found repaired thoroughly, the sections showing a structure very similiar to that of gills of control animals.

Perna viridis was maintained in a medium containing 10 ppb and 20 ppb copper for a period of one week before transferring them to raw sea water to faciliate depurative process. The results obtained are shown in Fig.16. It is clear from the sections that the presence of such low concentration of copper brought about cellular damage to the gills as in the case of digestive tubules. In the case of gills , the flexing of chitinous rods has probably resulted in the bulging of brachial blood vessels . On the whole the gill tissue showed

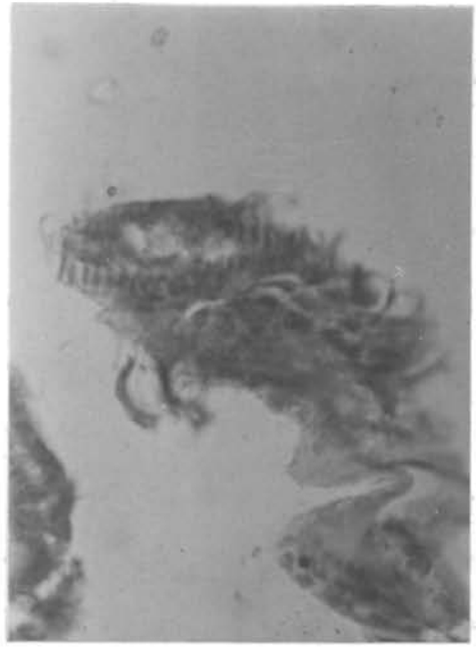


Fig. 17 *C.S. of gills of P. viridis exposed to combination of 10 ppb copper and 20 ppb mercury for 1 week showing disintegrated gill lamellae and wandering haemocytes.*

disintegration which was found to get repaired quickly on return to raw sea water .

Fig.17 shows the damage caused to the gills exposed to combination of 10ppb copper and 20ppb mercury. The gill lamellae was found to be disintegrated especially those cells carrying cilia . Wandering haemocytes were found in gill tissue . Here also maintenance in raw sea water resulted in the gill lamella regaining semblance of control tissue . Although the brachial vein was found to be enlarged, it was not clear whether the damage caused was irreparable.

In the case of gills of animals exposed to 10 ppb copper and 50 ppb mercury there was extensive damage. The cells especially the abofrontal ones were found dislodged . The brachial veins were found obliterated and wandering haemocytes were found inside the lumen of the gill.

Depurative process did help in regaining some normalcy of filamentar cells. But inter filamentar space and brachial vein did not register any substantial recovery.

A series of experiments were conducted to find out exposure of Perna viridis to culture media containing 20 ppb copper and 20 ppb mercury. Results evidenced from sections of tissue show that damage caused on gill filaments were of greater degree than that resulted when animals were exposed to lower concentrations. Gill filaments showed extensive damage. Shrinking of cells resulted in general distortion of gill filaments. Maintenance in normal

sea water helped in regaining certain degree of normalcy in structure, although regaining of functional responses are unknown

In the case of gills of those animals exposed to 20ppb copper and 50ppb mercury, the damage was so extensive resulting in total dismembering of cells . It was difficult to make out the structure of damaged proximal portions of gill filaments. Not withstanding this external damage the gill filament was found to regain normalcy in exposure to clean sea water . It is not clear whether the animals depurated possessed gills with comparable degree of damage after exposure to toxicants.

III .4 .3 STRUCTURAL ASSAY OF TISSUE

A series of tables were prepared to find out whether enlargement of cells of digestive tubules as a consequence of prolonged exposure to toxicants can be employed as a useful tool in histological monitoring of pollution effects. Data are presented in tables 1, 2, 3, 6, 7, 10, 11.

The data show that the variation in cell number in unit area of digestive tubule and gills was significant when compared with that of control. This clearly shows that there was enlargement of cells of digestive tubules which would have resulted in edamatous thickening of the tubules belonging to P. viridis exposed to various concentrations of copper and mercury . A comparison in variation of cell-number in digestive tubules of P.viridis exposed to 10 ppb copper and 50 ppb mercury showed that

TABLE 1. *Perna viridis* : Number of cells in unit area (mm^2) of digestive tubules and gills in the case of individuals exposed to heavy metal toxicants (copper and mercury in ppb) in individual and combination for four weeks.

TISSUE	DIGESTIVE TUBULES								GILLS							
Treatment	Number of fields examined															
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Control	12	7	14	10	11	6	8	10	6	4	5	6	5	6	5	6
Copper 10	7	5	10	8	6	9	6	6	6	4	6	5	6	7	5	7
Copper 20	7	2	1	3	4	2	5	5	2	1	2	1	1	2	1	1
Mercury 20	7	6	5	7	6	6	6	7	3	2	5	6	2	3	5	2
Mercury 50	5	4	3	9	6	4	5	11	6	5	3	6	4	3	6	14
Copper 10 + Mercury 20	7	12	10	9	9	5	6	8	2	3	4	4	3	2	3	3
Copper 10 + Mercury 50	14	6	10	11	16	6	6	4	5	3	4	6	3	4	2	4
Copper 20 + Mercury 20	8	10	10	9	8	7	6	6	5	5	4	3	4	4	3	4
Copper 20 + Mercury 50	12	7	7	8	10	7	6	7	5	3	2	3	2	2	3	4

TABLE 2. *Perna viridis* : Inference on variation in cell number of treated animals on exposure to heavy metal toxicants (copper and mercury in ppb) for four weeks with that of control arrived at employing WILCOXON SIGNED RANK TEST.

Tissue	Treatments compared	T^-
Digestive tubules	Control, Copper 10	1 *
Gills	Control, Copper 10	10 **
Digestive tubules	Control, Copper 20	0 *
Gills	Control, Copper 20	0 *
Digestive tubules	Control, Mercury 20	0 *
Gills	Control, Mercury 20	0 *
Digestive tubules	Control, Mercury 50	1 *
Gills	Control, Mercury 50	4 *

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

* Significant

** Not significant

TABLE 3. *Perna viridis* : *Inference on variation in cell number of the digestive tubules and gills of treated animals on exposure to heavy metal toxicants (copper and mercury in ppb) in combination for four weeks when compared with that of control arrived at employing* WILCOXON SIGNED RANK TEST

Tissue	Treatments compared	T^-
Digestive Tubules	Control, Copper 10 + Mercury 20	1 *
Gills	Control, Copper 10 + Mercury 20	0 *
Digestive Tubules	Control, Copper 10 + Mercury 50	6 **
Gills	Control, Copper 10 + Mercury 50	0 *
Digestive Tubules	Control, Copper 20 + Mercury 20	3 *
Gills	Control, Copper 20 + Mercury 20	1 *
Digestive Tubules	Control, Copper 20 + Mercury 50	3 *
Gills	Control, Copper 20 + Mercury 50	2 *

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

* - Significant

** - Not significant

TABLE 4. *Perna viridis* : Histological status of cells by rank analysis in digestive tubules and gills of Perna viridis exposed to heavy metal toxicants (copper and mercury in ppb) for four weeks.

Treatment Stages of degeneration	Control	Copper 10	Copper 20	Mercury 20	Mercury 50	Copper 10 + Mercury 20	Copper 10 + Mercury 50	Copper 20 Mercury 20	Copper 20 + Mercury 50
a	0	1	2	3	0	3	0	0	0
b	0	2	3	0	1	3	0	0	0
c	0	2	0	2	2	0	2	3	2
d	0	1	3	1	1	3	2	3	1
e	0	1	3	0	2	3	0	3	2
f	0	2	0	1	2	0	3	3	2
g	0	3	2	2	3	3	2	0	0
h	0	0	0	1	3	3	3	2	2
i	0	0	0	0	0	2	2	1	2
j	0	2	3	2	3	2	3	3	2
k	0	1	2	2	3	0	0	0	0
l	0	3	2	2	1	3	3	3	2

Stages of degeneration cited :

a) reduction in height of epithelial cells
b) presence of infiltrated haemocytes
c) increase in number of vacuolated cells
d) presence of sloughed off cells
e) presence of degenerated epithelial cells
f) increase in height of epithelial cells
g) dilation of branchial vein
h) swollen endothelial cells
i) infiltrated haemocytes in gills
j) presence of damaged or distorted cilia
k) swollen epithelial cells
l) degenerated cells in gills

rank value :

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

TABLE 5. *Perna viridis* : Spearman's correlation coefficient for different concentration based on rank analysis of histological status of cells of digestive tubules and gills exposed to heavy metals toxicants (copper and mercury in ppb) for four weeks.

Sl. No.	Treatments compared	r
1.	Control, Copper 10	0.5385
2.	Control, Copper 20	0.5525
3.	Control, Mercury 20	0.5490
4.	Control, Mercury 50	0.5333
5.	Control, Copper 10 + Mercury 20	0.6067
6.	Control, Copper 10 + Mercury 50	0.5525
7.	Control, Copper 20 + Mercury 20	0.5787
8.	Control, Copper 20 + Mercury 50	0.6154

Significance at 5% level $r \geq .5035$

TABLE 6. *Perna viridis* : Number of cells in unit area (mm^2) of digestive tubules and gills in case of individuals exposed to heavy metal toxicants (copper and mercury in ppb) in individual and in combination for one week.

TISSUE	DIGESTIVE TUBULES								GILLS							
Treatment	Number of fields examined															
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Control	20	13	12	11	12	18	14	16	4	5	6	5	6	4	6	6
Copper 10	14	16	10	12	7	8	10	4	4	3	2	5	4	6	3	2
Copper 20	16	12	11	14	6	8	4	10	7	5	4	3	3	4	4	3
Mercury 20	7	8	5	6	7	5	6	7	2	3	2	2	1	3	3	3
Mercury 50	4	12	6	6	4	9	7	7	4	4	4	3	4	2	5	5
Copper 10 + Mercury 20	17	10	11	16	12	13	14	15	4	2	3	5	4	3	5	6
Copper 10 + Mercury 50	15	14	8	16	19	9	9	10	6	7	6	8	5	6	6	4
Copper 20 + Mercury 20	17	10	12	7	11	15	15	8	4	5	4	3	5	5	3	3
Copper 20 + Mercury 50	6	6	6	9	10	5	4	5	6	2	1	3	5	2	5	6

TABLE 7. *Perna viridis* : Number of cells in unit area $(mm)^2$ of digestive tubules and gills in case of individuals exposed to heavy metal toxicants (copper and mercury in ppb) in individual and combination for a week and subsequently depurated for one week.

TISSUE	DIGESTIVE TUBULES								GILLS							
Treatment	Number of fields examined															
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Copper 10	5	12	14	7	10	17	14	4	5	4	5	2	5	4	2	3
Copper 20	10	8	8	9	6	6	8	5	8	5	5	4	4	5	5	4
Mercury 20	16	17	14	12	10	18	16	12	3	5	5	4	3	4	5	5
Mercury 50	8	10	10	10	8	12	12	12	6	5	5	4	5	3	5	6
Copper 10 + Mercury 20	12	15	16	18	14	15	18	14	5	5	4	4	6	5	5	6
Copper 10 + Mercury 50	16	12	10	18	20	10	8	10	5	6	6	7	4	4	3	6
Copper 20 + Mercury 20	10	20	18	10	12	16	20	10	3	4	5	5	9	9	7	5
Copper 20 + Mercury 50	14	8	9	10	8	10	10	10	4	5	5	5	3	6	6	6

TABLE 8. *Perna viridis* : *Inference on the cell number of gills exposed to heavy metal toxicants (copper and mercury in ppb) for a week followed by a week depuration in the basis of rank analysis arrived at employing PAGE'S STATISTIC*

Sl.No.	Treatment	Value of L
1.	Copper 10	103.5
2.	Copper 20	105
3.	Mercury 20	111
4.	Mercury 50	109.5
5.	Copper 10 + Mercury 20	104
6.	Copper 10 + Mercury 50	91
7.	Copper 20 + Mercury 20	102.5
8.	Copper 20 + Mercury 50	103

Significance at 5% level $L \geq 104$

TABLE 9. *Perna viridis* : Inference on the cell number of digestive tubules exposed to heavy metal toxicants (copper and mercury in ppb) for a week followed by a week depuration on the basis of rank analysis arrived at employing PAGE'S STATISTIC.

Sl.No.	Treatment	Value of L
1.	Copper 10	102.5
2.	Copper 20	103.5
3.	Mercury 20	107.5
4.	Mercury 50	111
5.	Copper 10 + Mercury 20	101
6.	Copper 10 + Mercury 50	101.5
7.	Copper 20 + Mercury 20	104.5
8.	Copper 20 + Mercury 50	111

Significance at 5% level $L \geq 104$

TABLE 10. *Perna viridis* : *Inference on variation in the cell number of the digestive tubules and gills of treated animals on exposure to heavy metal toxicants (copper and mercury in ppb) for one week when compared with that of control, arrived at employing* WILCOXON SIGNED RANK TEST.

	Tissue	Treatments compared	T^-
1.	Digestive Tubules	Control, Copper 10	13 **
2.	Gills	Control, Copper 10	1 *
3.	Digestive Tubules	Control, Copper 20	1 *
4.	Gills	Control, Copper 20	1 *
5.	Digestive Tubules	Control, Mercury 20	0 *
6.	Gills	Control, Mercury 20	0 *
7.	Digestive Tubules	Control, Mercury 50	0 *
8.	Gills	Control, Mercury 50	0 *

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

* - Significant

** - Not significant

TABLE 11. *Perna viridis* : *Inference on variation in the cell number of the digestive tubules and gills of treated animals on exposure to heavy metal toxicants (copper and mercury in ppb) in combination for one week, when compared with that of control 1* WILCOXON SIGNED RANK TEST.

	Tissue	Treatments compared	T^-
1.	Digestive Tubule	Control, Copper 10 + Mercury 20	1 *
2.	Gill	Control, Copper 10 + Mercury 20	0 *
3.	Digestive Tubule	Control, Copper 10 + Mercury 50	6 **
4.	Gill	Control, Copper 10 + Mercury 50	10 **
5.	Digestive Tubule	Control, Copper 20 + Mercury 20	1 *
6.	Gill	Control, Copper 20 + Mercury 20	1 *
7.	Digestive Tubule	Control, Copper 20 + Mercury 50	1 *
8.	Gill	Control, Copper 20 + Mercury 50	1 *

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

* - Significant

** - Not significant

TABLE 12. *Perna viridis* : Histological status of cells by rank analysis - *Perna viridis* exposed to heavy metal toxicants (copper and mercury in ppb) in individual and in combination for one week.

Treatment Stages of degeneration	Control	Copper 10	Copper 20	Mercury 20	Mercury 50	Copper 10 + Mercury 20	Copper 10 + Mercury 50	Copper 20 + Mercury 20	Copper 20 + Mercury 50
a	0	0	0	3	0	3	0	0	3
b	0	0	0	0	0	2	0	0	0
c	0	2	2	3	3	1	3	3	3
d	0	3	1	3	2	2	2	2	3
e	0	2	2	3	2	3	2	2	3
f	0	3	2	0	3	0	2	3	0
g	0	3	2	0	1	0	3	0	2
h	0	3	2	3	2	2	2	3	2
i	0	0	0	0	0	2	0	2	2
j	0	3	3	3	2	3	2	3	3
k	0	2	2	2	2	3	2	3	3
l	0	0	0	3	0	3	0	3	2

Stages of degeneration cited :

a) reduction in height of epithelial cells
b) presence of infiltrated haemocytes
c) increase in number of vocuolated cells
d) presence of sloughed off cells
e) presence of degenerated epithelial cells
f) increase in height of epithelial cells
g) dilation of brachial vein
h) swollen endothelial cells
i) infiltrated haemocytes in gills
j) presence of damaged or distorted cilia
k) swollen epithelial cells
l) degenerated cells in gills

rank value :

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

TABLE 13. *Perna viridis* : Spearman's correlation coefficient for different cocentration based on rank analysis of histological status of tissue of digestive tubules and gills exposed to different concentration of heavy metals (copper and mercury in ppb) in individual and in combination for one week.

Sl.No.	Treatments compared	r
1.	Control, Copper 10	0.5595
2.	Control, Copper 20	0.5787
3.	Control, Mercury 20	0.6154
4.	Control, Mercury 50	0.5542
5.	Control, Copper 10 + Mercury 20	0.5542
6.	Control, Copper 10 + Mercury 50	0.5175
7.	Control, Copper 20 + Mercury 50	0.5752
8.	Control, Copper 20 + Mercury 50	0.5805

Significance at 5% level $r \geq 0.5035$

the variation in cell number was not significant.

In the case of animals exposed to various concentrations of copper and mercury for one week it was noticed that both digestive tubules and gills recorded enlargement of cells (Table 10). Higher concentrations of mercury was found to bring about more change than that of copper.

Combined effects of copper and mercury on distribution of cells of digestive tubules and gills are presented in Table 11. In general there were variations inthe number of cells in the gills of those animals exposed to 10 ppb copper and 50 ppb mercury. On the contrary exposure to 20 ppb copper and 50 ppb mercury for one week resulted in noticeable variation in cell size of digestive tubules.

To find out whether characteristics revealed by histological assay of digestive tubule and gills using visual indices such as variation in vacuolated cells , sloughed off cells , degeneration of cells , dilation of brachial veins etc. can be used as useful tools to define histological status of tissue, have shown that in major cases the tissues showed tendency to degenerate with reference to above characteristics and degree of degeneration was comparable between animals exposed for one week and one month. It was indicated that prolonged exposure need not bring about concomitant degeneration of tissues as a function of time.

Table 8 presents data on the repair of gill tissue with

reference to the number of cells in unit area by animals allowed to depurate after exposure to copper and mercury singly and in combination.

The method employed is PAGE STATISTIC. The L value of 104 and above indicates that the rate of repair was significant . If this is taken into consideration it seems that only animals exposed to 20 ppb of copper , 20ppb mercury and 50ppb mercury , 10ppb copper and 20ppb mercury could efficiently repair the damaged tissue. The animals subjected to combinations of copper and mercury could not perform repair in the same pace within the experimental time of seven days .

Table 9 presents similiar data on the digestive tubule . Speedy repair of tubules happened in the case of those animals exposed to 20 ppb copper 20 ppb and 50 ppb mercury and combination of 20 ppb copper and 20 ppb mercury and 20 ppb copper and 50 ppb mercury . Curiously enough those animals exposed to 20 ppb copper and mercury alone were the combination of copper and mercury. Twenty and fifty ppb of mercury the rate of repair was slow . So was the case of animals exposed to 10ppb copper.

III.5 DISCUSSION

Studies on histology and histopathology have been proved to be a very useful tool in assessing pollutant induced injury to whole animals It is known that there will be structural and

functional alterations in individual cell types or group of cells at an early stage of response before alteration in cellular structure could manifest at organismic level.(Moore M.N. 1980). Normally injured cells undergo structural alterations . These alterations can have at least two phases . Reversible alterations or alterations leading to total disruption of cellular function and hence death of cell which can be called as irreversible change (Trump and Arstilla, 1975). Viarengo et al. (1982), evaluating the general and specific stress indices in mussels inhabiting an environment with define pollution gradient found that the digestive gland of animals sampled from heavy metal polluted area had a high concentration of low molecular weight thioneine like copper binding protein . It is possible that accumulation of cellular secretions of intra cellular origin is a response of increased activity by those intrusions responsible for such secretions. High vacuolation of digestive cells is known to be one of the manifestations of stress response which is indicative of increased lysosomal number.

The cells of the digestive epithelium belonging to the tubules have shown degeneration and structural alterations at various degrees . Workers on histopathology have identified structural changes in digestive cells involving atrophy or epithelium thinning . There is a tendency in the case of workers to generalise such changes to stressors effects mainly xenobiotics or prolonged starvation (Bayne et al., 1978; Pipe and Moore 1985; Moore et al., 1989) Structural assay of cellular damage have shown that there was enlargement of cells of

digestive tubules resulting in bulbous epithelial structure or total atrophy resulting in thinning. Enlargement of cells results in overall increase in volume of cells because of formation of enlarged or giant lysosomes . This subsequently leads to atrophy of digestive cells . The functional aspect being confined to autolytic or autophagic activity brought in by enlarged lysosomes.

A very interesting finding of the present investigation is that damage caused by low levels of xenobiotics , administered for shorter durations could be repaired. It may be stated here that one of the tissues used for the investigation was digestive tubule which has excretory, secretory and absorptive function, performing the duties of hepatopancreas of a crustacean. Death of cells, production of cells, degeneration and regeneration of cells are cardinal features of decapod crustaceans (Scylla serrata). Therefore, the findings in the present instance that there is a possibility of effective repair of cells clearly indicates that this capacity of digestive tubules must be an inherent one. Another possible aspect would be the total replacement by healthy cells in those tubules where extensive damage has happened owing to toxicity . However , it is not possible to explain the role of time factor , the capacity of regeneration etc. in the light of limited information available from histopathological studies. Severe degeneration of epithelial cells of digestive diverticula has been cited as an important after effect of exposure of bivalves to copper. The

prescience of thoroughly damaged cells in digestive tubules is an indication of atrophy which would eventually lead to sloughing off of cells . Brown coloured secondary and tertiary lysosomes are known to be conspicious features of digestive cells of the tubules. (Auffrett, 1988) Rasmussen et al. (1983 a & b) noticed necrosis and infiltration of severely damaged tubules by haemocytes. It is quite likely that these are injuries of internal organs brought about by chronic exposure to polluted environment. Changes in the morphology of the gill filaments , atrophy of the ciliated epithelial cells and damage of chitinous rods are patholigical indices of gill filaments of bivalves exposed to heavy metals and thermal stress (Sunila, 1986). Discussing on the effects of these damages on the general performance of bivalves, Sunila(1988) has pointed out that damage of gill filaments would have an effect either on respiration or food transport. She, however, says that nature of damage of cells could be different , thus the endothelial cells can become granular when exposed to copper , whereas it can render cells highly vacuolated when exposed to silver. According to her the inflammatory reaction in the gill is characterised by dilation of brachial vessels. The findings by Sunila(1988) that nature of damage of gills by heavy metal toxicity could be different in animals living in coastal waters and those exposed in the laboratory opens up an issue which needs further clarifications.

Commenting on the nature of repair of degenerative tissue, it was opined that the architecture of the tissues can vary on

degeneration (Sunila, 1988). However, no conclusive evidences have been obtained on this in the case of gills examined during the present investigation.

CHAPTER IV

HISTOCHEMISTRY

IV.1 INTRODUCTION

Histochemistry is a very effective tool for the direct diagnosis of chemical changes occurring in tissues at cellular levels. Moore (1991) remarked that the necessity to find out scale of aquatic environmental pertubrations "has focused attention on the urgent need for sensitive and precise diagnostic tools , or biomarkers with a predictive capability in assessment of toxic contaminant impact." Moore,(loc cit) has suggested molecular detection systems for a broad spectrum of cellular constituents. Practically this involves detection and identification of a cellular constituent or inclusions which are prone to exhibit qualitative and quantitative changes in response to stressors employed externally through the culture media.

It is known that mussels accumulate environmental contaminants in their tissues particularly those of digestive glands. The digestive gland is a major organ which maintains a contact with the environment, since this is the site where much of the digestive and absorbtive processes take place by utilization of materials such as food and water. Cells of the gills and the digestive cells are multifunctional and the latter is involved in uptake of food and subsequent intercellular digestion. The cellular reactions that take place within the digestive tubules will be identifiable chemically since these are

the sites of the secretory, digestive and excretory processes.

The normal method of assay in histochemistry involves development of colour so as to flag the biochemical compound used as the biomarker. In the present investigation lipofuschin and fatty acids were the biomarkers. Staining techniques developed and employed were sufficient to assess intensity of histochemical reactions evidenced by variations in colour. Totaro et al., (1985) suggested that these evidences could be considered to be the effect of lipoperoxidative action of free radicals induced by heavy metals . It may also be possible according to them that formation of lipofuscin granules could also be interpreted as a cellular defense mechanism.

IV . 2 REVIEW OF LITERATURE

Lysosomes , the oft called suicidal bags , with its store of innumerable enzymes and a multitude of functions have in recent years have risen to a topic of continuous research . In the light of recent investigations this organelle has proved to be the pivotal structure behind the performance of a wide range of physiological functions in the body (Moore 1988). Therefore, it would be most apt to consider lysosomes as an ideal, starting point for investigations of generalised stress responses to invertebrates.

Innumerable literature is available on the effects of environmental stressors on lysosomes both in invertebrates and

vertebrates. The cytochemistry of lysosomes has been investigated extensively (Moore, 1976,1979; Hawkins 1980 ; Moore and Farrar,1985; Moore et al.,1987) and it has been found to be one of the main targets of many toxic effects of contaminants. Pathological alterations in the lysosomal structure has helped to identify the adverse effect of toxicants on organisms especially in mussels inhabiting the coastal areas. The molluscan tissue which are rich in lysosomal concentration , are in fact store houses of reserves and intracellular digestion (Owen 1972a). Many cytochemical methods to detect lysosomal alterations have been evolved which have become very handy for environmental biomonitoring in both marine molluscs and fishes. (Moore et al., 1987; Lee et al., 1989 ; Kohler 1989, 1990).

Hawkins (1980) has classified three categories of lysosomal reactions 1) changes in lysosomal contents, 2) changes in fusion events and 3) changes in membrane permeability.

Lysosomal alterations can be induced by several factors, chemical and nonchemical. Chemical factors include effect of heavy metals, polycyclic aromatic hydrocarbons and the like. Non chemicals include hypoxia, hyperthermia, osmotic shock and dietary depletion (Moore 1985).

Studies in Mytilus edulis (Harrison and Berger,1982)show copper to be sequestered in lysosomes. It was also found that the copper concentration in digestive gland was directly related to the concentration in water to which the mussels were exposed.

Viarengo et al., (1984), while studying the detoxification of copper in metal exposed mussel expressed the opinion that it could occur in two forms. 1)Increased synthesis of copper thioneins 2) accumulation of metals in insoluble forms in lysosome eliminated by endocytosis. This reflects on the possible role of lysosomes in copper detoxification , particularly elimination of copper thioneins in metal exposed mussels.

In the digestive glands of marine mussels metallothioneins and lysosomes play an interrelated key role in homeostasis (Viarengo et al., 1987). In Mytilus edulis and Ostrea edulis demonstration of the compartmentation and immobilisation of metals in membrane bound vesicles have been shown (George et al.,1976;Coombs and George 1978; George et al., 1978). Secretory cells of kidney of Mytilus edulis have shown to sequester zinc(II) and iron(III) in lysosomes.(George et al., 1976). Iron and lead are seen to accumulate in amoebocytic brown cells of Mytilus edulis in the form of cytoplasmic granules believed to be lysosomes.(Moore and Lowe 1977).

Hydrocarbons are seen to reduce the latency of lysososomal enzymes (Moore and Clarke 1982). Another effect noticed was a pronounced increase in the permeability of the limiting membrane of lysosomes (Allison 1969; Moore 1979; Moore et al.,1978b). Further, it has also been opined that lipophillic hydrocarbons induce dearrangement of lipoprotein structure. The degree of dearrangement bears a direct relation to the molecular configuration of the pollutant (Moore and Farrar 1985). The

structural differences in anthracene and phenanthrene and the differences in their mode of action on the lysosomal membrane and endoplasmic reticulum in the digestive glands of Mytilus edulis are worked on by Nott et al.,(1985). Other notable effects include presence of large autophagic lysosomes in Mytilus edulis (Moore and Clarke, 1982). Modulation of cell morphology by lysosomes is also noted in estuarine clam, Rangia cuneata (Marsh et al., 1981).

Changes in salinity also tends to effect alterations in lysosomal vacuolar system (Pipe and Moore 1985). Further changes in lipid level and formation of enlarged secondary lysosomes are also found to cause changes in digestive cell architecture (Lowe 1988). When marine mussels are exposed to contamintants, the lysosomes in digestive glands show rapid pathological alterations (Lowe 1988 ; Moore 1988). These include swelling of digestive cell lysosomes , accumulation of neutral lipid in lysosomes, increased fragility of lysosomal membrane and excessive build up of lipofuschin in lysosomal compartment. These are accompanied by atrophy of digestive epithelium. Whether atrophy of cells follow autophagic process is uncertain.

Among the many evidences used as biomarkers of lysosmal pathology indicating stress due to adverse effects on organisms, lipofuschin build up in digestive cells and fatty acid degeneration along with accumulation of neutral lipids serve as very important indices.

The lipoprotein membrane undergo lipid peroxidation and condensation with lysosomes resulting in the formation of lipofuschin rich residual bodies. (Brunsk and Collins 1981). Another suitable version of formation of lipofuschin production is the consideration of transition metals to be radicals which react with lipoproteins to cause peroxidative phenomena (Sohal 1981, Totaro et al., 1987). Further , it has also been stated that this endogenous pigment derived fron lipid or lipoprotein precurssors by progressive oxidation looses its fatty characteristic and darkens colour. Several factors induce the formation of lipofuschin granules like normal aging and dietary, toxic or stressful environmental factors.

Elevated intracellular or intralysosomal concentrations of metals, such as copper or mercury may contribute to enhance lipofuschin content, as these metals are important in mediating the toxic effect of oxygen (Halliwell 1981) . Exposure of mussels to copper results in increased formation of lipofuschin in digestive cells. (Viarengo et al.,1987).

Lipofuschin is a fluorescent pigment which is commonly referred to as age pigment. The presence of copper has shown to increase the level of lipofuschin in Corollospora martima. Besides all other transitional elements which contain unpaired electrons and qualified as radicals serve to induce lipofuschinogenesis in C. martima.(Pisanti et al.,1988). It has also been proved that rise in metal concentration increases the

production of lipofuschin.

Immobilization and detoxification of heavy metals are effected by cellular compartmentation which is facilitated inside pigmented membrane limited granules present in the tertiary lysosomes.(George et al .,1982). Viarengo et al.,1988 a,b, states copper induces formation of lipofuschin . The metal then gets bound to pigment granules in a relatively stable form and gets eliminated by exocytosis of the residual bodies.

Heavy metal effects lipid per oxidation in tissues of Mytilus galloprovincialis was noted by Viarengo et al., (1990). While describing the structures induced by contaminants in structure of digestive epithelium of Mytilus edulis Lowe and Clarke (1989) have stated abnormal accumulation of lipids in digestive and basophilic cells. Sarasquete et al., (1990) while comparing the histopathological alterations in digestive gland of marine bivalves exposed to copper and cadmium reports of accumulation of unsaturated lipids and pathologically enlarged lysosomes and lipofuscin granules. Capuzzo and Leavitt (1988) have reported accumulation of neutral lipids as a result of exposure to xenobiotics. Though available literature on the levels of fatty acids in organisms in relation to stress is scarce , it is beyond doubt that the fatty acids are important stores of energy and reserve food material in the cell. Exposure to environmental stressors lead to disturbances in cellular physiology leading to degradation of fatty acids and accumulation of neutral lipids.

Toshiko Daikko et al.,(1982) has shown that the fatty acid concentration in guppies show a decrease as a result of sea water adaptation. In mussels it has been noted that fatty acid degeneration leads to accumulation of neutral lipids.(Moore, 1988).

Capuzzo and Leavitt(1988) carried out lipid class analysis of Carcinus maenas populations from waters polluted with aromatic hydrocarbons and reported increase in triglycerol and neutral lipid contents. Whether the increase in lipid content under xenobiotic stress is due to inability of the organism to catabolize lipids or due to an increase in synthesis is not certain.

The objective of the present study was to compare the production of lipofuscin pigment in digestive tubules of P.viridis exposed to various concentrations of copper and mercury over varying lengths of time. It was also thought that the study would help to understand the relationship between duration of exposure and the load on the pigment production . Though numerous methods to evaluate the level of peroxidation and accumulation of lipofuscin are known , thiobarbituric acid test (Placer et al.,, 1966; Kikugawa et al.,,1985) flurometric analysis (Kuk and Enesco 1981), a simple staining technique to detect its presence (Schmorl's reaction for lipofuschin, Lillie 1965) was adopted.

IV.3 Materials and Methods

This part of the thesis centered around delineating the histochemical effects on selected tissues of Perna viridis when exposed to sublethal concentrations of mercury and copper individually and in combination for a period of one week followed by one week exposure to raw sea water and a prolonged exposure for four weeks, to different concentrations of copper and mercury individually and in combination.

The details of the test animals , testmedia , laboratory conditioning of animals , toxicants etc. has already been given earlier (Section 3.3).

IV.3.1HISTOCHEMICAL STUDIES

IV.3.1.1 Exposure for 4weeks

Perna viridis, exposed to different concentrations of mercury and copper individually and in combination for a period of four weeks were utilised for the study. The procedure followed for exposure of animals to the different test concentrations was the same as explained in the previous chapter.. After termination of the exposure period , the animals were dissected and the soft tissues comprising of the digestive tubules and the gills were washed and processed for takfng sections using the cryostat and a simple rotary microtome.

IV.3.1.2 Exposure for one week to toxicants followed by one week exposure to raw sea water.

Here again ,the basic methodology remained the same with only the duration of exposure lasting for a week. After exposure, animals from each test concentration was removed and fixed in suitable fixative. The remaining animals were then exposed for one week to raw sea water. Samples from each concentration exposed to raw sea water for a week were then processed for taking sections using a cryostat and a simple rotary microtome.

The following procedure was adopted for sectioning tissues in cryostat.

In the crysotat small pieces of digestive gland were dissected from the exposed mussels(size 5×5×5mm). They were placed on aluminium chucks with 5 piece of tissue in a straight row across the centre. The chuck was placed for one min.in a small bath of hexane (aromatic hydrocarbon free;boiling range 67-70^{O}C), which had been pre cooled to -70^{O}C in liquid nitrogen in order to quench the tissue.

The chuck plus the quenched ,solidified tissue were sealed by double wrapping in aluminium foil and stored at - 30^{O}C for sectioning.

Tissue sections,10μ thick were prepared in Bright's cryostat with a cabinet temperature of less than -25^{O}C. Sections

were transferred to room temperature .Sections were stored in cryostat until required, but not longer than 4 hrs.

Chemical composition of the fixative used :-

Formal Calcium

Formalin -100ml

Calcium acetate-20gms

Water to 1000 ml.

Staining technique employed:

Schmorl's reaction (Pearse 1972)

1) Duplicate cryostat sections were fixed for 15 mins in Calcium formal at 4 ^{o}C.
2) Sections were then rinsed in distilled water and then immersed in the reaction medium (1%ferric chloride and 1%KCN in ratio of 3:1)
3) Sections were stained for 5 mins.in the solution.
4) Rinsed in ascetic acid for 1 min.
5) Rinsed in distilled water and mounted in aqueous mounting media

Staining technique using paraffin sections:

The staining technique to stain paraffin sections for lipofuschin granules is essentially the same with slight modification. It is as presented below :

1. Bring sections to water.

2. Treat with freshly prepared solution of 30ml of 1% $FeCl_3$, 4ml of freshly prepared 1% Potassium Ferricyanide and 6ml of distilled water for 10 minutes.
3. Wash in running water.
4. Dehydrate, clear and mount in D.P.X.

Lillie's sulfuric Nile blue technique for fatty acid.

Reagent

Acid Nile blue sulphate

Nile blue sulphate-0.05g

Distilled water-99ml

Con. sulfuric acid-1ml.

Method

1) Bring paraffin sections / cryostat sections post fixed in calcium formal to water.
2) Stain in Nile blue sulphate for 20 mins.
3) Wash in running water for 10 mins.
4) Mount in glycerin jelly.

IV.4 RESULTS

IV. 4. 1 Lipofuscin activity in Perna viridis

The intensity of colour developed on treatment for identifying lipofuscin content of digestive tubules has yielded

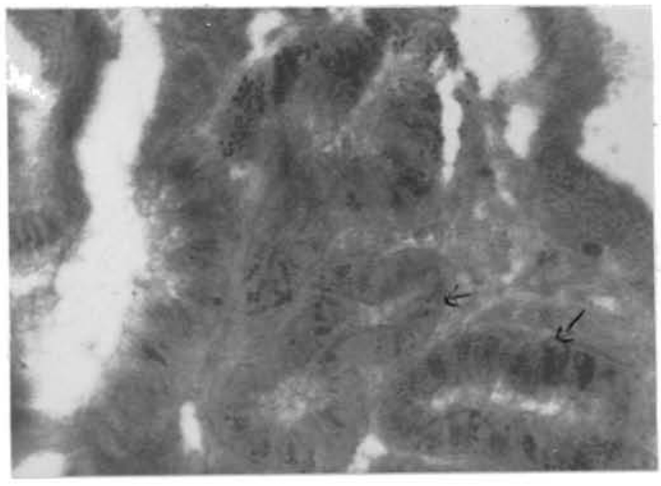
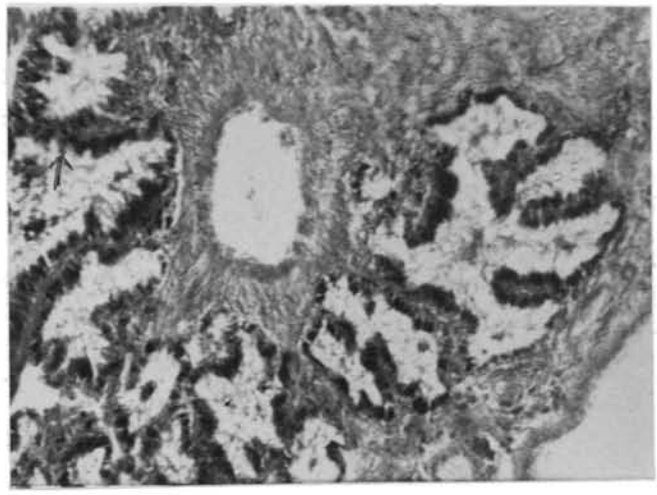


Fig. 1 *C.S. of Digestive tubules of P. viridis maintained under normal conditions for 4 weeks showing high lipofuscin activity x 400.*

Fig. 2 *C.S. of Digestive tubules of P. viridis exposed to 20 ppb mercury for 4 weeks showing dense lipofuscin accumulation x 200.*



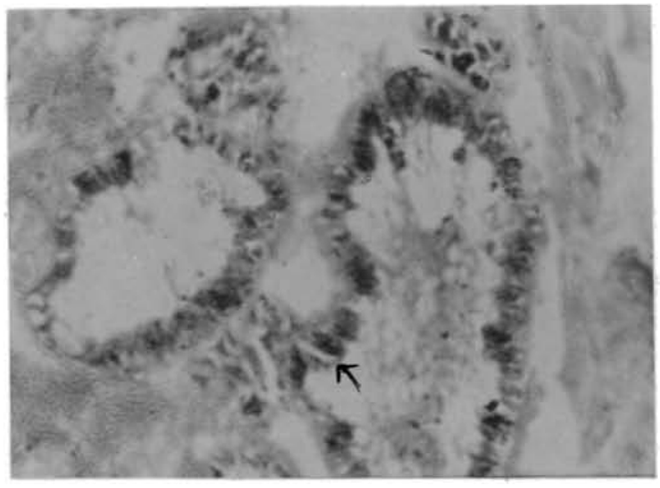
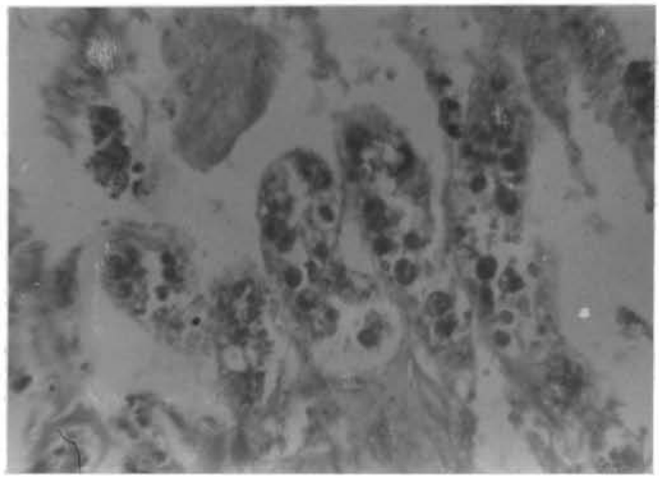


Fig. 3 *C.S. of Digestive tubules of P. viridis exposed to 50 ppb mercury for 4 weeks showing less intense lipofuscin granules x 400.*

Fig. 4 *C.S. of Digestive tubules of P. viridis exposed to a combination of 10 ppb copper and 20 ppb mercury indicating high lipofuscin activity with dislodged cells from lamina x 400.*



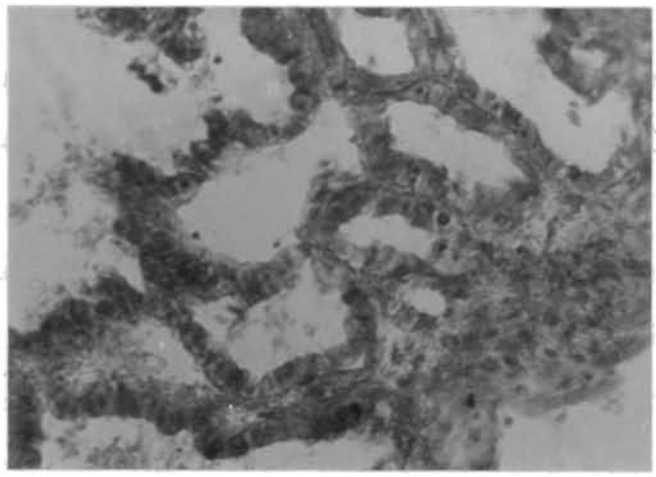
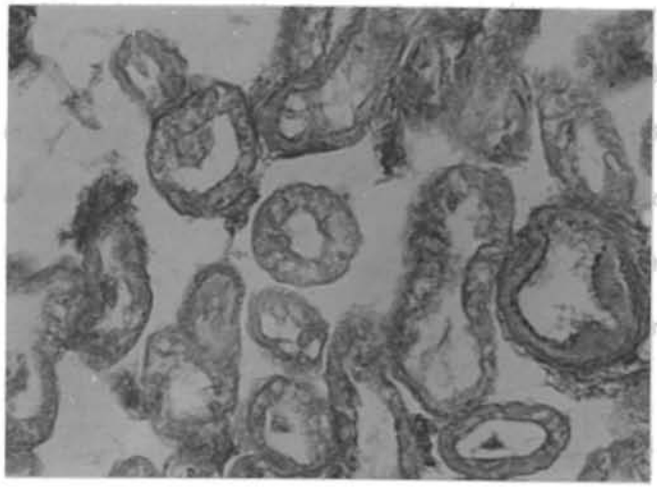


Fig. 5 *C.S. of Digestive tubules of P. viridis exposed to combination of 20 ppb copper and 50 ppb mercury for 4 weeks showing intense lipofuscin activity x 200.*

Fig. 6(a) *C.S. of Digestive tubules of P. viridis exposed to 20 ppb copper for 4 weeks showing less intense lipofuscin granules x 400.*



interesting results. Photomicrographs depicting tissues with various intensity of colour and hence lipofuschin activity are presented in this section.

IV.4.1.1 Lipofuschin activity in digestive tubules of <u>Perna viridi</u> subjected to 4 weeks exposure.

Fig.1-6a shows lipofuschin content in the cells of gastric tubules. It is clear from the Sections that animals exposed to 20 ppb copper showed more lipofuschin activity than those exposed to 10 ppb. However, the fact that simple stress like captivity can result in lipofuschin activity is evidenced by highly coloured tubules of control animals .

Exposure to 20 and 50 ppb mercury brought in concomitant results. Those experimental animals maintained in a medium containing 20 ppb mercury showed more activity after one month than those under 50 ppb mercury . This indicates a possibility of biphasic reaction of mercury on lipofuschin activity .

Exposure to combined action of copper and mercury also brought about comparable results. However, 20 ppb copper and 50 ppb mercury showed more intense activity . In the case of those animals exposed to 10 ppb copper and 20 ppb mercury, dislodging of cells from basal lamina has resulted in obtaining photographs of blue spherules like structures indicating cells with high lipofuschin activity.

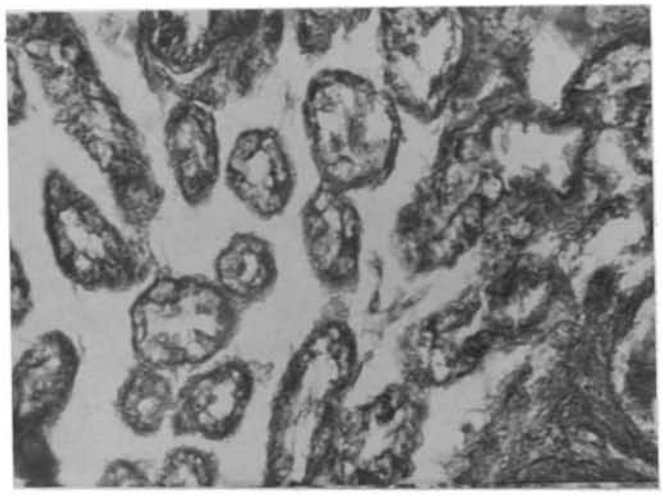


Fig. 66) C.S. of Digestive Tubules of P. viridis exposed to 20 ppb copper for 1 week showing high lipofuscin activity x 400.

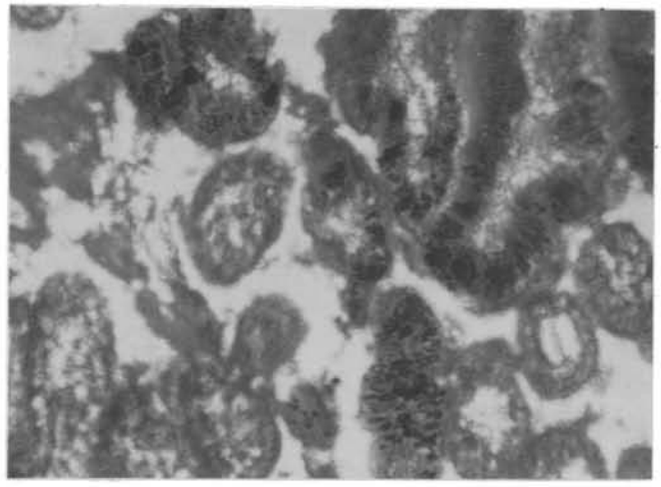
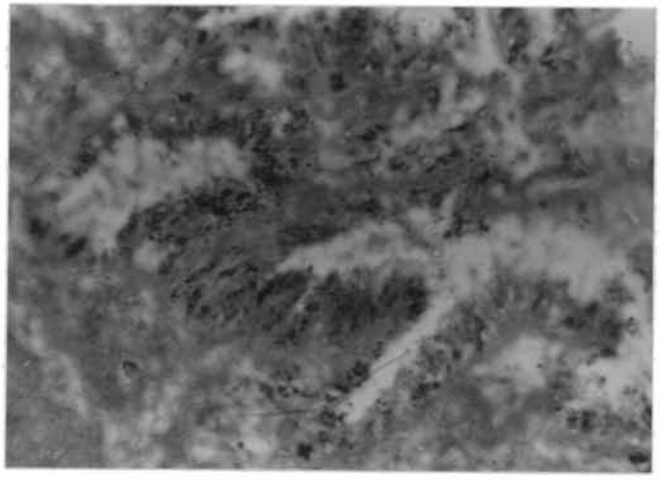


Fig 7 (a) *C.S. OF Digestive tubules of P. viridis exposed to 20 ppb mercury for 1 week showing intense accumulation of lipofuscin granules x 400.*

Fig 7 (b) *C.S. of Digestive tubules of P. viridis which was exposed to 20 ppb mercury for 1 week and subsequently exposed to raw sea water for a week showing reduced lipofuscin activity x 400*



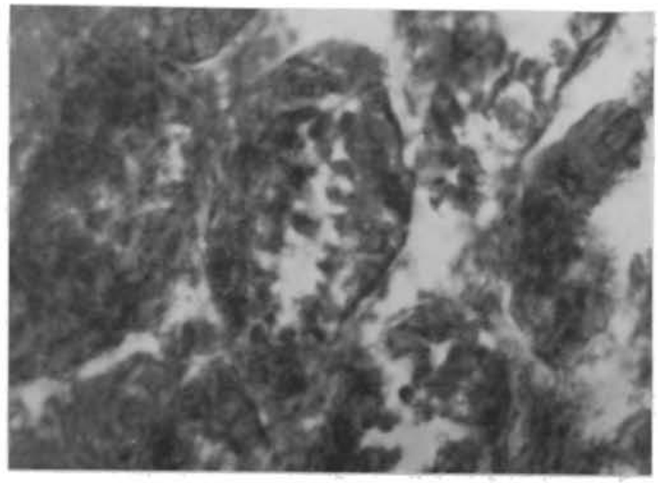
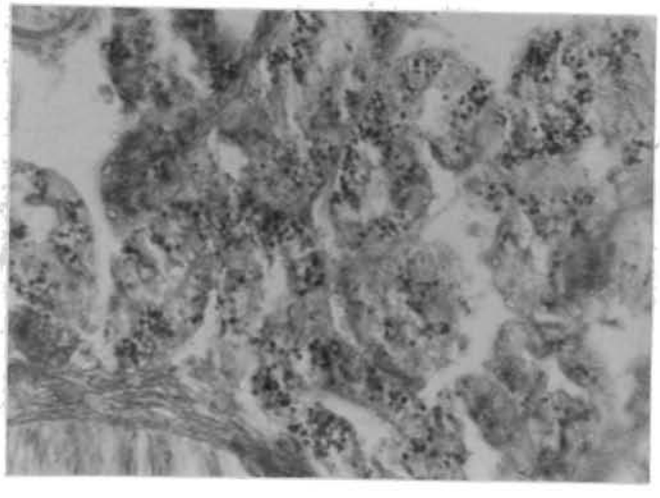


Fig 8 (a) *C.S. of Digestive tubules of P. viridis exposed to a combination of 10 ppb copper and 20 ppb mercury for a week with intense lipofuscin granules x 400*

Fig 8 (b) *C.S. of Digestive tubules of P. viridis which was exposed to combination of 10 ppb copper and 20 ppb mercury and subsequently exposed to raw sea water showing highly reduced lipofuscin granules x 400.*



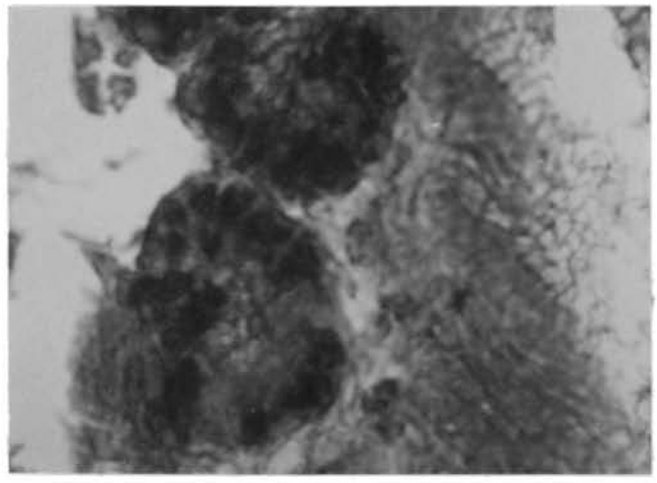
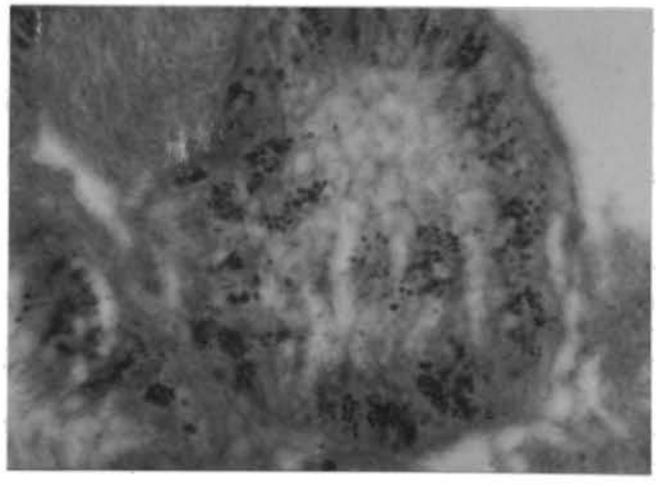


Fig 9 (a) *C.S. of Digestive tubules of P. viridis exposed to combination of 20 ppb copper and 50 ppb mercury showing dense accumulation of lipofuscin granules x 400.*

Fig 9 (b) *C.S. of Digestive tubule of P. viridis which was exposed to a combination of 20 ppb copper and 50 ppb mercury showing reduced lipofuscin activity.*



IV.4.1.2 Lipofuscin activity in digestive tubules of Perna viridis subjected to 1 week exposure followed by 1 week depuration.

A set of experiments were conducted to analyse the effects of short term exposure on the mobilisation of lipofuscin in thedigestive tubules of Perna viridis.

Exposure to copper did not evince any drastic difference in the accumulation of lipofuscin , no matter whether the concentration of the copper in the external medium was 10 or 20 ppb . On the contrary , presence of 20 ppb mercury showed high dense lipofuscin distribution in digestive tubules. Curiously enough, maintenance under 50 ppb mercury, did not result in such a high distribution of lipofuscin.

In the case of Perna viridis exposed to a combination of 10 ppb copper and 20 ppb mercury and 10 ppb copper and 50ppb mercury the lipofuscin activity was noted to be nearly the same. However, lipofuschin activity in those animals exposed to 20 ppb copper and 20ppb mercury showed more lipofuscin activity than those exposed to 20ppb copper and 50ppb mercury.

The experiments on the depurative process involved as in the case of histopathological studies, maintenance of Perna viridis after one week's exposure to toxicants, in raw sea water. These animals were subjected to histochemical examination after a

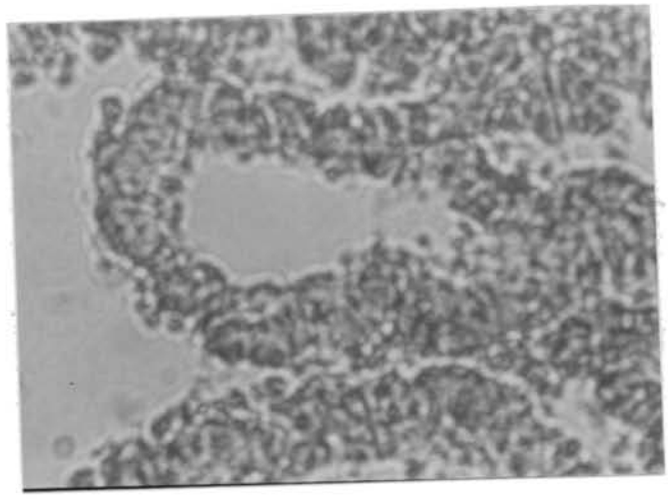
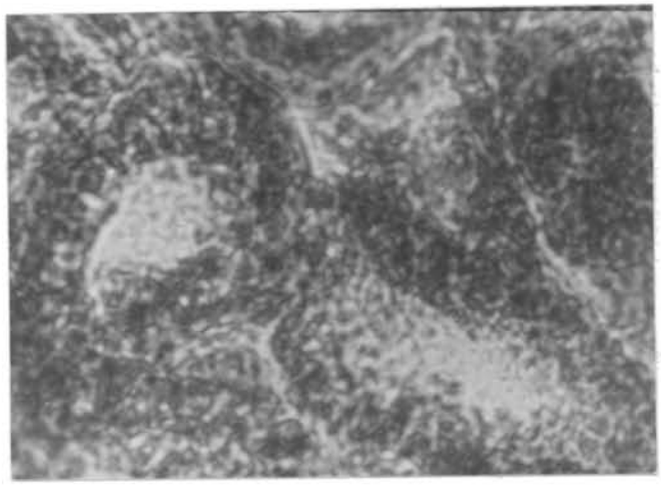


Fig 2 (a) C.S. of Digestive tubules of *P. viridis* exposed to 20 ppb copper for a week showing restricted fatty acid activity ×400

Fig 2 (b) C.S. of Digestive tubules of *P. viridis* which was exposed to 20 ppb copper for a week and subsequently exposed to raw sea water for a week showing intense fatty acid activity ×400



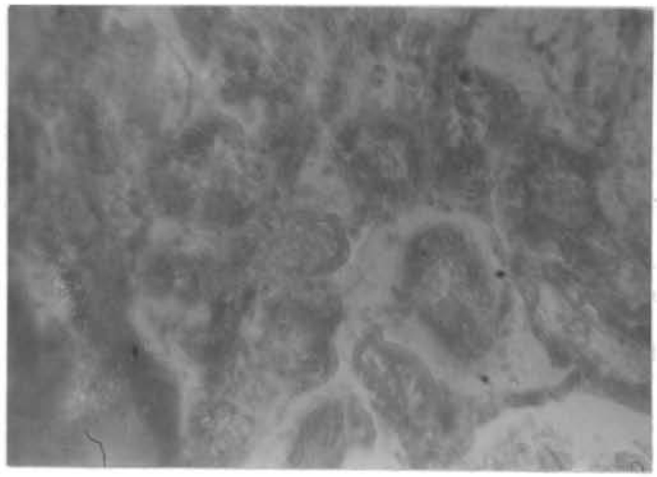


Fig 3 (a) C.S. of Digestive tubules of *P. viritis* exposed to 50 ppb mercury for a week showing dense fatty acid droplets ×400.

Fig 3 (b) C.S. of Digestive tubules of *P. viritis* which was exposed to 50 ppb mercury for a week and subsequently exposed to raw sea water showing increased fatty acid activity ×400.

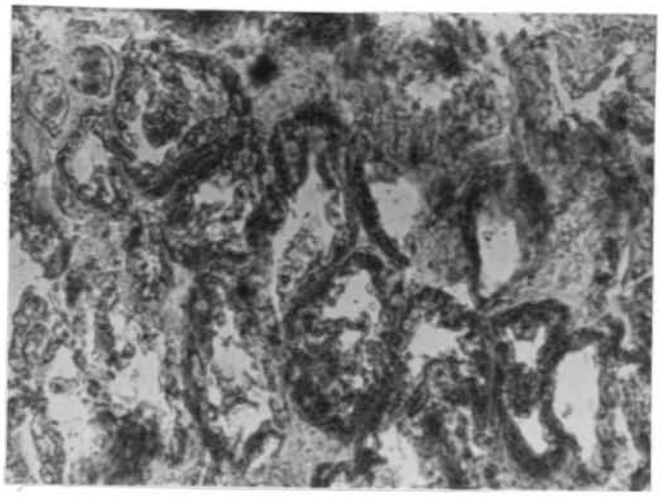
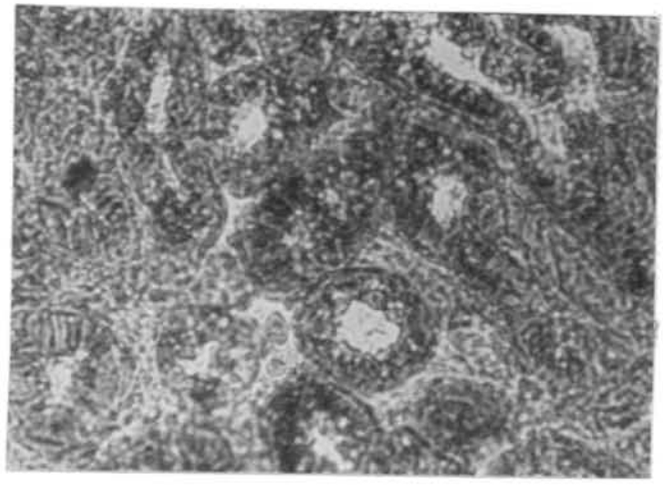


Fig 4 (a) C.S. of Digestive tubules of P. viridis exposed to a combination of 10 ppb copper and 20 ppb mercury showing traces of fatty acid activity x 400.

Fig 4 (b) C.S. of Digestive tubules of P. viridis which was exposed to a combination of 10 ppb copper and 20 ppb mercury for a week and subsequently exposed to raw sea water for a week showing fatty acid activity x 400.



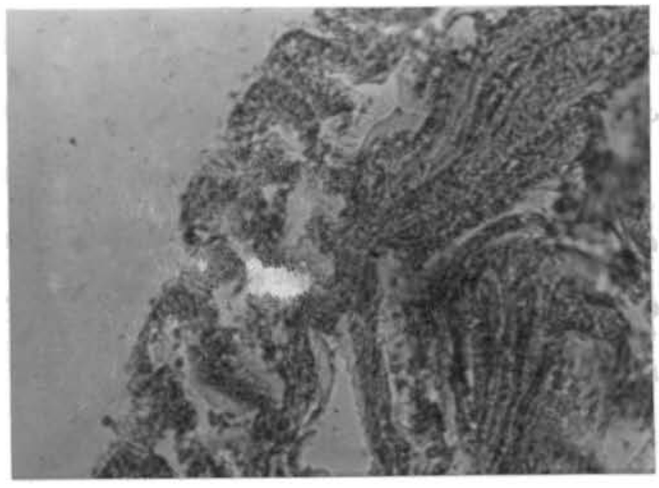
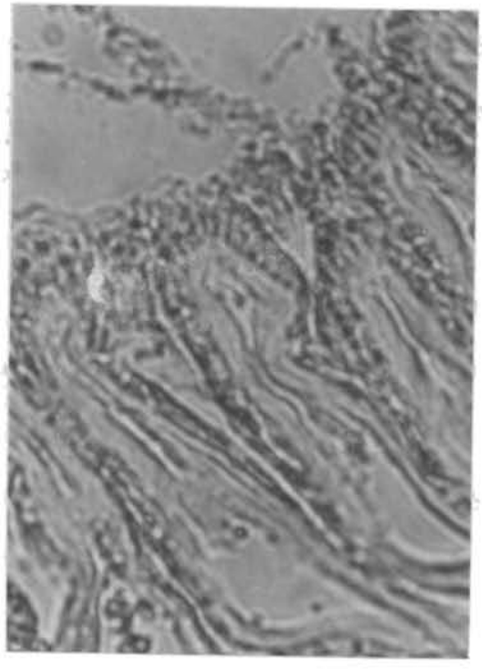


Fig 5 (a) *C.S. of gills of P. viridis exposed to 20 ppb copper showing fatty acid activity x 400.*

Fig 5 (b) *C.S. of gills of P. viridis which were exposed to 20 ppb copper for one week and subsequently exposed to raw sea water indicating fatty acid activity x 400.*



week and results obtained are depicted in photomicrographs.

It is clear that reduced lipofuschin activity was generally an index of absence of toxicant stress in the environment. This was specifically clear in the case of those animals exposed to lower concentration of copper and mercury. Reduced activity, of lipofuschin however , was not evidenced in case of those animals maintained in 10 ppb copper and 50 ppb mercury. On the other hand this was very low in those animals which were exposed to 20 ppb copper and 50 ppb mercury.

IV. 4 .2 Fatty Acid activity in Perna viridis

As in the case of lipofuschin , fatty acids also has been used as a biomarker to indicate stress effects. When the animals were exposed for one month in various concentrations of copper and mercury or their combination the section did not develop any colour . However , the control animals recorded fatty acid activity. So it is assumed that the fatty acid content of the digestive tubule which are the so called hepatopancreas of bivalves did not contain fatty acids in conspicious quantities after exposure to toxicants for a month .

A series of experiments were conducted to find out the effects of fatty acid distribution consequent to heavy metal exposure. The results obtained are shown in figures,2a&b;3a&b . In the case of copper exposed animals fatty acid activity was restricted , whereas in animals exposed to 20 ppb and 50 ppb

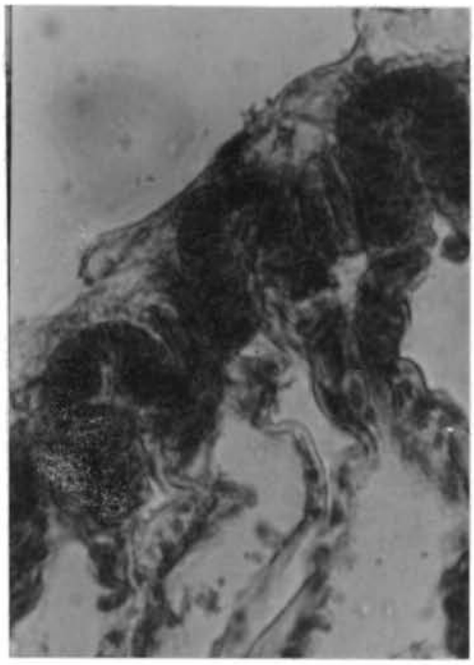
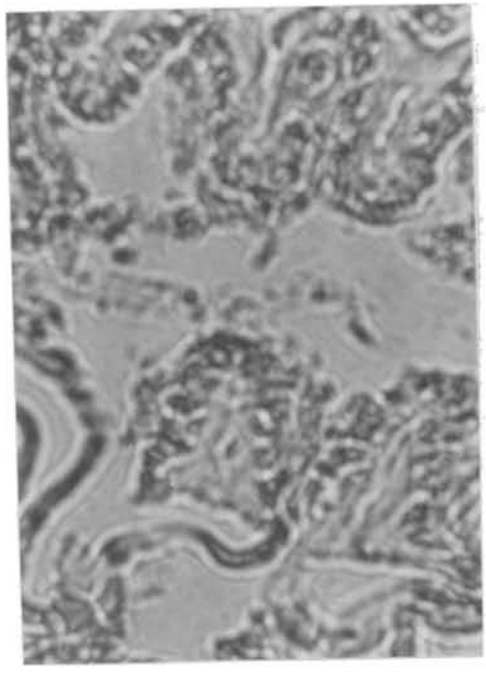


Fig 6 (a) C.S. of gills of *P. viridis* exposed to 50 ppb mercury for one week indicating presence of fatty acid x 400.

Fig 6 (b) C.S. of gills of *P. viridis* which was exposed to 50 ppb mercury for one week and subsequently exposed to raw sea water indicating reduced fatty acid activity x 400.



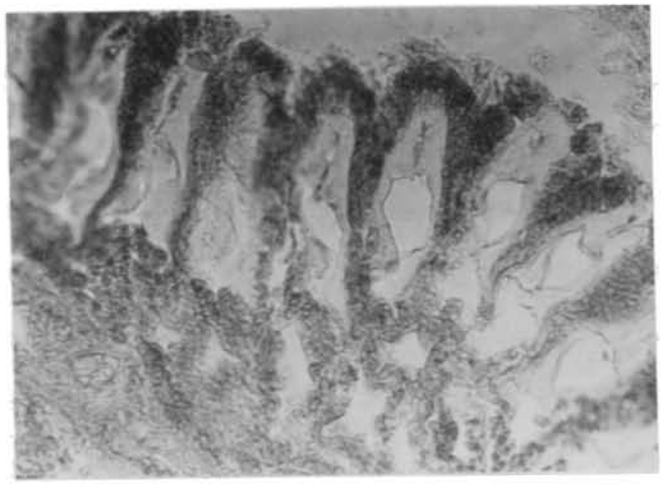
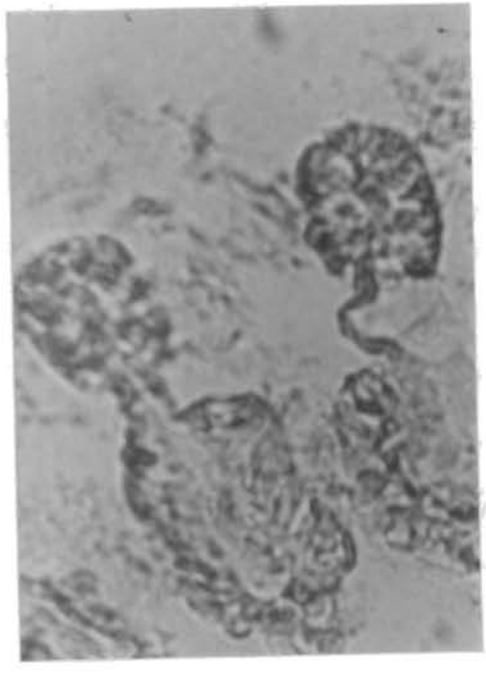


Fig 7 (a) *C.S. of gills of P. viridis exposed to combination of 10 ppb copper and 50 ppb mercury showing increased fatty acid activity x 400.*

Fig 7 (b) *C.S. of gills of P. viridis which was exposed to combination of 10 ppb copper and 50 ppb mercury showing reduced fatty acid activity x 400.*



mercury intense reactions were noticed evidenced by intense colouration . Exposure to combination of copper and mercury irrespective of concentration also showed similar trend .

A series of experiments to analyse the fatty acid content of digestive tubules of animals allowed to depurate heavy metals after one week exposure to copper and mercury was also done . The depurative process seems to increase the rate of fatty acid metabolism in gastric tubules evidenced by deep blue colouration in sections of animals exposed to various concentrations of copper and mercury.

A series of histological sections,for a histochemical examination for fatty acid distribution or activity in the gillwere taken . A curious phenomenon noticed from the results was that fatty acid activity in relation to transport or metabolism was maximum in the gills when the animals were exposed to toxicants like copper and mercury. The duration of exposure was one week . The gills of those animals exposed to 10 ppb copper, and subsequently allowed to depurate lasting one week,were totally devoid of fatty acids . Such a drastic variation was, however, not noticed by those animals allowed to depurate after an exposure to 20 ppb copper. Exposure to mercury at 20 ppb and 50 ppb level produced results similar to those noticed in the case of 10 ppb copper exposed animals . Combined action of copper and mercury did not yield any clear cut picture regarding fatty acid distribution in gills . It was noticed that

exposure to 20 ppb copper and 50 ppb mercury produced high activity of lipids evidenced by characteristic blue hue in gills. A similar reaction was noticed when the medium contained 10 ppb copper and 20 ppb mercury. After the depurative process which lasted for one week in both the sets of gills there were traces of fatty acid activity.

IV.5 DISCUSSION

Using lipofuscin as a biomarker with biochemical techniques is a promising field that has received attention from recent workers. It is known that the cellular organelles are involved in degradation of food particles taken up by endocytosis into the cell and in this method lysosomes are the most important sub-cellular organelles. Sternleib and Goldfischer (1976), Viarengo et al. (1981),and Moore et al.(1984) have demonstrated that lysosomes are the most important sites of metal compartmentalisation of cell. Loss of membrane stability results in the disruption of lysosomes resulting in leakage of hydrolytic enzymes into the cell. The formation of lipofuscin granules is connected with lipid peroxidation process stimulated by accumulation of lysosomes which are the most important sub-cellular organelle involved in metal detoxification. The relationship between quantity of lipofuscin in digestive cells and the quantity of heavy metals taken by bivalves is amply demonstrated by the findings of Viarengo et al. (1988b) that a

considerable quantity of copper is trapped in lipofuschin granules which are always present in lysosomes of copper exposed mussels. It is clear that the biological half life, ie. time for which biologically required heavy metals are retained in the biochemical constituent of invertebrates vary from metal to metal. This is normally shorter in the case of highly essential metals especially copper which has got a biological half life of seven to eight days. On the contrary , unwanted metals like cadmium, lead, and mercury have a longer biological half life In the case of cadmium and mercury this is as long as seven to eight months (Menon personal communication) Therefore, analysis of lipofuschin content by histochemical methods could give meaningful results only in the case of those heavy metals which have a shorter biological half life since this indicates the rapidity with which such heavy metals are warded off the tissue by biological processes.

It is known that copper is able to alter the red- ox balance in the cells and thereby stimulate lipid peroxidation in gills and digestive glands. It is possible that by stimulating lipid peroxidation copper may alter the physiology of lysosomes and enhance the amount of lipofuschin which can bind the metal in relatively stable form in these organelles. (Viarengo et al., 1988.).

A relationship between histological alterations and histochemical reactions could be significant since it is known

that increase in lipid accumulation is associated with enlargement of secondary lysosomes which bring about structural alterations in digestive epithelium and morphology of gastric tubules.(Lowe 1988). Moore et al.(1988) have opined that accumulation of lipid is associated with lysosomal dysfunction which as evidenced by histology.

Although it is not possible to quantify histochemical changes by rank analysis the latest analytical techniques by image analysis (Chassard,1991), it would be possible to distinguish gradation in colouration with reference to regions of digestive tubules. This probably would help in clear estimation of lipofuschin content in the tissue, which in turn could be used as index of pollution effects.

CHAPTER V

ACCUMULATION AND DEPURATION OF HEAVY METALS

V.1 INTRODUCTION

Recent evidences indicate that free ions are biologically the most available inorganic species of trace metals in sea water. It is also known that the concentration of free ions is controlled not only by metal concentration but also by the inorganic and organic complexation. Inorganic complexation is dependent on salinity and pH and the organic complexation varies from complete prevention of metal uptake to no effect. Although diet is the main source of heavy metals for higher vertebrates, water is the principal source in bacteria, phytoplankton and seaweed. In the case of mercury, water is known to be the most important route of uptake. May be for other metals food is the significant route. Rate of uptake of heavy metals can vary between metals and between species. In the case of molluscs uptake and transfer of metals is mainly controlled by pinocytosis (Bryan, 1984).

The metals on entry into the body get detoxified by various mechanisms. These include "binding to non specific high molecular weight proteins " , "specific low molecular weight proteins of metallothionein type " , " immobilisation in intracellular inclusion of different types by incorporation in shell, skeleton,etc. Such detoxified metal can be thrown out of

the body by diffusion or excretion; as fluids or granules ; via alimentary tract , exoskeleton or through eggs.

Copper and mercury has been used as toxic metals to study the effects of relatively high concentration on rate of uptake and depuration by Perna viridis. Copper is an important metal utilised for accumulation studies mainly because it has a well defined toxicity to a particular organism and its speciation in various systems is generally well characterized. Mercuric chloride is also known to permeate lipid bilayer membranes much faster than free metal ions.

In this chapter , the rate of accumulation and depuration of copper and mercury by P. viridis has been worked out. The animals were exposed either individually or in combination of these metals for various duration. The quantity of heavy metals accumulated or depurated was estimated using Atomic Absorption Spectrophotometer and cold vapour Atomic Absorption Spectrophotometer.

Localisation of heavy metals by staining procedures and X-Ray analysis have yielded useful results. Among this X-Ray and electron microscopy have found to give more informative results useful for energy kinetics. However, in the present case the requirement was to identify a useful method which could be cheap and effective to establish the possibilities of viewing whole tissue sections so as to understand the regions where metal ions tend to get localised in the case of animals subjected to chronic

exposure under laboratory conditions.

V.2 REVIEW OF LITERATURE

It is a known fact that mussels are capable of accumulating heavy metals in their tissues from polluted waters or from natural processes in unpolluted areas . Attention has been received by scientists on the uptake and storage rates of essential and non essential metals. Various theories have been proposed on different methods for storage of metals of marine animals. Coombs (1980) pointed out that copper is stored as inorganic precipitate ie. in a non toxic form. George et al.(1982) opinied that it is stored in membrane limited vesicles; while Viarengo et al.(1981) and George (1983a,b,) have noted heavy metals stored in lysosomes. Kagi and Nordberg (1979) have proved metals to be trapped in cytosolic cystein rich proteins called metallothioneins. A wealth of literature is available on the accumulation of heavy metals in invertebrate tissue (Graham,1972 ; Simkiss, 1976; George et al., 1975; Alexander and Young, 1976; Coombs and George 1978; Goldberg, 1980;).

Heavy metals ,halogenated hydrocarbons and petroleum hydrocarbons accumulating in the marine and estuarine waters in

the world are the most potential deleterious contaminants contaminating our biota and human consumers of sea food(Martin and Richardson, 1991). Of the many contaminants stated -heavy metals contamination in bivalves and its effects are well documented (Wesley and Raj, 1980; Mathew and Menon,1983; Prabhudeva and Menon, 1987;Krishnakumar et al., 1987; Webber, 1990; Webber et al., 1990; Mathew and Menon, 1992; Brock, 1992; Naimo, 1992). This property is solely due to the unique ability of bivalves to accumulate heavy metals in their tissue from environmenmental waters (Lakshman and Nambisan, 1989; Osuna et al., 1990; Rivas et al., 1990;Prabhudeva and Menon, 1991 a & b; Manoharan, 1991 Mersch et al., 1992; Busch et al., 1992;Prabhudeva and Menon 1993). Bioaccumulation capacity of marine animals have been reviewed by Bryan (1979; 1984); Phillips (1980) and Simkiss et al., (1982). This bioaccumulation in tissue occurs as a consequence of ingestion of inert or living particles contaminated with trace metals (Amiard et al., 1991). Uptake of metals is accompanied by simultaneous elimination of metals and it is when rate of uptake exceeds elimination that accumulation occurs . These toxicants are concentrated in the tissues at a concentration many time higher than ambient waters (Buikema Jr. et al ., 1982).

A number of factors influence metal uptake from the environment (Moc and Neilson, 1991). The well defined factors are environmental factors on metal chemistry in sea water, cellular homeostasis mechanisms, duration of metal exposure, mode of entry and route of uptake and storage of metals. (Harrison,

1979; Bouquegneau and Gills, 1979; George and Viarengo, 1985). The rate of elimination is equally important because its biological half life in the animal can be determined, which can serve as a warning about the persistence and potential for cumulative biological effects of a chemical (Buikema et al., 1982).

Accumulation and depuration studies on heavy metals have been well documented in literature. Pasteels (1968) demonstrated the presence of two metalloproteins , ferritin and peroxidase pinocytosed in the gill epithelia.

Ishi et al., (1985 b) reports the presence of ferric, copper and sulphur in granules of oyster tissue. It was also observed that spherical granules of trace metals appear extracellularly by the side of microvilli and develop into larger granules while moving into the lumen of the kidney. Viarengo et al ., (1988) reports copper to accumulate as insoluble copper thioneins in digestive gland lysosomes in Mytilus galloprovincialis.

Silver is found to accumulate in two forms in gastropods (Martoja et al., 1985), when less abundant it accumulates as silver-protein and when more abundant as sulphide in lysosomes. Electron probe microanalysis of digestive glands of scallop showed two types of cells which could store elements like cadmium, molybdenum, boron, zinc, copper, iron, calcium, sulphur, manganese (Dufrancais, 1985).

In estuarine bivalve, Villorita var cochenensis it is reported that metals like copper ,zinc and cadmium were found to accumulate more in abundance in soft tissue (Babukutty and Chacko, 1992), while lead, nickel and silver have been accumulated in bivalve molluscs around the coasts of U.S. That the eastern oyster, C. virginica is able to monitor levels of nine heavy metals along the coast lines is reported by Lytle and Lytle (1990). An illustrative account of the different factors influencing bioaccumulation of heavy metals collected from the coast of Taiwan is given by Huns-Tsu-Chang (1988). Contrary to the belief that coastal population are more contaminated than off shore populations the reverse was reported by Kroencke (1987). Piskwell and Steinert (1988) reports the accumulation of tin in digestive glands of oysters and mussels . Langston and Zhou (1987) while studying the accumulation -depuration of cadmium in bivalve, Macoma balthica has stated that either metallothioneins or metallothionein like proteins are involved in the elimination of the metal. Similarly, Chabert (1984) states that bioaccumulation of cadmium is high during early days followed by a steady state and finally a low rate. Vincent et al.(1988) gives a detailed account of accumulation of cadmium in relation to time , dosage and temperature of sea water along with structural modification of gill and digestive gland . Salinity is an important factor in metal accumulation (Wright and Zamuda, 1987; Sivadasan, 1987). Seasonal variations is another factor influencing metal accumulation in V. cyprinoides , P.

viridis and M. casta (Lakshmanan and Nambisan, 1983). Temperature has profound influence on rate of metal accumulation in bivalves especially copper (Regoli et al.,1991). Simkiss and Watkins (1988) have noted that oscillating temperature induces metal fluxes and enhances bioaccumulation .

While studying trace metals in sediments and bivalves from a large river estuarine system, salinity appeared to have secondary effects on metal concentration in sediments and bioavailability to bivalves. In C. viriginica and Mya arenaria it was noted that copper accumulation was inversely proportional to salinity and positively related with total copper and copper ion activities. Variable salinity influenced bioaccumulation of copper by Sunetta scripta (Latha et al., 1982). It has been stated that a certain relation exists between the level of metal in water and level in tissues (Phillips, 1980; 1976). Rate of uptake was found to have linear relation to concentration in water (Nambisan et al., 1977). Sexual maturity and spawning is also believed to influence accumulation of metals like zinc, copper, cadmium and iron (Coimbra and Carraca, 1990).

Metal interactions play a profound influence in accumulation . It can be antagonistic , synergistic or additive. The effect of these interactions on accumulation and their effects on the individuals are reported by Sprague and Ramsay (1965); Eaton (1973); Mac Innes (1981); Phillip (1990); Murukami et al.,(1976); Mohan et al.,(1986a) ; Baby (1987); Negilski (1981); Prabhudeva and Menon (1990);Menon,(1993).

Metallothioneins also play a very important role in accumulation (Viarengo, 1980 ; Roesijadi and Hall, 1981; Viarengo et al., 1985; Harrison and Lam, 1985; Beblamo and Langton, 1990).

The effect of heavy metal can be expressed in terms of alterations in the biochemistry of different subcellular compartments. The more pronounced reactions can be noticed on 1) Plasma membrane 2) Intermediate metabolism due to interactions with cytosolic components 3) changes in nuclear metabolism 4) Significant increase in low molecular weight proteins like Metallothioneins 5) Structural and biochemical changes in lysosomes. 6) Changes in protein and ATP synthesis.

The objective of the present study was to compare the metal load accumulated in the tissue of animal, P. viridis subjected to a long term exposure of four weeks and a short term exposure of one week. The load of metal retained within the tissue of animals exposed to toxicants for a week, followed by a week depuration's has also been found out to get an insight into the time taken for the elimination of half the accumulated metal in tissue. The fact that metals in solution can have more pronounced effect on the organisms living in it than the metal comtaminated food given to it is also proved . Finally a simple staining process for the detection of metals accumulated has also been carried out.

V.3 MATERIALS AND METHODS

This part of the experiment centred around obtaining information on the quantity of metals, copper and mercury , accumulated within the tissue as a result of exposure to sub lethal concentrations of mercury and copper individually and in combination for a period of four weeks and one week followed by one week depuration . The load of metal accumulated in the whole tissue of animals exposed to metal contaminated algae was also worked out. Finally , an illustration of the metal accumulated in the gastric tubules and gills of these exposed animals have been done by a simple staining technique.

The details of test animals, test media, laboratory conditioning of animals , toxicants, nature and duration of exposure was as mentioned earlier.

After the experiment three mussels each were taken to determine the total soft tissue content of copper by F.A.A.Spectrophotometer. The mercury concentration in the exposed mussel was estimated using Mercury Analyser.

Digestion procedure for Copper

The digestion procedure for copper is basically that of Martincic *et al.* (1984). Tissue samples were placed in 100 ml Kjeldahl flask covered with glass funnel. To a known weight of known sample 5-10 ml concentrated $HnO_3$and $HClO_4$ were added. After

preheating digestion for a period of 3 - 6 hrs. flasks were gently heated to avoid pumping and the heating continued until the organic matter was completely destroyed. This was indicated by a clear solution in flask. Lower or higher sample weights require less or more HNO_3 and $HClO_3$. The solution was cooled and diluted to a specific volume.(25 ml).

The copper concentration was determined in a Perkin Elmer 2830 model Flame Absorption Spectrophotometer using air acetylene flame. The samples were aspirated directly into the flame and corresponding readings were noted. Standards of metal solutions were prepared using A.R. grade $CuSO_4$ $.5H_2O$.

Digestion procedure for Mercury.

The digestion procedure for mercury was that of BITC 1976; Pentreath(1976).

A known weight of tissue sample was weighed and taken in oxidation flasks of Bethge apparatus. 2.5 ml of a cold mixture of concentrated HNO_3 and concentration. H_2SO_4 in ratio of 4:1 (V/V)was added to the flask . It was heated cautiously at first collecting the distillate in the reservoir. When the mixture starting darkening a little of the distillate was run from reservoir to flask. The procedure was continued maintaining a slight excess of HNO_3 acid in oxidation flask;until solution ceased to darken and fumes of H_SO were evolved. The solution was allowed to cool and made upto 50 ml. using distilled water.

Mercury was analysed by cold vapour absorption technique For this a Mercuric Analyser was used. $SnCl_2$ was used as reducing agent. Air free from mercury was used as carrier gas. A standard graph was prepared from standard $HgCl_2$ solution.

From the readings metal concentrations, in tissue samples were calculated and expressed in con. per gm. body weight.

V.3.2 Accumulation studies on *P. viridis* exposed to metal contaminated algae

A known volume of algal solution (250 ml) was filtered and the residue containing algal cells was transferred to 250 ml of 10 ppb copper solution. The solution (stock solution) was well shaken and kept for 12 h.

About 10 mussels of size 35-40mm. and previously acclimatised to laboratory conditions as mentioned earlier , were exposed in fiber glass troughs of capacity 10 litres, containing five litres of fully aerated sea water, to which a known quantity of stock solution (10 ml) was added.

The experiment was repeated in triplicate for two days (48 h). A set of control animals was also maintained. Water was changed every day to avoid contamination by pseudofaeces.

After the experiment, animals from all the troughs were taken and subjected to histochemical studies as mentioned

earlier. Bioaccumulation of copper in the exposed and control animals were measured after determining the metal content of the soft tissue by Flame Atomic Absorption Spectrophotometer.

V.3.3 Staining technique for metal localisation

For histochemical studies relating to metal localisation the following embedding and staining procedures were adopted. Chemical composition of the fixative used:-

Carnoy sodium- sulphide mixture

Ethanol 70% - 80 ml

Chloroform - 15 ml

Glacial acetic acid - 5 ml

to which 500 mg sodium sulphide crystals were added.

Fixative was kept in well stoppered dark bottle in refrigerator.

1) The tissues were left in the fixative overnight(12hrs).
2) The tissues were then dehydrated in changes of absolute ethanol for 3 hrs.
3) The tissues were then cleared in xylene, and then embedded in paraffin wax.

Ribbons of 8 µmm thick serial sections were cut from these blocks. Mounted slides were prepared using these ribbons. However, half the number of slides were prepared using ribbons which were exposed to moist hydrogen -sulphide gas for 30

mins. before spreading them on albuminised slides. The rest were prepared using those ribbons which were normal or unexposed to hydrogen sulphide.

4) Slides were then hydrated and subjected to treatment with Timm developer solution.(90mins.)
5) Slides were then taken out , washed thoroughly in distilled water and counterstained in Mayer's haem-alum and cytological stain like Eosin.
6) Slides were then dehydrated in ethanol grade , cleared in xylene and mounted in D.P.X.

V.4 RESULTS

V.4.1 ACCUMULATION STUDIES

A series of experiments were conducted to study the effects of varying concentration of copper and mercury on rate of accumulation and depuration by P.viridis. Copper sulphate and mercuric chloride were the source of copper and mercury. Two series of experiments were conducted of different durations. One series involved experiments which lasted for one week , during which animals were left in varying concentrations of copper and mercury and the animals were sampled for total body burden at the conclusion of the experiment.-

TABLE 14. *Perna viridis* : Accumulation :- Concentration in ppb of copper (as copper sulphate) in whole tissue (ug g^{-l} dry wt.) after animals were exposed to copper (individually for a period of one week.

Treatment (Copper)	Replicates			Mean	SD *
Control	11.7	11.8	11	11.5	0.36
10	379.1	379.92	380.80	380.0	0.68
20	233.6	233.5	234.9	234.0	0.66

TABLE 15. *Perna viridis* : Accumulation :- Concentration in ppb of mercury (as mercuric chloride) in whole tissue (ug g^{-l} dry.wt) after animals were exposed to mercury (individually) for a period of one week.

Treatment Mercury	Replicates			Mean	SD *
Control	Undetectable			--	--
20	9.4	9.92	9.52	9.63	0.22
50	190	190.2	189.38	189.866	0.34

* SD - Standard Deviation

TABLE 16. *Perna viridis* : Accumulation:- Concentration in ppb of copper (as copper sulphate) in whole tissue (ug g^{-1} dry. wt.) when animals were exposed to copper (individually) for four weeks.

Treatment (copper)	Replicates			Mean	SD *
Control	11.7	11.8	11	11.5	0.36
10	43.61	42.79	44.1	43.5	0.54
20	44.4	45.1	44.5	45	0.45

TABLE 17. *Perna viridis* : Concentration in ppb of mercury (as mercuric chloride) in the whole tissue (ug g^{-1} dry wt.) when animals were exposed to mercury (individually) for four weeks.

Treatment (mercury)	Replicates			Mean	SD *
Control	Undetectable			--	--
20	187.4	187.9	188.8	188	0.54
50	390.8	391.7	392.3	391.6	0.62

* SD - Standard Deviation

The rate of accumulation of copper by P.viridis on exposure to ten and twenty ppb copper is presented in Table -14. The conspicious feature of rate of accumulation was that the animals accumulated more copper when the external concentration was low. The difference in the rate of uptake was about 40 % which resulted in reduced load in the tissue of those animals which encountered 20ppb copper.

In another series of experiments animals were exposed to, four weeks under the same concentration . The rate of uptake was very slow. which resulted in lower levels of copper after four weeks. There was no difference in loads accumulated in tissue between animals exposed to,twenty and ten ppb copper. The quantity accumulated was only around 20% of what was accumulated by the same animal when exposed for one week.

In the case of mercury , animals maintained in the media containing 20 and 50 ppb, the rate of accumulation was drastically different. Those kept in 20 ppb accumulated a quantity which was about 20 times less than those exposed to 50 ppb.

On the contrary , those animals which were exposed to the same concentration of mercury in medium for four weeks showed a uniform rate of uptake. Animals kept in 50 ppb mercury accumulated around two times the quantity accumulated by those kept in medium containing 20ppb mercury.

The rate of accumulation of mercury when they were

TABLE 18. *Perna viridis* : Accumulation:- Concentration of mercury (as mercuric chloride) in whole tissue (ug g^{-1} dry.wt) when animals were exposed to mercury in combination with copper for a period of one week.

Treatment	Replicates			Mean	SD *
10Cu + 20Hg	2.1	2.2	1.7	2	0.22
10Cu + 50Hg	161.72	161.9	161.24	161.61	0.28
20Cu + 20Hg	63.5	64.8	63.7	64	0.37
20Cu + 50Hg	14.49	13.4	14.95	14.28	0.65

TABLE 19. *Perna viridis* : Accumulation Concentration in ppb of mercury (as mercuric chloride) in whole tissue (ug g^{-1} dry.wt) when animals were exposed to mercury in combination with copper for a period of four weeks.

Treatment	Replicates			Mean	SD *
10Cu + 20Hg	3.8	3.6	4.6	4	0.43
10Cu + 50Hg	3.0	2.4	2.1	2.5	0.37
20Cu + 20Hg	140.7	142.0	141.8	141.5	0.57
20Cu + 50Hg	92.2	94.4	94.4	94.0	0.57

* SD - Standard Deviation

TABLE 20. *Perna viridis* : Depuration:- Concentration in ppb of copper (as copper sulphate) in the whole tissue (ug g^{-1} dry wt.) of animals when exposed to raw sea water for a period of 7 days after being exposed to copper (individually) for a period of 7 days.

Treatment (copper)	Replicates			Mean	SD *
Control	11.7	11.8	11	11.5	0.36
10	150.1	150.8	152.1	151	0.83
20	90.7	91.3	91	91	0.44

TABLE 21. *Perna viridis* : Depuration:- Concentration in ppb of mercury (as mercuric chloride) in whole tissue (ug g^{-1} dry wt.) of animals when exposed to raw sea water for a period 7 days after exposure to mercury (individually) for a period of seven days.

Treatment (mercury)	Replicates			Mean	SD *
Control	Undetectable			--	--
20	8.75	7.3	7.5	7.85	0.64
50	140.62	141.1	140.5	141.41	0.89

* SD - Standard Deviation

introduced in combination are presented in Tables 18-19. Rate of accumulation of mercury was substantially high when this was applied in combination with copper during the first seven days. On the contrary , when animals were retained for 28 days ,in the medium containing 20 and 50 ppb mercury , the quantity of mercury accumulated in the tissue was very low.

The picture was totally different when 20 and 50 ppb mercury was supplied along with 20 ppb copper . Here it was noticed that in seven days time high levels of copper accumulated brought about low accumulation of mercury , when the medium contained 50ppb mercury. Prolonged exposure in the same concentration lasting for 28 days resulted in constant increase in mercury concentration in the tissue.

V.4.2 DEPURATION

To find out the rate at which mercury was depurated from the tissue by P. viridis when the metal was applied singly or in combination with copper , a set of experiments were conducted by exposing the animal to raw sea water for seven days.

The quantity of copper thrown out from the tissue was not found to show any relation with the internal concentrations. The copper levels was reduced from 381-151 $\mu g\, g^{-1}$ after seven days.

Similarly the body burden of 234 $\mu g\, g^{-1}$ of copper was reduced to 91 $\mu g\, g^{-1}$ The biological half life ie. the time taken to reduce the internal concentration by 50 % through biological process was

TABLE 22. *Perna viridis* : Depuration:- Concentration of mercury (as mercuric chloride) in whole tissue (ug g^{-l} dry wt.) of animals when exposed to raw sea water for one week after exposure to mercury in combination with copper for one week.

Treatment	Replicates			Mean	SD *
10Cu + 20Hg	1.2	1.1	2.2	1.5	0.50
10Cu + 50Hg	114.24	114.6	115.98	114.94	0.75
20Cu + 20Hg	51.1	52.1	52.8	52	0.70
20Cu + 50Hg	12.5	13.2	13.3	13	0.36

TABLE 23. *Perna viridis* : Number of days required for half the accumulated metal copper (as copper sulphate) to be lost as a result of biological processes.

Treatment (copper)	Conc. of metal accumulated in whole tissue (ug g^{-l} dry wt.) after 7 days	Conc. of metal accumulated in whole tissie (ug g^{-l} dry wt) after depuration of 7 days.	Half life
10	380	151	5.2
20	234	91	5.13

* SD - Standard Deviation

TABLE 24. *Perna viridis* : Number of days required for half the accumulated metal mercury as mercuric chloride to be lost as a result of biological processes.

Treatment	Conc. of metal accumulated in whole tissue (ug g^{-1} dry wt.) after 7 days	Conc. of metal in whole tissue (ug g^{-1} dry.wt) after depuration for 7 days	Half life
20Hg	9.615	7.85	24
50Hg	189.866	141.414	16.48
10Cu 50Hg	161.62	114.94	14
20Cu 20Hg	64	52	28
20Cu 50Hg	14.28	13	52

TABLE 25. *Perna viridis* : Accumulation : Concentration in ppb of copper (as copper sulphate) in whole tissue of animals fed on copper dosed algae for 48 hours.

Treatment (copper)	Replicates			Mean	SD *
Control	11.7	11.8	11	11.5	0.355
10	12.9	12.6	12	12.5	0.37

* SD - Standard Deviation

around 5 days in both the cases.

In the case of mercury the rate of depuration was very slow irrespective of internal concentration. Reduction in concentration of mercury in tissue was rather quick when the internal concentration was high. Seven days depurative process resulted in only a slight lowering in the internal concentration of mercury. On the other hand , those animals maintained in 50 ppb mercury depurated mercury at a higher pace. This resulted in different biological half life which was 24 days in the former case and 16 days in the latter.

The rate of depuration of mercury when the external medium contained both copper and mercury was also analysed. A striking feature of the results obtained was ,very low rate of depuration of mercury when tissue concentration of mercury was low. Thus the biological half life of mercury was found to shift drastically and a minimum period of 14 days was recorded when the internal concentration was comparatively high. The rate by which mercury was depurated was very slow , when internal concentration was low. Animals which contained 14$\mu g g^{-1}$ of mercury depurated only 1$\mu g g^{-1}$ in seven days time; biological half life stretching to 52 days.

V.4.3 ACCUMULATION OF COPPER IN P.VIRIDIS FED ON COPPER CONTAMINATED ALGAE

An experiment was performed to find out accumulation of copper by P.viridis when animals were fed with copper

TABLE 26. S.Salina : Accumulation : Concentration in ppb of copper (as copper sulphate) in copper dosed algae in ug g^{-l} wet wt.) for a priod of 48 hours.

Treatment (copper)	Replicates			Mean	SD *
10	111	112.4	112.6	112.4	0.71

* SD - Standard Deviation

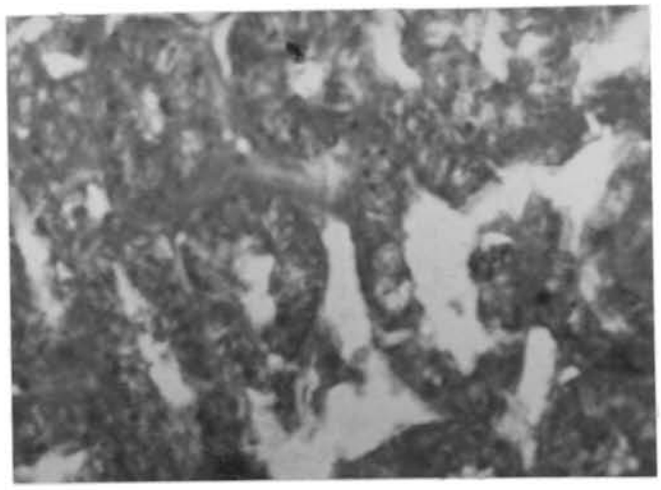
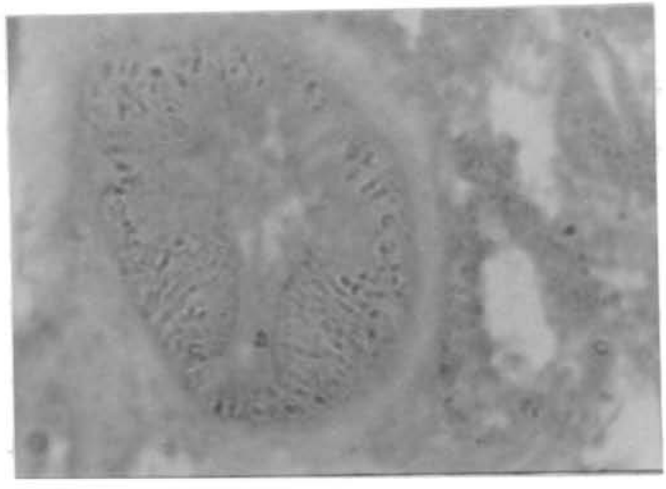


Fig. 1 *C.S. of Digestive tubules of P. viridis fed on copper contaminated algae showing reduced lipofuscin granules x 400.*

Fig. 2 *C.S. of Digestive tubules of P. viridis fed on copper contaminated algae showing absence of metal granules x 400*



contaminated S. saline .

The cells were suspended in medium containing 10 ppb copper for 48 hours. The algal cells containing absorbed and adsorbed copper were given as feed for P.viridis. Even after 48 hours the uptake of copper was found to be low. Tissue sections of P.viridis fed on copper contaminated algae when stained for metal localisation did not give satisfactory results. On the other hand, lipofuschin activity was noticed in the gastric tubules.

V.4.4.METAL LOCALISATION

A histological method was adopted to find out metal containing granules in gills and gastric tubules of P. viridis. The photomicrographs of tissue examined for metal granules are presented in this section. The digestive tubules of 20 ppb copper exposed animals showed the presence of dense granules in the basal lamina as well as in the basophilic and acidophilic cells. The fact that wandering cells also contained metal granules probably indicate that they are dislodged cells.

In the case of those animals exposed to 20 ppb mercury extensively damaged gastric tubules contained numerous metal containing granules. The presence of 50 ppb mercury in the culture medium resulted in copious production of metal laden granules by Perna viridis in four weeks time. A combination of copper and mercury, however, did not result in such conspicious

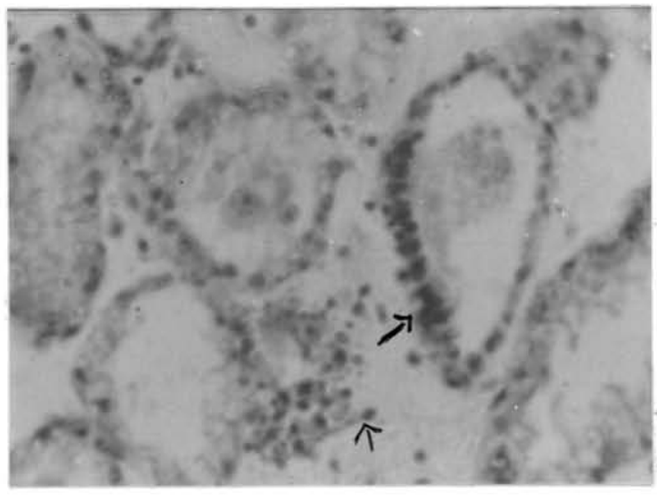
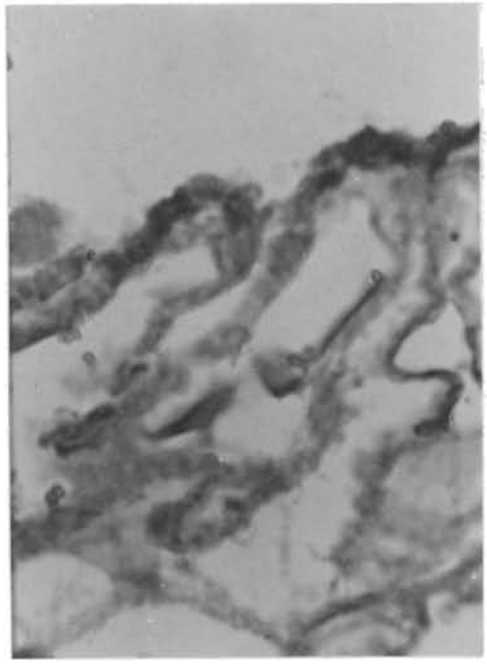


Fig 1 (a) *C.S. of Digestive tubules of P. viridis exposed to 20 ppb Cu for 4 weeks showing accumulation of dense metal granules in basal lamina and dislodged cells* ×400

Fig 1 (b) *C.S. of gills of P. viridis exposed to 20 ppb copper showing accumulation of metal granules* ×400



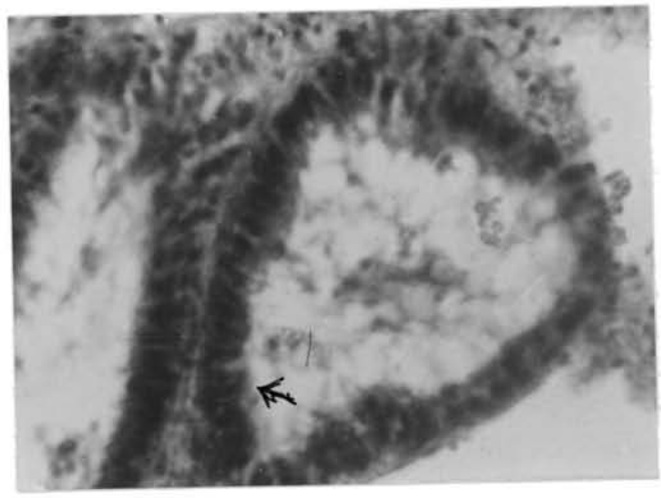
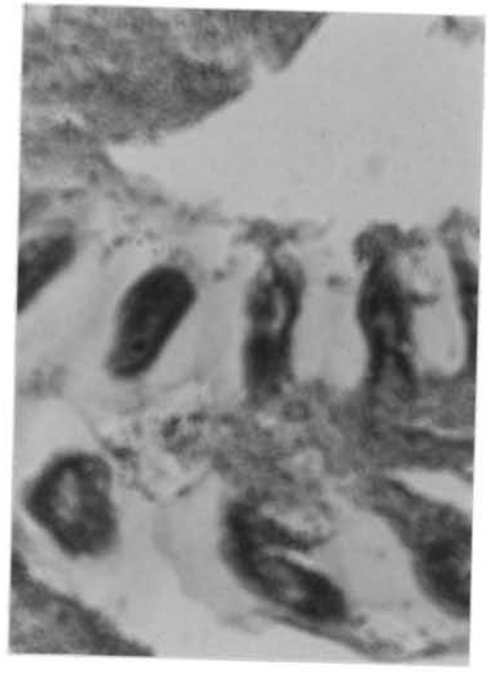


Fig 2 (a) C.S. of Digestive tubules of *P. viridis* exposed to 50 ppb Hg for 4 weeks showing densly laden metal granules. x 400

Fig 2 (b) C.S. of gills of *P. viridis* exposed to 50 ppb Hg for 4 week showing dense accumulation of metal laden granules. x 400



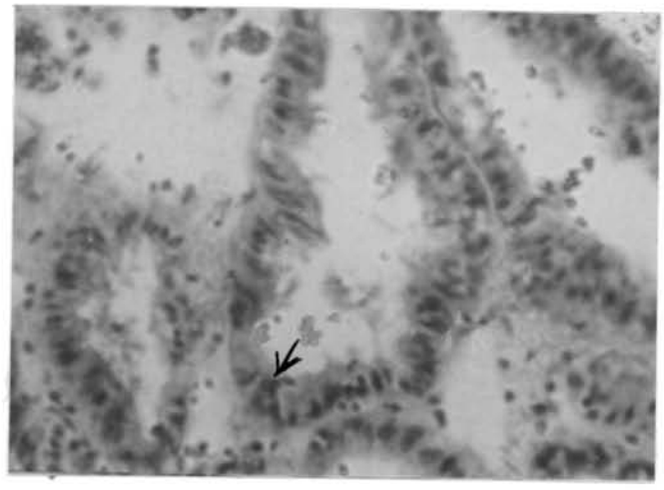
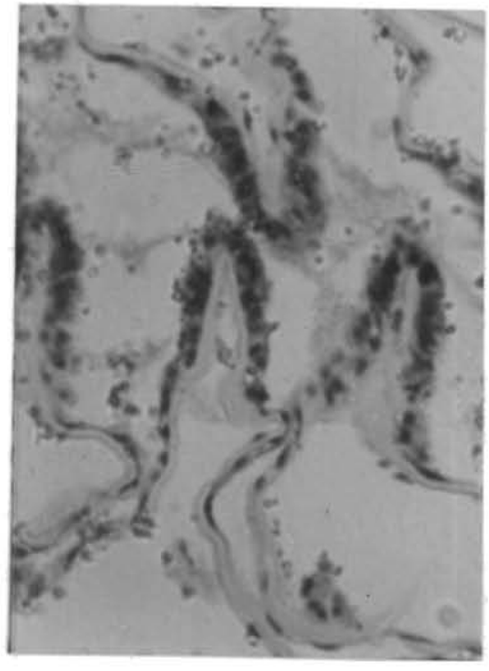


Fig 3 (a) *C.S. of Digestive tubule of P. viridis exposed to combination of 10 ppb Cu and 50 ppb Hg showing presence of dense metal granules in basal lamina and epithelial cells* ×400.

Fig 3 (b) *C.S. of gills of P. viridis exposed to combination of 10 Cu and 50 Hg showing metal laden granules x 400.*



development of metal containing granules. Of the four combination employed only those animals maintained in the culture media which contained 10 ppb copper and 50ppb mercury contained had digestive tubules with basal lamina, acidophilic and basophilic cells containing metal granules.

In the case of gills also similar metal containing granules were identified. However, deeply stained granules were noticed for their preponderance when the animals were exposed to 20 and 50 ppb mercury and combination of these two.

V.5 DISCUSSION

Experimental studies have shown that bivalves have a capacity of accumulating considerable quantities of heavy metals in their tissue. Authors studying the kinetics of mercury in bivalves maintained under laboratory conditions have found small individuals responding rapidly to ambient levels of mercury . Cunningham and Tripp(1975b), Fowler et al. (1978) found that uptake of mercury from solution by smaller individuals is much more rapid than in larger ones in the case of C. virginica and M. galloprovincialis . Miehnen et al.(1969) found that rate of mercury lost is also faster in the case of smaller individuals . Commenting on this aspect , Phillips (1980) remarked that smaller individuals, time integrate ambient mercury levels over a

shorter period compared with large bivalves of the same species. It is likely that since the rate of uptake of mercury when present in water is controlled by tissue water contact ratio , the smaller organism will have a relatively higher surface area of contact which resulted in increased uptake . It is not clear whether this would be so in the case of metals where the main route is through food and other suspended solids .

Drastic internal concentration difference was noticed in the rate of uptake of mercury when present individually. The increase in uptake was nearly 20 fold when there was a doubling of concentration in culture medium . Biologically most available state of metals in sea water are still uncertain but include organic complexes of mercury and free ions of some metals In most invertebrates water movements come to near or within the body have some relevance on metal uptake.

Present findings show that prolonged exposure does effect the uptake of mercury. In four weeks time the increase in mercury concentration was nearly 20 times more than that of animals exposed for one week. On the contrary, increase in concentration did not show concomitant rise in the case of those animals exposed to 50 ppb mercury. Baby(1987) exposed P. indica to very low concentrations of mercury for twenty one days and based on uptake rates he has identified three distinct uptake rates. In the case of those animals exposed to 4 ppb of mercury the trend was found to be true as identified by Roesijadi (1982) in the case of M. edulis. However, in the case of M. edulis the three

phases were better exemplified in the rate of accumulation in gills .Roesijadi (loc cit) attributed accumulation of mercury in phases to changes in outward transport ie. through incorporation in other tissues and organs . Cunningham and Tripp (1975) and Roesijadi and Hall (1981) suggested internal organ transport of mercury from gills. However, the findings of Baby,(1987) showed intermediate transportation of mercury into other tissues or seemlier trends in uptake by other tissue, since the concentration by gill, mantle and other tissues showed comparative trends after seven days (Baby and Menon, 1993 in press). Commenting on uptake of mercury by gills and whole tissue by P. indica in the presence of cadmium and zinc , Baby and Menon (1993) found that presence of cadmium did not have any influence on the net uptake of mercury while that of zinc did. It was clearly proved in the present study that at relatively high concentrations of copper the uptake of mercury came down. In the presence of 5 $\mu g/L$ zinc with 1 $\mu g/L$ mercury, the load of mercury was less in both gills and whole tissue . The inhibition became very evident from seven days onwards. The rate of depuration was found to be controlled by the internal load of mercury. This is in confirmity with the findings of Baby and Menon(1993) in P. indica. Similar findings are available in literature also. Ashanullah, et al.,(1981) observed that cadmium and copper enhanced accumulation in zinc by Callianasah australianensis , where the presence of zinc and cadmium decreased the net uptake of copper. Jackim et al.(1977) reported that the presence of 500 $\mu g/L$ of zinc reduced accumulation of

cadmium in M. edulis while Fowler and Benayoun (1974) showed that zinc at 180 µg/L had no effect on accumulation of cadmium by M. galloprovincialis. Elliot et al.(1986) concluded that cadmium accumulation by marine animals is retarded when zinc is present in high concentrations.

Triquet et al.(1986) proved that ability of mussels to limit the bioaccumulation of copper and zinc varied from organ to organ and decreased with higher level of contamination and longer periods of exposure. This is in confirmity with findings of the present study where an increase in copper concentrations did not show any increase on prolonged exposure . As a matter of fact the body burden of copper was found to be drastically reduced on prolonged exposure. It is interesting in this context the opinion of Triquet et al.(1986)where it is stated that even at highest sublethal levels of copper in mussels tissue were not much higher than that in natural levels. This opens up an interesting aspect of employing mussels as bioindicators of heavy metal pollution. Since in the case of essential metals on short term exposure the levels of trace metals in organisms could be largely independent of their concentration in the ambient sea water.

Another very interesting finding of the present study was that related to the upake of mercury in the presence of copper. It was noticed that uptake of mercury was practically stopped in the medium of 10 ppb copper, whereas increase in copper in the medium and duration of exposure resulted in higher uptake of

mercury. On the contrary, presence of 20 ppb copper and 50 ppb of mercury resulted in a very low uptake of mercury. Therefore, it is clear that the chemical nature of heavy metals, concentrations and duration of exposure will have an important role to play on the uptake process of metals by P. viridis.

It was found that the uptake of mercury in the presence of selenium changed the distribution of mercury in Astrea rubens(Sorenson and Bjerregard,1991)). Mainly mercury was found to get accumulated in the peripheral tissues. Similarly on exposure to mercury,augmented selinium concentration in M. edulis (Pellitier 1986; Sorenson and Bjerregaard 1991), however,no effect was noticed in the case of A. granosa(Patel et al., 1988).

Prabudev and Menon (1993) found that in the case of P. indica the gastric diverticula accumulated maximum concentration of copper . The rate of accumulation was found to be controlled by external concentration, only when this exceded a limit. This is in confirmity with the present result and that of Delhai and Cornet (1975), Simkiss et al.,(1982). Discussing on the rate of depuration of copper the same authors stated that those animals which were allowed to depurate after being left in media containing low concentration of copper, the rate was slow. This was specially so when the period of depuration was seven days . The findings of Phillips (1976 a and b) that copper concentrations in mussels varied with seasons,mainly because of wet weight changes caused by build up and loss of gonadal materials need not neccessarily reflect normal depuration rate of

mussels. Phillips (loc cit) hypothesised that there might be specific copper transporting sites which have evolved because of biological importance of copper to Mytilus. It is interesting to note in this connection the findings of Langston and Zhou(1987) that, accumulation distribution and elimination of cadmium in Macoma balthica was independent of presence of metallothioneins and metalloproteins . Prabhudeva and Menon(1993) very clearly established the influence of external metal concentration on accumulation. It was proved that there could be clear cut concentration dependant depuration and uptake when to external concentration varied between .5-4ppb. Prabhudeva and Menon loc cit proved that irrespective of previous accumulation history the quantity of copper thrown out was always more when the body burden was high. They also proved irrespective of salt forms of metals the rate of depuration was controlled by actual quantity of copper rather than the salt forms.

Very little information is available on the rate of accumulation and depuration in heavy metals when the animals are exposed to combinations of heavy metals. Prabhudeva(1987) found that in the presence of mercury the uptake of copper was reduced. Eliot et al. (1986) working on metal combination uptake found that cadmium accumulation can be influenced by presence of zinc. Negliski et al. al. (1981) found that both cadmium and zinc reduced copper accumulation in shrimp Callianassa australianensis. Phillips (1976 b) found reduction in copper in presence of zinc in M. edulis . According to Devineau and Triquet

(1985), antagonistic interactions between metals can influence the process of biological uptake . Moulder(1980) found that in the presence of copper in the medium resulted in a significant reduction in mercury uptake in G. dubeni. The present findings showed that the presence of copper reduced the uptake of mercury when copper concentration ranged from 10-20 ppb. This was especially so when copper concentration was 20 ppb . This must be the result of antagonistic interaction between copper and mercury. Discussing on the accumulation and depuration of copper Viarengo et al. (1985) demonstrated an increase in the level of copper binding proteins during the first 6 days of detoxification The increase regained normalcy only after 12 days. During the present study it was proved that prolonged exposure to low levels of copper does not result in increased accumulation of copper. This must be an indication of the adaptive mechanism, in Perna to regulate the uptake of copper in the presence of realistic concentration of copper in culture medium .

Results on the metal localisation obtained during the present investigations suggest the view that mussels have the capacity of storing metals in granular form . Biochemical and electron microscopic studies have shown that 80 % of iron in digestive gland is stored in inorganic granules in the case of Abran (Hyne, 1992). Similarly granules containing iron, copper and phosphate were found in kidney also. Simkiss and Mason(1983) opined that in gastropods metal containing granules accumulated in digestive glands. Digestion of food provides the main source of metal uptake and both digestive glands and kidney provide a

route for excretion of granules . Cyclic uptake and release of copper , zinc and mercury from granules in cells in the digestive glands of shrimp , P.semisulcatus and M. dobsoni have been reported by Al-mohanna and Nott(1989) and Mary(1993).

SUMMARY

The thesis is essentially presented as seven chapters among which three chapters cover histopathology , histochemistry and accumulation and depuration rates of heavy metals. The preface highlights the relevance of the study.

The general introduction deals with aspects connected with heavy metal toxicity in relation to structural and functional aspects of biology of bivalves.

The chapter on histopathology gives concise information on the present status of histopathological studies on intoxicated species of mussels by way of introduction and review of literature. Important works relating to histopathology which have direct relevance to the present study are reviewed and cardinal findings highlighted. The materials and methods used are explained under the concerned section. The highlights of the results obtained from histopathological alterations are the differences between extent of damage of gills and gastric tubules in animals experiencing different concentrations of heavy metals for varying periods and variations in area of damage. The results are discussed in the light of available literature .

The chapter on histochemistry contains information on the present status of this relatively recent biomonitoring method, developed and supported by a school of thought . Essentially the methods involve identifying qualitatively and to some extent

quantitatively by staining and image analysis, the occurrence of biochemical material which are indices of stress reactions at tissue levels. Certain biochemical components essentially lipids have been identified as biomarkers to distinguish stress profiles. Many recent publications are available wherein information on the findings based on histochemistry to prove the relevance of such studies . This chapter explains the materials and methods and results obtained . Important findings were variation in lipofuschin activity of tissues of animals exposed to copper and mercury. It seems that the methods are most reliable to identify copper stress brought about by mercury. The results are discussed in the light of available literature.

The capacity of bivalves to accumulate heavy metals have been well understood. Therefore, analysis of body burden of marine bivalves exposed to heavy metals with reference to concentration have been utilised to understand the relationship between exposure and rate of accumulation. The total load in the tissue is estimated to analyse the relationship between concentration and duration of exposure. Numerous papers are available on the rate of accumulation and depuration of heavy metals by marine bivalves of tropical and temperate regions. Exhaustive literature is reviewed and presented in this chapter. The standard procedure employed to assess the heavy metal concentration in tissue is given. The most important finding was that exposure to realistic concentration of copper and mercury results in increased uptake of these metals , when the duration

of exposure was one week. However, continuous exposure to copper did not result in concomitant rise in copper load even after 4 weeks. On the other hand , clear cut increase in mercury load was found to occur when the animals encountered mercury in the medium for 4 weeks. The presence of copper along with mercury was found to influence both the rate of uptake and depuration of mercury by P. viridis. It was found that staining procedures could be employed to find out whether there is any localisation of containing heavy metal granules in the tissue. The results are discussed in the light of information available in the literature. Wherever necessary, tables and photographs are provided to exemplify the results and assumptions .

The chapter on references lists out various papers consulted during the course of the study.

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