STUDIES ON THE EFFECT OF TOXIC HEAVY METAL MERCURY ON THE PHYSIOLOGY AND BIOCHEMISTRY OF AN ESTUARINE CRAB *SCYLLA SERRATA* (FORSKAL)

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In partial fulfilment of the requirements for the award of the Degree of

DOCTOR OF PHILOSOPHY IN ENVIRONMENTAL TOXICOLOGY UNDER THE FACULTY OF ENVIRONMENTAL STUDIES

By

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Certificate

This is to certify that the thesis entitled **"Studies on the effect of toxic heavy metal mercury on the physiology and biochemistry of an estuarine crab *Scylla serrata* (Forskal)"** is an authentic record of the research carried out by Ms. Shaganas Banu, B., under my supervision and guidance at the School of Environmental Studies, Cochin University of Science and Technology, in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Environmental Toxicology, under the faculty of Environmental Studies, 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 of any university.

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DECLARATION

I hereby declare that the thesis entitled **"Studies on the effect of toxic heavy metal mercury on the physiology and biochemistry of an estuarine crab *Scylla serrata* (Forskal)"** is a genuine record of the research carried out by me under the supervision and guidance of Dr. Rajathy Sivalingam., Reader, School of Environmental Studies, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.

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Shaganas Banu

Shaganas Banu, B.

Dedicated to

my loving family . . . . . .

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Pollution is the changing of a natural environment, either by natural or artificial means, so that the environment becomes harmful to the living things normally found in it. Most often than not, this refers to the input of toxic chemicals into the environment as a consequence of human activities. Pollution as a general term is associated with unfavorable alterations in the ecology, resulting in deleterious effects on human health and resources. It is an insidious and growing process, which manifests itself only when the outflow of effluents/insult exceeds the capacity of the receiving ecosystem of environment to recover. The various causes of pollution are the explosive growth of population, increasing urbanization, rapid industrialization, and indiscriminate use of fertilizers, chemicals, and pesticides, and lack of general awareness on environmental issues. Pollution results in human health hazards and destruction of aquatic food resources such as fishes, prawns, crabs, lobsters, molluscs and so on.

In recent years, concern has increased over the issues related to heavy metal pollution, as all heavy metals are potentially harmful to most organisms at some level of exposure. The release of increasing quantities of heavy metals and their salts into the terrestrial and aquatic environment, and their accumulation in living and non-living systems endangers the life of all living organisms. The nature of environment is of critical importance when considering homeostatic mechanism of an animal. Whereas terrestrial animals are generally exposed to metals

through their diets, or through the air they breathe, aquatic organisms are exposed to dissolved and particulate metals in the ambient medium.

Heavy metal pollution of estuarine ecosystems has become the focus of attention in recent years. Organisms living in estuaries are subjected to natural periodical fluctuations in the environment and are generally able to adapt to these environmental stresses (Spaargaren, 1974). However, increased demands placed on estuarine biota when industrial wastes and sewage are discharged into the estuaries may prove lethal if a sub- lethal pollutant is combined with a stressful environmental change.

The mechanism of metal uptake has not yet been fully elucidated. The available evidence indicates that metals cross the cell membrane essentially by a passive transport process although endocytosis is also suggested to take place (Viarengo, 1985). Studies by Simkiss (1983) suggest that the metal complex goes through biological membranes as a lipophilic compound. Moreover, in crustaceans, metals such as Hg are able to disrupt the ionic balance, altering the permeability, characteristics of the cell membrane. Thus, the effect of passive ion movements as well as the active transport process either by directly inhibiting the activities of Na, K- dependent ATPase or as a secondary effect by reducing the availability of ATP (Bouquegneau and Gilles, 1979) affect the physiology of the organisms. When metals cross the cell membrane, the metals react with cytosolic components, and are usually complexed in many ways (by sulphydrylic binding, chelation, salt formation etc.) to cytosolic compounds such as high affinity ligands, substrates, products of enzymatic activity, or enzymes themselves. The form of the heavy metal (ionic form, oxidized, reduced, complexed by organic substances, adsorbed to inorganic or organic particulate materials, acting singly or in combination with other cations) to which organisms are exposed is

extremely important in its overall toxicity to aquatic organisms and its uptake by them. Metals are taken up by aquatic organism usually across respiratory surfaces, and strongly bound by sulphydryl groups of proteins. Because of this ability, there is a tendency for them to be fixed in tissue and not to be excreted. In other words, they have a biological half-life. Metals also change the structure and enzymatic activities of proteins and cause toxic effects evident at the whole organisms level.

Certain organisms have long been known to accumulate pollutants from the ambient water. Many molluscs and crustaceans have been known to accumulate a wide range of metals. Different heavy metals may be accumulated by aquatic biota, sometimes at levels far above those found in the surrounding medium, thereby enabling the use of selected organisms for monitoring the levels of the metals in water bodies. They have several advantages over the classical methods of water or sediment analysis. The greatest advantage is that the biological availability of pollutant is measured directly. In addition to this, such animals would produce a time-averaged index of pollution availability. The much higher concentrations of pollutants in the body, compared with those in the surrounding water, make it easier to analyze the samples, and assess the more evident biochemical, histopathological, ionic balance, and enzymatic changes caused by pollutants.

Industries and the regulatory authorities concerned in collaboration with the environmental management group increasingly recognize the need for biological monitoring to know the changes, either deterioration or improvement, in environmental quality. Numerous and varied biological responses have been suggested as potential techniques for monitoring biological impact of waste discharged to the aquatic environment.

The present investigation will eventually open up a very interesting aspect of toxicology, the understanding of which would help gain a better knowledge on the effects of mercury on the physiological, biological and histological aspects of an organism.

Industrial effluents containing toxic heavy metals such as mercury, cadmium, lead, zinc, copper and arsenic are constantly discharged into our costal environment causing serious threat to aquatic organisms, and posing potential public health risk. It becomes thus necessary to understand the physiological and biochemical responses of the organisms living there, especially fishes, molluscs and crustaceans, to environmental contaminants such as heavy metals. This kind of study may help provide information on the status, trends, and sources of risk to the aquatic ecosystem.

There have been several studies on heavy metal pollution and their effects on aquatic organisms. Such studies have thrown information on the accumulation pattern of heavy metals in different tissues of the body, and their effects in the form of altered physiology of the organism as well as pathological changes in the tissues, organs and organ systems. The amount of the toxicants tends to get magnified even as they are transformed to the higher trophic levels, and in many a case man himself becomes the ultimate victim of pollution processes.

In the present investigation, the heavy metal chosen is mercury. Mercury is the only common metal that is liquid at room temperature.

In nature mercury occurs as metallic mercury and as HgS and $Hgcl_2$ in the earth's crust. Natural sources include volcanic action and erosion of mercury-containing sediments. Anthropogenic sources consist of mining, transporting and processing of mercury ores, dumping mercury containing wastes, exhaust from metal smelters, paper pulp, paints,

batteries, lamps, switches, caustic soda, medicine and instruments. The chlor-alkali plants consume twenty five percent of the total production of mercury, and the rest is used in electrical equipments, paints, thermometers, sphygmomanometers, dental products, and in agriculture. Methyl and ethyl mercury have been extensively used in seed dressings. The major sources of water contamination by mercury are effluents from chlor-alkali plants, which use mercury for producing chlorine and caustic soda. Application of mercury in agricultural purposes also contaminates water bodies. Over 70 mercury compounds are known to have been used to control seed-borne and soil-borne fungal diseases. They get washed with rain and irrigation waters and pollute the waterways. Mercury metals and mercury compounds are also used in a variety of industrial applications, and also as components of anti-fouling agents. Burning fossil fuels or sewage sludge, municipal waste, mercury-containing fertilizers are also important sources of mercury contamination. The release of Hg compounds from industrial liquid wastes has been shown to greatly influence the adjacent environment of the source. It is learnt that about 180 tons of mercury and mercury compounds are introduced into the Indian coastal environment per annum (Patel and Chandy, 1988), but by now the figures might have certainly gone up. Industrial effluents contain heavy loads of metals, which are toxic to aquatic organisms even at lower concentrations. The high toxicity of mercury compounds was known for a long time and its presence as a pollutant in many water bodies was also detected in India especially near chlor- alkali plants (Thangappan, 1972; Chandra, 1980). In recent years considerable research has been going on to study the effects of sub-lethal concentrations of heavy metals, particularly mercury, on the physiology of decapod crustaceans. Such studies are also useful to assess the effects of low concentrations of metals during prolonged periods of exposure, and also to assess the safe levels of pollutants in marine and estuarine water bodies. Gradual elimination of

valued species through prolonged exposure by low concentration of any pollutant may be more serious than instant kills, because in the former the symptoms are less obvious and have to be traced to the source on time to permit recovery of the environment and survival of the organism. This also helps in monitoring the quality of coastal waters and seafood for human consumption. In view of the above facts, protecting and preserving the environment from the perils of pollution has become an international concern.

Pollution by mercury is widespread in the aquatic environment, with a clear tendency toward an increase (Nriagu and Pacyna, 1988; Botton, 2000; Gonzalez et al., 2002). It is one of the common, persistent and toxic pollutants in the aquatic environment (Gonzalez et al., 2002). Freshwater and marine organisms and their predators normally contain more Hg than terrestrial animals. Crustaceans have been reported to accumulate heavy metals used as bioindicators (Perez, 1999;Chou et al., 2002). Particularly, many toxic effects have been reported in larvae, post larvae, and embryos (Mac Rae and Pandey, 1991; Selvakumar et al., 1996; Rajathy, 1991;Rodriguez and Medesani, 1994;Lopez Greco et al., 2002). The great affinity that mercury has to sulfur groups, like other heavy metals have, is well known, causing changes in the structures and functions of many proteins (Viarengo and Nott, 1993). On the other hand, mercury is a non- essential cation that tends as a consequence to progressively get accumulated in exposed organisms (Rainbow, 1988). Embryonic development, perhaps the most complex stage during the life cycle of an organism, can be affected by mercury through disruption of several processes; e.g., the inhibition of metabolic pathways and the assembling of microtubules and microfilaments, leading to failures in the energy balance, cellular differentiation, and cellular migration, among other events (Itow et al., 1998).

The pattern of toxicity of the metal varies with the chemical form in which it exists. Mercury mainly exists in three forms viz., elemental, inorganic and organic. Of these organic mercury, especially in the alkyl form of methyl or dimethyl mercury, is the most toxic because it is easily absorbed by the body and is deposited in vital organs like brain. The human body poorly ingests mercury as element, and lungs can weakly absorb mercury only in vapor (oxide) form. Mercury vapor can diffuse through alveolar membrane and reach the brain whereby the vapor may interfere with coordination. The ultimate effects of mercury in the body are inhibition of enzyme activity and cell damage. Inhibition of a large variety of enzyme systems by mercury has been reported (Landis and Ming-Ho Yu, 1999). Mercury is known to affect the metabolism of mineral elements such as Na and K, and is also known to inhibit active transport mechanism.

1.1. Scope of the Present Study

Again, it is very important to have a more definite and clear understanding of the effects of a given pollutant on an aquatic ecosystem, and to be made aware of this problem from as many perspectives as possible. Shellfish can be used as an ideal and reference candidate for monitoring the intensity of pollution in general or in a given area as they are consumed by a large percentage or section of human population without knowing the sources of collection of the organisms, i.e., if they were collected from polluted or non-polluted areas.

Backwaters of Kerala are highly productive ecosystems supporting a rich brackishwater fishery, and serving as necessary ground for a variety of fish and shellfish species. They serve not only as food and natural resources on which human society depends, but are also the livelihood for a majority of population. This output can be sustained only if the ecosystems on which they survive are not abused, and the organisms are

not overexploited. The environmental health of these waters calls for immediate attention as the changes are immediately reflected on the health of the organisms.

Heavy metals are normal constituents of the aquatic environment in trace quantities. When the levels increase they act as cumulative poisons to estuarine and marine organisms. Pollution monitoring carried out in recent years have revealed elevated levels of many heavy metals in the marine environment. Therefore, the deleterious effects should be studied more seriously so that corrective or defensive methods could be developed. Though numerous toxicological studies have been carried out on finfishes in general and crabs in particular, a scrutiny of literature clearly reveals the gap in our understanding of the biochemical changes taking place in the organism exposed to heavy metals, which pollute the estuarine and marine habitats of the crabs.

1.2. Objectives of the Present Study

1. To evaluate the toxicity of three sub lethal concentrations of mercury, viz., 0.009 mg/l, 0.02 mg/l, and 0.04 mg/l on the mud crab, *Scylla serrata* through bioaccumulation, and depuration studies,

2. To characterize the biochemical responses to the sub-lethal stress of mercury in chelate muscles, abdominal muscles, hepatopancreas and gills,

3. To study the activity pattern of acid and alkaline phosphatases in mercury-exposed crabs,

4. To evaluate the induced changes in these tissues through histopathological studies,

5. To study the electrolyte status since it may serve as a sensitive physiological index, and

6. To characterize protein profile and the metal binding protein in chelate muscle, abdominal muscle, hepatopancreas, and gill tissues by SDS - PAGE, electrophoresis.

1.3. Area of the Study

The backwaters of Kerala, located between Azhikode and Alleppey (09°30'-10°10'N, 76°15'-76°25'E) form a large area (appx.250 sq.km) of shallow, semi-enclosed body of brackishwater running parallel to the coastline. Several rivers such as Periyar, Pamba, Meenachil, and Muvattupuzha, and a network of canals empty themselves into the backwaters discharging large quantities of freshwater during the monsoon months. The backwater system has two permanent openings to the sea, one at Cochin and the other at Azhikode. At Cochin the connection is about 450 m wide and forms an entrance to the Cochin harbour.

From historic time, this coastal land had attracted industrial and habitation investments resulting in the development of infrastructural facilities for the support of the overgrowing needs of the population. They too are constantly subjected to the consequences of the processes that are characteristic of any over- polluted land. These lacustrine environments are complex and highly variable and richly productive due to high nutrient content. At the same time this biologically unique system is significantly fragile with diverse and unique habitats. The coastal zone is of critical importance to man since it is the area where production, consumption, and energy exchange processes are most intense. The ecological conditions of this region are being much disturbed as a result of anthropogenic substances reaching the habitat.

The backwaters receive large quantities of sewage from Cochin and nearby areas. The bar- built Cochin estuarine system receives industrial effluents from various factories, which include metallurgical, fertilizer, chemical, rare earth, newsprint, and refinery. The effluents, treated and untreated, from these industries reaching the backwaters cause heavy metal enrichment of the aquatic environment. The disposal of industrial wastes and sewage is often carried out without critical appraisal of the losses incurred. Usually no consideration is given with regard to the deleterious environment impact on the receiving water body. The shipping facilities, trade effluents, land drainage, and also the inorganic compounds such as pesticides, insecticides and fungicides, and also organo chlorine pesticides used indiscriminately in this area, also reach the estuary on its way to sea. Further, there are several coconut husk-retting areas in the backwaters, and several prawn peeling sheds disposing off their wastes into the estuary.

The Cochin backwater system, still, is one of the most productive and biologically active areas. Infantile phases of a number of fishes and shellfishes are spent in this environment. Among the shell fishes, the mud crab, *Scylla serrata*, is an important resource and is fished extensively from this backwater region.

Kerala has been one of the leading maritime states in India in the exploitation and export of mud crabs (Raj, 1992). The backwater systems are well known for their rich population of mud crabs. According to Suseelan (1996) an average of above 25000 tons of crabs are landed annually from coastal marine sector. The importance of live mud crabs as an export commodity has opened up great opportunity for crab farming in many countries including India. Crab gathering, culturing, and fattening provide income to many in developing countries because of the fast turn over, low operating coast, high survival rate, and good market demand for

the end products. Economic analysis of mud crab farming practiced in most of the East Asian countries have established that crab culture is highly profitable in comparison to other forms of aquaculture due to increasing price of crabs in the international market, and low production cost.

Vypin

This stretch of land, an island, situated a few meters northwest of the main land is notorious for sewage pollution and occasional fish kills. Tidal flushing is very heavy here. Increased human activities, oil spills due to plying of fishing vessels etc. are common near this station. Moreover, major industrial units located upstream on the banks of river Periyar also contaminate the water. It is the easy availability of water, which attracted many industries to the banks of this river. Fertilizers and Chemicals Travancore limited (FACT), Thottekat Distilleries, Travancore Cochin Chemicals, India Aluminium Co., Hindustan Insecticides Ltd., Cominco Binani Zinc, United Catalyst, Periyar Chemicals, India Rare Earths etc. are the major factories contributing to chemical pollution of Periyar in one way or the other. Effluents from these industries are highly acidic or alkaline, and contain oxygen depleting substances and objectionable sediments. Oil, grease, metals, fluorides, phosphates etc. are commonly seen in the effluents. Solid wastes generated from some industries contain toxic heavy metals such as mercury, lead, cadmium, chromium etc. Besides these, wastes from slaughterhouses and tanneries also reach this water mass and add to pollution.

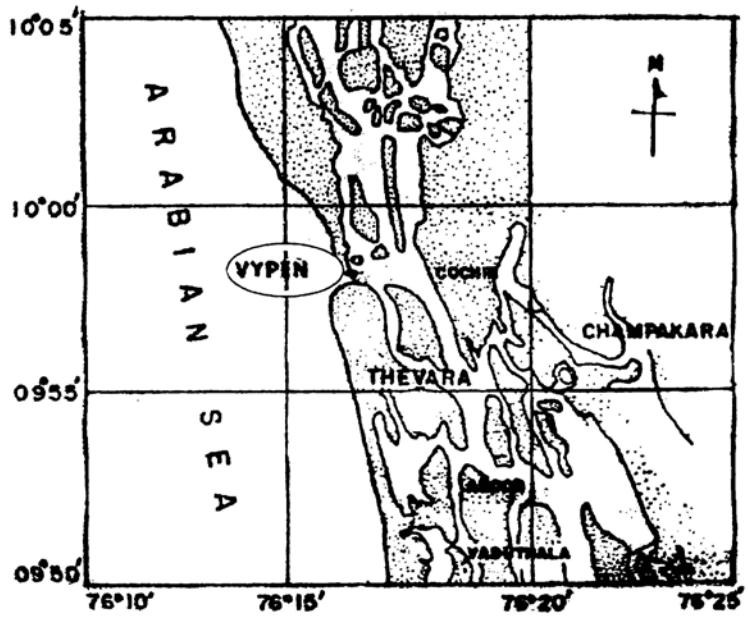


Figure 1. Station selected for the present study

1.4. Test Animal

Scylla serrata (FORSKAL) (Figs. 2 and 3)

This dark green crab, ***Scylla serrata,*** with deep green colored carapace is omnivorous but feeds predominantly on shrimp, crabs, bivalves and fishes.

It belongs to:

Phylum	-	Anthropoda
Subphylum	-	Crustacea
Class	-	Malacostraca
Subclass	-	Eumalocostraca
Order	-	Decapoda
Suborder	-	Dendrobrachiata
Family	-	Portunidae
Subfamily	-	Genus
Genus	-	*Scylla*
Species	-	*serrata*

This crab has biological as well as economic importance, being a choice seafood item having a good 'pull' in the international seafood markets. This animal has been selected because of its economic importance, and previous bioassay studies have proved that this is an ideal test animal for conducting long-term toxicity studies, as it is available through out the year.

Live specimens of the estuarine crab ***Scylla serrata*** were collected from crab farms in 'Vypin', Cochin backwaters. The crabs were being reared in crab farms, in clean seawater with a salinity of 18±1 ppt. Following collection; the animals were carefully transported, with least disturbance, to the laboratory in a healthy and live condition. The crabs were maintained for a couple of days in natural habitat waters to remove the pseudofaecal materials, and then transferred to 10- liter capacity fibre troughs filled with sea water of 18±1 ppt salinity, and acclimated for 7 days. Care was taken to ensure that there was no 'crowding effect'.

Figure 2. Estuarine crab of *Scylla serrata*

Figure 3. A crab farm

1.5. Bioassay studies

LC_{50} values

The effect of mercury on the survival of ***Scylla serrata*** was assessed following the static renewal type of bioassay method. Acute toxicity studies were conducted to determine the 96 hr LC_{50} value of mercury following the procedure given in Standard Methods for the examination of water and waste water (APHA, 1980). Since the toxic potentials of mercury are not known, exploratory tests were conducted to determine the approximate concentration range to be used for 96hr LC_{50} studies. Ninety six hour LC_{50} value of mercury was found to be 0.09mg/l. Based on the results of the exploratory test, a series of 3 sub-lethal concentrations of mercury, i.e., 0.009, 0.02, 0.04 mg/l was selected. While selecting concentrations, the concentrations that produced 0 and 100 per cent mortality were fixed as the lowest and highest, respectively. To fix the 96hr LC_{50} value, six animals were exposed to each concentration.

Table 1. Physical and chemical characteristics of the test medium

Salinity	18 ± 1ppt
Temperature	28 ± 1^{0}C
Oxygen	4.4 ± 0.2 ml/l.
*p*H	7.5 ± 0.3

Dissolved concentration of mercury at 0.009 mg/l, 0.02 mg/l, and 0.04 mg/l

Chapter 2

BIOACCUMULATION AND DEPURATION STUDIES

2.1. Introduction

Pollution, in general, is severe in semi-enclosed marginal seas and wastewaters bordering highly populated industrialized zone. The entry of toxic and carcinogenic pollutants into the food webs at various levels has generated greater public health concern as it affects the fishing industries in several ways. Shellfishes are capable of accumulating metal ions continuously from the environment and such bioaccumulation results in having concentrations of metals in the organism higher than the ambient levels in the surrounding environment (Bryan, 1964,68; Ahsanullah et al., 1981,84; Davies et al., 1981). It has been demonstrated that metal accumulation in tissues is directly linear to exposure concentrations. Moreover, accumulated metal ions induce severe pathological changes (Doughtie and Rao, 1983; Rao and Doughtie, 1984).

Environmental contamination by metals has increased in recent years due to excessive use of metals in agriculture and industry. As a result of their bio-concentration, immutable and non-degradable properties, these metals constitute a major source of pollutants. Among these metals cadmium, lead, and mercury are non-essential whereas copper, iron, manganese and zinc are essential elements. Heavy metals are very toxic because as ions or in compound forms they are soluble in water and may be readily absorbed into living organism. After absorption

these metals can bind to vital cellular components such as structural proteins, enzymes and nucleic acids and interact with their functioning.

Unlike many contaminants, heavy metals are normal constituents of the aquatic environment. Man has turned this environment into sinks and repositories by discharging large quantities of wastes, which contain heavy metals and these metals, essential or not, are potentially toxic to the living organisms. Aquatic organisms tend to accumulate heavy metals from the environment and are adapted to handle natural fluctuations in intake brought about by slight changes in their availability in water or food. Such a process of accumulation of pollutants by organisms is termed as bioconcentration or bioaccumulation. Bioconcentration refers to the ability of an organism or population of organisms of the same trophic levels to concentrate a substance from an aquatic system. Bioaccumulation refers to the ability of an organism to not only concentrate, but to continue to concentrate essentially throughout its metabolic life time so that the ratio of tissue to ambient concentration, once calculated, would be continuously increasing during its life time.

Besides bioavailability, the accumulation of trace metals in organisms at a defined exposure varies with the species, age, sex, size, physiological status of the organisms as well as physical factors such as temperature and salinity (Phillips,1980).The accumulated metal concentration depends on the net difference between rates of uptake and excretion whether in tissue or body levels, and the quantum of metals that can be held within a tissue depends on the nature and extent of the metabolic processes of metal detoxification available (Roesijadi, 1992; Roesijadi and Robinson, 1994). Decapod crustaceans absorb trace metals from food sources, and additionally, in the case of aquatic species, via permeable body surfaces such as gills, without resorting to active transport mechanisms. In general, watery medium and food represent the

major routes of metals uptake by marine invertebrates, the order of priority varying with many factors including species, food type, relative concentrations of metal in food and water, physico-chemical parameters of the aquatic medium, etc. It is observed that the body metal contents are summations of the contents of the constituents in tissues or organs (Depledge and Rainbow, 1990). The accumulated metal concentration depends on the nature of metal detoxification and extends to the metabolic processes (Viarengo, 1989; Roesijadi and Robinson, 1994).

Some crustaceans, at concentrations below threshold level, can regulate body levels of essential metals such as copper and zinc. Accumulation of these metals begins only after the organisms are faced with high concentration in the surrounding medium (Rainbow, 1988; Rainbow and White, 1989). In contrast, body levels of non-essential metals such as mercury, cadmium and lead were not found to be regulated by crustaceans (Krishnaja et al., 1987; Pastor et al., 1988).From a physiologist's point of view, bioaccumulation is the phenomenon that is relatively easy to detect but rather difficult to explain at the cellular level. In a number of cases, it provides a way to identify cellular sites of activity either by electron probe micro- analysis (Brown, 1977) or by cell fractionation (Coombs and George, 1978). It is implied that physiological processes exists specifically for regulating and removing such interfering cations so that the deposits represent the end product of a toxification system.

Scylla serrata, the estuarine crab, has high nutritional values and is a delicacy. Most of the estuarine habitats of these crabs contain heavy metals. However, there are only meager studies relating to bioaccumulation of heavy metals in general and mercury in particular, by estuarine crabs. In the present investigation bioaccumulation of mercury in different tissues of *Scylla serrata* was studied by exposing the

organisms to three sub-lethal concentrations of mercury.The rate at which accumulation occurs in an organism depends not only on the availability of the pollutant, but also a whole range of biological, chemical and environmental factors. The ultimate levels reached are governed by the ability of the organisms to excrete the pollutant or alternately store it. Thus, even though in a number of cases there is evidence that the accumulation of the xenobiotic metal by the organism is proportional to the concentration in the external medium, this is true only in the case of non-essential metals.

Heavy metals in the aquatic environment when present in excess become toxic. The system, which gets affected in living organisms due to the toxic effect, extends from interference with various activities to impairment of vital physiological function such as respiration. Organisms, which happen to accumulate the metals, do also have the capacity to depurate. Regulation of excess metal concentration in animal tissues is accomplished through several means. In the laboratory, under experimental conditions, the regulatory efficiencies of organisms have been observed by several workers and these may be summarized as: temporary absorption, storage and their release by hepatopancreas, losses across the gills, losses through antennal gland in the form of metal binding proteins- metallothioneins, and accumulation of metals in intracellular electron-dense granules within membrane limited vesicles (Miramand et al., 1981; Brouwer,et al., 1984; Bryan et al., 1986). Depuration data are helpful in estimating the time required for residues to be reduced to non-detectable concentrations. Bryan et al. (1987) reported that half of the accumulated tin was depurated between 50 and 100 days. From this it is conceivable that longer exposure in clean water will be much more effective.

Information on the efficiency of important biota to depurate accumulated metal is essential and the present investigation was undertaken also to find out the regulatory efficiency of *Scylla serrata,* and to trace the elimination of accumulated metal mercury through different tissues during the depuration period.

2.2. Review of Literature

Trace metals are accumulated by marine invertebrates in higher body concentrations, sometimes at several orders of magnitude higher than the concentration in an equivalent weight of the surrounding seawater. A number of environmental variables, i.e., physico-chemical parameters such as pH, temperature, dissolved oxygen, salinity, hardness of water, sediment chemistry, seasonal changes, presence of organic substances and EDTA, influence bioavailability, uptake and toxicity of heavy metals in organisms. The reports of such studies include those of Dhavale (1990), WHO (1992), Steenkamp et al. (1995), Srilakshmi and Prabhakararao (2000), and Mitra et al. (2000). Laporte et al. (1997) studied the combined effects of water, pH, and salinity on the bioaccumulation of inorganic mercury and methyl mercury in the shore crab, *Carcinus maenas*. Zinc and cadmium accumulations at different salinities were studied in fiddler crabs *Uca rapax* and *Carcinus maenas* by Zanders and Rojas (1996), and Chan et al.(1992). Effects of molting and metal uptake in crabs were studied by Chan et al. (1993a).

Sex and size related tolerance and accumulation of metals in crabs were studied by a number of researchers, and these include studies on *Oziotelphusa senex senex* by Radhakrishnaiah and Renukadevi (1990), and Radhkrishnaiah et al .(1991), and in *Barytelphusa guerini* by Sarojini et al. (1990) and Sastre et al. (1999). Accumulation and flux of inorganic mercury and methyl mercury

across the gills and intestine of the blue crab were studied by Jean et al.(2002). Bioaccumulation of mercury and methyl mercury, arsenic, selenium and cadmium in freshwater invertebrates and fish were studied by Mason et al. (2000). Ananthalakshmi kumari et al. (1990) studied the toxicity of mercury in male and female field crabs *Paratelphusa hydrodromous,* and levels of arsenic, chromium, copper, lead, magnesium, manganese, selenium, vanadium and zinc concentrations were determined in various organs such as hepatopancreas, gills, stomach and muscle of the blue crab, *Portunus pelagicus* by Al-Mohana et al.(2001). Accumulation of cadmium and mercury in blue swimmer crab and an estuarine crab Scylla serrata was reported by Ross et al. (2001), and Rajathy, (1991). Accumulation and depuration of mercury in blue crabs by Evans et al. (2000), and heavy metals accumulation in the rock crab, *Thalamita crenata* by Meng-Hsien Chen et al. (2005). Accumulation of mercury and flux across the gills and intestine of the crab, *Callinectes sapidus* were investigated by SandrineAndres et al. (2002).

The hepatopancreas is the primary target organ involved in bioconcentration and biomagnification of the toxicants was reported, among others, by Shah et al. (2001) and Henry Charles, (1985). Bioaccumulation of heavy metals in some fauna and flora were reported by Ravindrakumarsingh et al. (2007).

2.3. Materials and Methods

Crabs of the intermoult stage were chosen for the experiment. The water used for the study was collected from the same place from where the crab specimens were collected. The salinity of the water used for the experiments was 18± 1ppt, the temperature 28±1^{0}C, the oxygen content 4.4±0.2ml/l, and *p*H 7.5±0.3. Healthy crabs were selected and acclimated for 7 days in the experimental tanks filled with filtered

water. Standards were prepared using Analar Grade mercuric chloride. All the standards were prepared in deionised water as recommended by APHA(1980).

Long-term accumulation study: Based on the 96hr LC_{50} values (0.09mg/l), 3 sub-lethal concentrations were chosen for the metal mercury i.e., low (0.009mg/l), medium (0.02mg/l), and high (0.04mg/l). Experimental media were prepared with filtered estuarine water. Test media were renewed once every 24 hr. Water was well aerated and the media were prepared afresh daily. A group of 18 animals was exposed to the 3 sub-lethal concentrations (6 each) of mercury for a period of 30 days, and an equal number of animals of the same size and weight served as controls, and were maintained in tanks filled with filtered water. After 30 days of exposure, all the 6 crabs from each concentration, and an equal number of controls were sacrificed and the tissues - hepatopancreas, gills, abdominal muscle tissue, and chelate muscle- were removed, washed properly in double distilled water, kept in an oven at 110^0C up to 24 hours, and then digested with acid as suggested by Agemian and Chau (1976). Mercury content was estimated by Atomic Absorption spectrophotometer (Perkin Elmer Model 2280).

In depuration studies a total of 18 crabs were exposed for a period of 30 days; 6 in each of the three sub lethal concentrations of mercury. Subsequently, the same crabs were transferred to clean water, without metal concentrations, and maintained for 30 days. At the end of 60^{th} day, the crabs were sacrificed and tissue samples of abdominal muscle, chelate muscle, hepatopancreas, and gill taken for analysis of metal mercury following the same method as applied for bioaccumulation studies.

2.4. Results

Bioaccumulation:

1. Gill tissue appeared to accumulate more mercury than hepatopancreas and muscle tissue in 30 days of exposure to the three sub-lethal concentrations of mercury. Very low levels of mercury were found in the tissues of the control crabs. Mercury concentrations in control animals were: gill (2.69 ±0.49μg/g), hepatopancreas (1.37±0.54μg/g), abdominal muscle tissue (0.09±0.07μg/g), and chelate muscle tissue (0.05±0.02μg/g) . After 30 days of exposure, the pattern of accumulation was the same in the treated crabs. The maximum accumulation was found to occur in gill tissues (5.04, 7.04 and 8.68) (Table 2, 3and 4, respectively, at three concentrations). Gill tissue was followed by hepatopancreas and muscle tissue in accumulation pattern. Abdominal muscle tissue and chelate muscle tissue showed the least accumulation rate. Variations in metal content in the tissues with increasing concentration were also noticed. Thus a dose dependent accumulation was observed in the present study.

2. Statistical Analysis: The data on mercury bioaccumulation in four different tissues at three sub-lethal concentrations of mercury were statistically analyzed using student's 't' test.

3. Accumulation of mercury in treated tissues was significantly higher (Table 2 and Fig. 4) in the crabs after 30 days of exposure at 0.009 mg/l concentration compared with the controls.

4. Accumulation of mercury in treated tissues was significantly higher (Table 3 and 4; Figs. 5, 6) in the crabs after 30 days of

exposure at 0.02mg/l and 0.04 mg/l concentrations when compared with controls.

5. It was statistically concluded that the mean accumulation level in treated tissues was higher than the levels in control tissues. Since 't' values (6.92, 13.40, 5.06, 5.52, 9.73, 9.60, 9.15, 11.51, 9.98, 7.34, 12.33, 13.01; Table 2, 3 and 4) were significant at 1% level, it was concluded that treated tissues had higher concentrations of mercury than the mean levels in the corresponding tissues of the controls.

6. Percentage of depuration level of mercury concentration was significantly higher (Table 5, Fig. 4) in the crabs after 30 days of exposure to mercury at low concentration (0.009 mg/l) compared with the accumulation level of the treated tissues.

7. Percentage of depuration levels of mercury concentrations were significantly higher (Table 6 and 7; Figs.5and6) in the crabs after 30 days of exposure at 0.02 mg/l, and 0.04 mg/l when compared with the control values.

8. Based on the results analyzed statistically using student's 't' test, it was concluded that the percentage of depuration levels in treated tissues were higher than the accumulation levels in the tissues. Since 't' values (14.96, 9.00, 48.84, 14.92, 19.90, 61.36, 103.57, 29.84, 24.25, 10.40, 51.63, 60.38; Table 5, 6 and 7) were significant at 1% level, it implies that the mean depuration levels are higher than the mean accumulation levels in treated tissues.

Table 2. Student's *t* – Test for Control Vs Accumulation at low concentration of mercury- 0.009 mg/l

***t* – Test : Two sample assuming equal variances**

Source (Abdominal muscle tissue)			Standard
Variable	Count	Mean	Deviation
Control	6	0.09	0.076
Accumulation	6	0.687	0.100
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		0.503	0.008
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	6.9211**	0.001	Reject Ho
Source (Chelate muscle tissue)			Standard
Variable	Count	Mean	Deviation
Control	6	0.057	0.025
Accumulation	6	0.510	0.053
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		0.453	0.002
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	13.4005**	0.000	Reject Ho
Difference: (Control)-(Accumulation)			
Source (Hepato pancreas)			Standard
Variable	Count	Mean	Deviation
Control	6	1.373	0.546
Accumulation	6	3.620	0.541
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		2.247	0.296
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	5.0604**	0.004	Reject Ho
Source (Gill)			Standard
Variable	Count	Mean	Deviation
Control	6	2.693	0.497
Accumulation	6	5.047	0.545
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		2.353	0.272
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	5.5256**	0.003	Reject Ho

Pooled data for experimental animals

Tissue	Control	Accumulation
Abdominal muscle tissue	0.09	0.68
Chelate muscle tissue	0.05	0.51
Hepatopancreas	1.37	3.62
Gill	2.69	5.04

**Significant at 1% level. (*t* value 4.604)

Table 3. Student's *t* – Test for Control Vs Accumulation at medium concentration of mercury - 0.02 mg/l

t – Test : Two sample assuming equal variances

Source (Abdominal muscle tissue)			Standard
Variable	Count	Mean	Deviation
Control	6	0.093	0.076
Accumulation	6	0.873	0.096
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		0.690	0.008
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	9.7365**	0.000	Reject Ho
Difference: (Control)-(Accumulation)			
Source (Chelate muscle tissue)			Standard
Variable	Count	Mean	Deviation
Control	6	0.05	0.02
Accumulation	6	0.693	0.051
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		0.510	0.004
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	9.6001**	0.000	Reject Ho
Source (Hepatopancreas)			Standard
Variable	Count	Mean	Deviation
Control	6	1.373	0.546
Accumulation	6	5.283	0.499
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		3.910	0.274
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	9.1521**	0.000	Reject Ho
Source (Gill)			Standard
Variable	Count	Mean	Deviation
Control	6	2.693	0.497
Accumulation	6	7.043	0.084
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		3.350	0.127
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	11.5138**	0.000	Reject Ho
Difference: (Control)-(Accumulation)			

Pooled data for experimental animals

Tissue	Control	Accumulation
Abdominal muscle tissue	0.09	0.87
Chelate muscle tissue	0.05	0.69
Hepatopancreas	1.37	5.28
Gill	2.69	7.04

**Significant at 1% level. (*t* value 4.604)

Table 4. Student's *t* – Test for Control Vs Accumulation at high concentration of mercury- 0.04 mg/l

t – Test : Two sample assuming equal variances

Source (Abdominal muscle tissue)			Standard
Variable	Count	Mean	Deviation
Control	6	0.093	0.076
Accumulation	6	1.067	0.133
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		0.883	0.012
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	9.9876**	0.000	Reject Ho
Source (Chelate muscle tissue)			Standard
Variable	Count	Mean	Deviation
Control	6	0.057	0.025
Accumulation	6	0.867	0.189
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		0.810	0.018
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	7.3468**	0.001	Reject Ho
Source (Hepatopancreas)			Standard
Variable	Count	Mean	Deviation
Control	6	1.373	0.546
Accumulation	6	6.213	0.405
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		4.840	0.231
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	12.3352**	0.000	Reject Ho
Difference: (Control)-(Accumulation)			
Source (Gill)			Standard
Variable	Count	Mean	Deviation
Control	6	2.693	0.497
Accumulation	6	8.683	0.441
Difference: (Control)-(Accumulation)			
		Mean	Pooled
		Difference	Variance
		4.990	0.221
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	13.0125**	0.000	Reject Ho
Difference: (Control)-(Accumulation)			

Pooled data for experimental animals

Tissue	Control	Accumulation
Abdominal muscle tissue	0.09	1.06
Chelate muscle tissue	0.05	0.86
Hepato pancreas	1.37	6.21
Gill	2.69	8.68

**Significant at 1% level. (*t* value 4.604)

Table 5. Student's *t* – Test for Accumulation Vs Depuration at low concentration of mercury - 0.009 mg/l

t – Test : Two sample assuming equal variances

Source (Abdominal muscle tissue)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	0.687	0.100
Depuration	6	5.653	0.566
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		4.967	0.165
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	14.9622**	0.000	Reject Ho
Source (Chelate muscle tissue)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	0.510	0.053
Depuration	6	4.667	0.797
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		4.157	0.319
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	9.0091**	0.000	Reject Ho
Source (Hepatopancreas)			Standard
Variable`	w	Mean	Deviation
Accumulation	6	3.620	0.541
Depuration	6	39.550	1.153
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		35.930	0.812
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	48.8463**	0.000	Reject Ho
Source (Gill)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	5.047	0.545
Depuration	6	48.567	4.904
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		42.520	12.174
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	14.9253**	0.000	Reject Ho
Difference: (Accumulation)-(depuration)			

Pooled data for experimental animals

Tissue	Accumulation	Depuration
Abdominal muscle tissue	0.68	5.65
Chelate muscle tissue	0.51	4.66
Hepato pancreas	3.62	39.55
Gill	5.04	48.56

**Significant at 1% level. (*t* value 4.604)

Table 6. Student's '*t*' – Test for Accumulation Vs Depuration at medium concentration of mercury- 0.02 mg/l

t – Test : Two sample assuming equal variances

Source (Abdominal muscle tissue)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	0.873	0.096
Depuration	6	8.233	0.633
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		7.360	0.205
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	19.9072**	0.000	Reject Ho
Source (Chelate muscle tissue)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	0.693	0.051
Depuration	6	6.817	0.165
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		6.123	0.015
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	61.3699**	0.000	Reject Ho
Source (Hepatopancreas)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	5.283	0.499
Depuration	6	48.417	0.520
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		43.133	0.260
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	103.576**	0.000	Reject Ho
Difference: (Accumulation)-(depuration)			
Source (Gill)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	7.043	0.084
Depuration	6	52.660	2.646
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		45.617	3.504
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	29.8481**	0.000	Reject Ho

Pooled data for experimental animals

Tissue	Accumulation	Depuration
Abdominal muscle tissue	0.87	8.23
Chelate muscle tissue	0.69	6.81
Hepatopancreas	5.28	48.41
Gill	7.04	52.66

**Significant at 1% level. (*t* value 4.604)

Table 7. Student's *t* – Test for Accumulation Vs Depuration at high concentration of mercury - 0.04 mg/l

t – Test : Two sample assuming equal variances

Source (Abdominal muscle tissue)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	1.067	0.133
Depuration	6	9.273	0.592
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		8.400	0.180
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	24.2532**	0.000	Reject Ho
Source (Chelate muscle tissue)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	0.867	0.189
Depuration	6	7.250	1.090
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		6.557	0.595
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	10.4099**	0.000	Reject Ho
Source (Hepatopancreas)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	6.213	0.405
Depuration	6	51.897	1.482
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		46.613	1.222
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	51.6349**	0.000	Reject Ho
Source (Gill)			Standard
Variable	Count	Mean	Deviation
Accumulation	6	8.683	0.441
Depuration	6	58.297	1.468
Difference: (Accumulation)-(depuration)			
		Mean	Pooled
		Difference	Variance
		51.253	1.081
		Prob	Decision
Hypothesis	t-Value	Level	0.050
Difference < 0	60.3877**	0.000	Reject Ho

Pooled data for experimental animals

Tissue	Accumulation	Depuration
Abdominal muscle tissue	1.06	9.27
Chelate muscle tissue	0.86	7.25
Hepatopancreas	6.21	51.89
Gill	8.68	58.29

**Significant at 1% level. (*t* value 4.604)

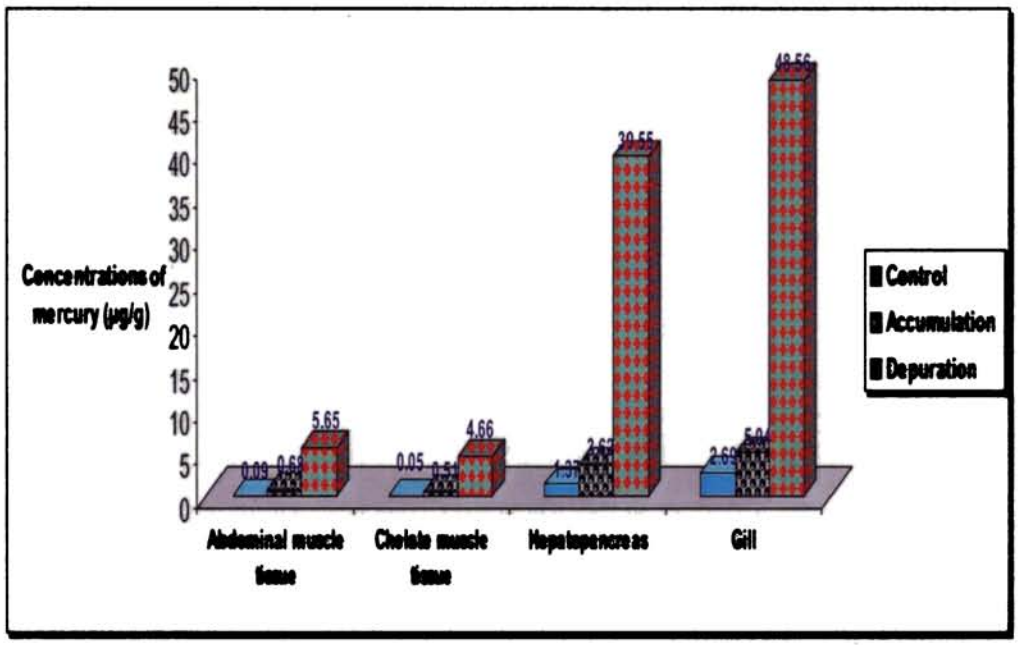


Figure 4. Accumulation and depuration in crabs exposed to sub lethal concentration of mercury at 0.009 mg/l.

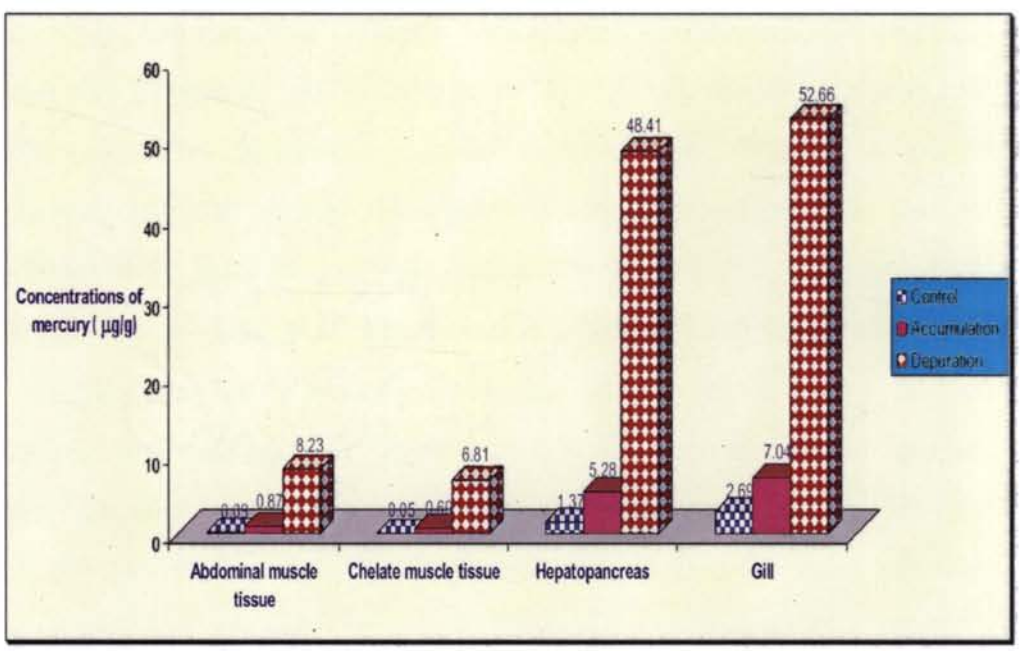


Figure 5. Accumulation and depuration in crabs exposed to sub lethal concentration of mercury at 0.02 mg/l.

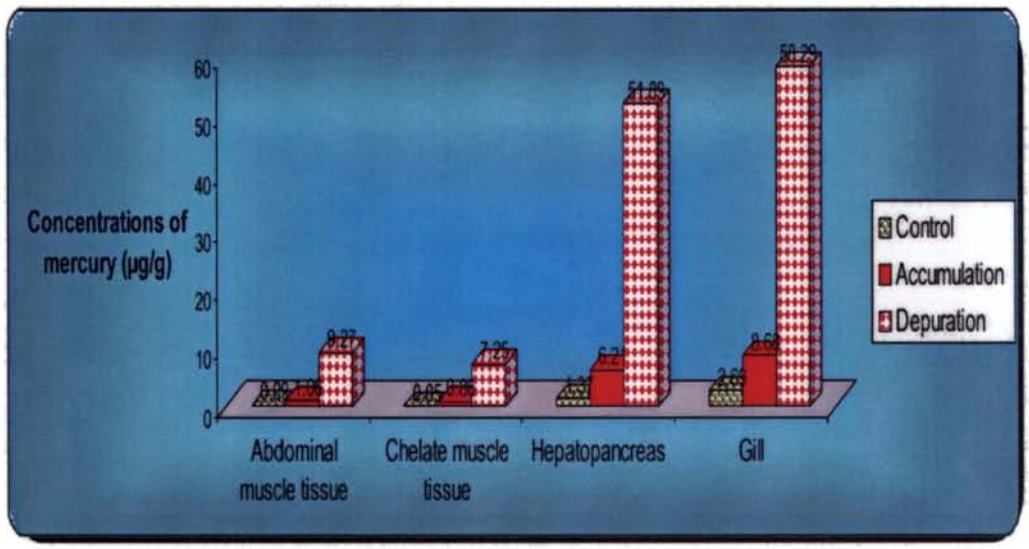


Figure 6. Accumulation and depuration in crabs exposed to sub lethal concentration of mercury at 0.04 mg/l.

2.5. Discussion

Bioaccumulation: In the present study a linear relationship between absorption of metals by tissues and external medium was observed. Of the four tissues selected, gill was found to accumulate high amount of mercury. Similar observations were reported in *Uca pugilator* exposed to mercury (Vernberg and Vernberg, 1972). The accumulation of mercury in gill was dose dependent. Similar results were obtained in *Carcinus maenas* and the shrimp *Palaemon serratus* (Papathanassiou and King, 1983, 1986; Ahsanullah, 1984; Amiard, et al., 1987) exposed to cadmium. Higher level of mercury accumulation in the gill was due to the fact that the gills are the likely sites for the absorption of heavy metals in crustaceans as suggested by Bryan (1964). Hepatopancreas appears to play a vital role in metal detoxification as reported by Lyon et al. (1983). Sheffy (1978) reported that hepatopancreas is also the main site for the accumulation of mercury apart from gill. In the present study also accumulation of mercury was found more in hepatopancreas, next to gill. The gills are the most permeable regions of the body, other factors being the large surface area for absorption and the large volume of water passing

over the gill surface (Anderson and Brouwer, 1978). The rate of uptake of metal slows down during the latter period of exposure to the metal and the following reasons may be attributed for the effect,

- Metal could have translocated to other tissues rapidly from gills, and
- Impairment to the structure of gills might have impeded the uptake of metals.

Comparatively less accumulation and depuration of mercury were observed in both the muscle tissues. Muscle tissue often showed a very slow degree of heavy metal uptake compared to other tissues (White and Rainbow, 1986a). Nussey et al. (2000) made similar observations that only less metal concentrated in muscle and skin in their study on the tissues of *Labeo umbratus*. Accumulation of mercury in muscle tissue was also reported in the American Oyster *Crassostrea virginica* (Cunningham and Tripp, 1975a), and in the muscle of the shrimp *Penaeus duorarum* due to cadmium (Nimmo et al., 1977).The accumulation of metal in the muscle may be due to the fact that the muscle is a tissue receiving a major portion of the blood supply and, if heavy metals were transported by blood, muscle tissue would be expected to accumulate those heavy metals. Crustaceans' muscle often shows a very low degree of heavy metal uptake relative to other tissues (White and Rainbow, 1986a) as has been evidenced in the present study. Bryan (1984) reported that in decapod crustaceans the absorption of metals from solution was almost certainly a passive process involving absorption on the cuticle and inward diffusion, probably attached to organic molecules.

In mercury accumulation studies involving four tissues a direct correlation to the concentration of metals in the medium was observed and this was in accordance with the observations noticed in other organisms

such as *Palaemon serratus* (Papathanassiou and King, 1986a).Muscle tissues accumulated the lowest level of the metal. Lowest amounts of metals were recorded in the muscle of *Callinectes sapidus* (Hutchenson, 1974), *Salvelinus fontinalis* and *Salmo gairdneri* (Haux and Larsson, 1982) corroborating the results of the present study. Muscle tissue occupies large volume of the body and therefore has greater chances of accumulating the metal. Since the muscle tissue constitutes a major portion of the weight of the organism it is reasonable to assume that the tissue accumulates considerable quantity of the metals. The low levels of metals in the muscle tissue are probably due to its inability to sequester the metal at higher concentration as is reported in *Palaemon elegans* (White and Rainbow, 1986a). Similar observations were reported by Jean et al. (2002) on the uptake of mercury, and on the effect of ligands across the gill and the intestine of the blue crab *Callinectes sapidus.* It is very common for metal concentrations in the hepatopancreas to be higher than those in other tissues because of the heavy involvement of this organ in detoxification.Gabaruko and Friday(2007) were reported that the accumulation rate of muscle tissues were very slow when compared with other tissues, such as gill and hepatopancreas.

Depuration: The percentage of depuration of mercury through gill increased with increase in the test concentrations, which the crabs were exposed to. Similar loss of zinc through gill was reported in the crab *Carcinus maenas* (Bryan, 1966), and mercury in the American system *Crassostrea virginica* (Cunningham and Tripp, 1975a). A similar trend in depuration of mercury was also noticed in the hepatopancreas through the present study. A dose- dependent elimination of mercury was noticed in the muscle also. But the rate of elimination was very low. The slow rate of excretion could be due to the formation of metal binding proteins. Several

authors have reported low elimination of cadmium during depuration in the lobster *Homarus americanus* (see,Mc Leese et al.,1981).

Removal of zinc was more effective by way of temporary absorption, storage and subsequent release by the hepatopancreas and also across the gill (Bryan, 1968). The losses across the gill after the depuration periods were, 48.56%, 52.66%, 58.29%, respectively in the three test concentrations. Similar elimination of zinc through gill (85%) was reported by Bryan (1966) in the crab *Carcinus maenas.*

The mud crab *S. serrata* is an esteemed food item. The present study on depuration has shown low elimination through muscle when compared with the same process in gill and hepatopancreas. Crustacean muscle often shows a very slow degree of heavy metal uptake compared to other body tissues (White and Rainbow, 1986a).

In some groups of animals the body concentrations are regulated against fluctuation in intake and one such group is the decapod crustaceans (Bryan et al., 1986). There was a higher rate of depuration of mercury in gills than in muscle in *Tilapia mossambica* exposed to 0.1± 0.003 ppm of mercury/l for 26 days (Tariq and D'Silva, 1993). The delayed and marginal loss of metals from the muscle tissue was probably due to the binding of some metals with muscle protein. Such binding could be irreversible. The retention of mercury in the muscles of *Tilapia mossambica* is suggested to be due to the possible linkage with protein and subsequent methylation of mercury (Tariq and D'Silva,1993). Similar feature was noted in the present investigation. The loss of metals in the tissues during depuration differs markedly. It was detected that more release of mercury had taken place from gill than from hepatopancreas. Similar observations were reported in *Macrobrachium* exposed to 0.008 ppm Hg/l for 21 days, and then placed in normal water for some period,

and reduction of 62%, 29% and 17%, respectively, was also recorded (Loganathan 1995).

Kureshy and D'Silva (1993) conducted experiments on *Tilapia mossambica, P. viridis and Villorita cyprinoides* from Dona Paula seashore in Goa on the uptake and loss of mercury, cadmium and lead in various tissues, and found mercury was highly toxic to the clams as compared to fish and mussles.

From this study it is concluded that: (i) the gills are the most likely sites for the adsorption of heavy metals in crustaceans due to direct contact of these organs with the test media, and also due to their more permeable nature; (ii) an organism has limited power of excretion and tends to detoxify and store metals, and the hepatopancreas, due to their detoxification function, acts as sponge mopping up the excess metal from the blood and then keeping the blood metal level fairly normal, and (iii) crustacean muscle often shows a very low degree of heavy metals uptake relative to other tissues It may also be concluded that the varying rates of bioaccumulation of heavy metals may probably be due also to the specific requirement for maintaining the osmoregulatory process. It is also suggested that *Scylla serrata* can be used as a test organism for evolving water quality criteria as it is comparatively more hardy.

Chapter 3

BIOCHEMICAL STUDIES-I: TOTAL PROTEINS, CARBOHYDRATES AND LIPIDS

3.1. Introduction

Any information on the biochemical composition is helpful in assessing the nutritive value of organisms. In the changed scenario of increased environmental pollution, changes in biochemical constituents of biota can be used as a convenient index or tool to assess the degree of impairment caused by the pollutants on the organisms. No doubt, certain heavy metals are quite essential in trace quantities, but when the concentration exceeds the required level, they disrupt the metabolic processes, and thus altering the physiological state of the organisms. Under metal-contaminated environmental conditions, the concentrations of heavy metals in organisms can increase considerably to which the aquatic organisms have not previously been exposed. Under such unfavorable conditions the organisms develop certain adaptive methods such as mobilization of energy from reserves to tide over the crisis and to protect themselves (Bryan et al., 1986).

All organisms maintain their "internal milieu" more or less constant by making use of a variety of regulatory mechanisms. When the levels of pollutants in the environment exceed, changes are reflected in the physiological and biochemical processes, and it may finally end up in mortality of the organisms. Analysis of such variations in the body functions based on laboratory investigation is an important aspect of pollution research. Metals are taken up by aquatic organisms usually across respiratory surfaces and strongly bound by sulphydryl groups of proteins. Because of this ability

there is a tendency for them to get fixed in tissues and will not be excreted. In other words, they have a long biological half-life. Metals also change the structure and enzymatic activities of proteins and cause toxic effects. Under these circumstances, a clear understanding of the physiological and biochemical changes taking place as a result of environmental contamination by heavy metals, in the local fauna will be very useful in formulating theories on the deleterious effects of heavy metals on aquatic organisms, and to make comparisons with data from other sources. This kind of study may also help to provide information on the status, trends and risk to the aquatic ecosystem. Even though many have investigated effects of environmental pollutants on the mortality of aquatic animals, very little is known about the changes in the physiological and biochemical processes in specific organisms and in specific tissues on exposure to toxic heavy metals. Therefore, an experiment was designed to ascertain the biochemical changes, if any, to tissue proteins, carbohydrates, and lipids in *Scylla serrata* exposed to three sub lethal concentrations of mercury at three time periods of 1, 7 and 15 days, and the results are presented in this chapter.

3.2. Review of Literature

When metals cross the cell membrane, they react with the cellular components in different ways. Metals can combine with different functional groups. Most of the heavy metals can cause serious impairment to the metabolic as well as physiological activities of the animals.

The general response of an organism to stress is the utilization of nutrient reserves to meet the metabolic requirements, which get enhanced of the normal values. This response to stress can be measured in terms of alteration in the balance between catabolism of carbohydrates, protein and lipid substrates (Bayne et al., 1985). The potentially toxic heavy metals can be detoxified intracellularly by partitioning into lysosomes (Moore, 1980) or by binding to the metal-binding protein, metallothionein (Bayne et al., 1985).

Protein induction, defined as an adaptive increase in the number of molecules of a specific protein or decreased rate of its degradation, is a possible selective means for regulating levels of specific protein (Rana and Kumar, 1980). Bharani Kumar et al. (1982) reported the long terms effects of organophosphate and organochlorine compounds on the biochemistry of the freshwater crab, *Oziotelphusa senex senex*. PCB induced blood sugar changes were observed in the blue crab, *Callinectes sapidus* by Colia nase and Neff (1982) The organochlorine insecticide tends to accumulate in the lipid-rich tissues of the biosystem due to their lipophilic nature as reported by (Agarwal, 1981; Bhakthavasthalam and Reddy, 1981). Bhagyalakshmi et al. (1984) had studied the effect of sub lethal concentrations of sumithion on some biochemical aspects of metabolism in *Oziotelphusa senex senex*. DDT induced blood sugar level changes on *Barytelphusa guerini* and *B. cunicularis* were reported by Fingerman et al. (1981), and Jawle (1985), respectively. Lipids serve as an alternate source of energy, in crustaceans particularly during stress (Chang and O'Connor, 1983.) Balaji et al. (1989) studied in vivo binding of exogenous copper to haemolymph fractions of *Scylla serrata* (Forskal). Villalan (1988) had observed heavy metal altered protein, lipid and carbohydrate levels in the crab *Thalamita crenata*.

Effects of cadmium chloride and mercuric chloride on biochemical changes in the tissues of fresh water crab *Barytelphusa guerini and Scylla serrata* were reported by Reddy et al. (1991) and Rajathy, (1991). Mule and Lomte (1994) studied the effect of mercuric chloride on protein content of body organs of the snail *Thiara tuberculata* . Cyril et al. (1993) reported biochemical changes in *Cyprinus carpio* in response to nickel and lead. Tulasi et al. (1992) had studied the biochemical changes in the haemolymph of *Barytelphusa guerini* exposed to lead. Effects of mercury and cadmium on proteins and enzymes in *Oreochromis mossambicus* were studied by Rema (1995). Effects of copper and mercury on the glycogen and protein

contents of liver and muscle of the fish *Macrones gulio* were reported by Asha (2001). Loganathan (1995) had studied changes in proteins, carbohydrates, and lipids in tissues of the freshwater prawn, *Macrobrachium malcomsonii* exposed to mercury and lead. Effects of nickel chloride on glycogen content in the gill, foot, mantle, digestive gland, and the whole body of the fresh water bivalve, *Parreysia* were studied by Choudhari et al. (2002). Parate (2003) studied changes in the total protein profile in the muscle and gills of the crab, *Paratelphusa* exposed to cypermethrin. Changes in carbohydrate metabolism in the nervous system of *Scylla serrata* were reported by Maheswari et al. (1996). Effects of dietary proteins on growth, food conversion, and digestive enzyme activities of *Macrobrachium rosenbergii* were reported by Lizy paulose (1996). Jehosheeba (2003) studied the biochemical effects of petroleum hydrocarbons on the fish *Oreochromis mossambicus*. Effects of naphthalene on lipid peroxidation of *Scylla serrata* were reported by Vijayavel et al. (2004).

The variations in haemolymph and muscle lipid profiles with molting and maturation are rather extensive (Millamena et al., 1984; Sai et al., 1984; Sriraman and Reddy, 1977). The variations in haemolymph lipids in relation to temperature were studied by Bricton et al. (1980) .The muscle lipids content in relation to seasonal changes was studied in *Callinectes sapidus* by Amsler and George (1984). Seasonal variations in the lipids of the digestive gland in the shrimp *Pleoticus mullerei* were reported by Jeckel (1991).

3.3. Materials and Methods

Estimation of Total Protein

Total protein was determined following the method of Lowry et al. (1951).

Principle:

The carbamyl groups of protein molecules react with copper and sodium reagent to form a biurette complex. This complex reacts with

tyrosine and phenolic compounds present in the protein and reduces the phosphomolybdate of the folin phenol reagent to a blue color.

Eighteen crabs were exposed to the three sub lethal concentrations of mercury for three time periods of 1, 7 and 15 days. Six crabs served as the controls. At the end of each time period, six crabs from the experimentals were sacrificed and tissue samples of abdominal muscle, chelate muscle, hepatopancreas, and gill were removed and processed for protein, carbohydrate and lipid analysis. The controls were also sacrificed on the 15^{th} day and tissue samples processed for biochemical analysis as done in the case of experimentals.

The samples were oven-dried at 110°C for 24h. A sample of 10 mg dry weight of each tissue was transferred into a test tube, added 5 ml of 1N NaOH , and warmed slightly for 10 minutes, but not over heating. After warming, the tubes were cooled and their volumes adjusted to 10 ml with distilled water, and then centrifuged for 5 minutes at 3500 rpm. From this 1 ml of the supernatant was taken, then 5 ml of alkaline copper reagent added and mixed well, incubated for 10 minutes, 0 .5 ml of Folin's phenol reagent was added, and mixed well. The optical density of the sample was determined spectrophotometrically at 500 nm, after 30 minutes. The concentration of total protein is expressed as mg/100gm dry weight of the tissues.

Estimation of Total Free Sugar Content

The total free sugar content was estimated by anthrone method (Roe, 1955).

Principle:

Sulphuric acid hydrolyses disaccharides and oligosaccharides into monosaccharides and converts monosaccharides into furfuryl

derivatives, which react with anthrone and produce the colored product.

A 10mg dry weight sample of each tissue was weighed out into a test tube, and hydrolyzed by keeping it in a boiling water bath for 3 h with 2.5 N Hcl , and cooled to room temperature, neutralized with solid Na_2Co_3 until the effervescence ceased. The volume was then made up to 10 ml and centrifuged at 3500 rpm for 5 minutes, the supernatant was pipetted out, and 1 ml of the aliquot was taken for analysis. To that added 4 ml of anthrone reagent, and boiled for 8 min in a boiling water bath. The optical density of the sample was determined spectrophotometrically at 620 nm. The concentration of total free sugar content is expressed as mg total sugar/100 g dry weight of the tissue.

Estimation of Lipid (Barnes and Blackstock, 1973)

(Sulphophospho vanillin method)

Principle:

The quantitative estimation of lipid by sulphophospho vanillin method depends on the reaction of lipids extracted from the sample using chloroform–methanol with sulphuric acid, phosphoric acid and vanillin to give a red complex.

Procedure

To a 10 mg dry weight sample of each tissue were added 10 ml of chloroform-methanol (2:1 v/v), and mixed well. Filtered the homogenate through a Whatman No.1 filter paper, added 2ml of sodium chloride solution (0.9% Nacl), shaken well, transferred the mixture to a small separating funnel, and allowed to stand overnight at 0°C, to obtain a clear biphasic layer. The lower phase contains lipids. Removed the lower

phase and adjusted the volume to 10 ml by addition of chloroform. A 0.5 ml sample of this extract was taken into a clean dry test tube, dried in a vacuum desiccator over silica gel, then dissolved in 0.5 ml of concentrated sulphuric acid, mixed well, and the test tubes were plugged with non absorbent cotton wool. Placed in a boiling water bath for 10 minutes and then cooled the tubes to room temperature.

To 0.2 ml extract of this acid digest, pipetted 5 ml of vanillin reagent, mixed well and allowed to stand for half an hour, and measured the developed color at 520 nm in a spectrophotometer.

3.4. Results

Protein

Abdominal muscle tissue

In controls, the protein level in the abdominal muscle tissue on the 1st day was 68.60±2.95 mg /100 gm (Table 8, Fig. 7), while the experimentals exposed to the three sub lethal concentrations of mercury showed the values as 55.13± 2.12, 49.95±2.07, and 46.15 ±1.98 mg/100 gm (Table 8, Fig. 7) on the first day. The values on the 7th day in the controls and the three experimentals were 65.12 ± 2.19, 52.37 ±2.06, 46.50 ±1.92 and 42.03 ±1.78, respectively. On the 15th day the values in the controls and the three experimentals were 62.09±1.89, 50.99 ±1.69, 44.28 ±1.43, and 40.11 ± 1.22, respectively (Table 8, Table 9, and Fig. 7). Significant changes in the total protein content were observed in all the three experimentals (F=519.71; critical value 3.223, Table 9), and also during the different time periods (F = 172.55, critical value 2.223, Table 9). Treatment versus duration (TxD) showed an F value of 137.91 and critical value 6.224(Table 9).

Chelate muscle tissue

In the case of controls the total protein content in chelate muscle tissues on the 1st day was 56.99 ±1.79 mg/100gm (Table 8, Fig. 8), where as in the experimentals the total protein content on the 1st day was 50.86 ±1.65, 44.72±1.54, 38.25 ± 1.49 (Table 8 , Fig. 8). The values in the control organisms on the 7th and 15th day were 55.00± 1.69, 51.23 ± 1.63 mg/100 gm (Table 8, Fig. 8). Significant changes in total protein content among the experimentals were also observed (F=2731.44; critical value 3.22, Table 9). Similarly, significant changes in total protein content were observed in all the treatments during the time periods (F= 582.50; critical values 2.22; Table 9). Treatment versus duration (TxD) showed an F value of 210.68, and critical value 6.22 (Table 9).

Hepatopancreas

Total protein content of the controls on the 1st day was 28.10± 1.35 mg/100gm (Table 8, Fig. 9). In the case of treated organisms the total protein contents on the 1st day were 26.88±1.33, 21.69±1.32, 20.83±1.30 mg/100gm (Table 8, Fig. 9). The values in the controls on the 7th and 15th days were 26.52±1.34, and 22.10±1.31mg/100gm (Table 8,Fig. 9). Similar changes in total protein content were observed among the experimentals (F=689.82; critical value 3.22; Table 9). Significant changes in the protein content were also observed in the experimentals at different time periods (F=282.56, critical value 2.22; Table 9). Treatment versus duration (TxD) showed an F value of 359.82; and critical value 6.22 (Table 9).

Gills

In the case of the controls the total protein content in the gill tissue on the first, seventh and fifteenth days were 18.90 ± 1.26 mg/100 gm, 14.70 ± 1.24, and 12.29 ± 1.20, respectively (Table 8, Fig. 10). The three sub lethal concentrations of mercury have produced significant changes in the

levels of total protein content (F = 671.28; critical value 3.225, Table 9). Similarly, the values in the time periods also showed significant changes (F = 31.95, critical value 2.22 Table 9). Treatment versus duration (TxD) showed an F value of 238.95; and critical value 6.224 (Table 9).

Table 8. Levels of total protein in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values expressed as mg/100 gm dry weight of tissue. Each value is a mean of six observations ± SD. Values in parenthesis are percentage change over control.

Tissues	Days	Control	0.009	0.02	0.04
Abdominal muscle tissue	1	68.60±2.95 (100%)	55.13±2.12 (80.36%)	49.95±2.07 (72.81%)	46.15±1.98 (67.27)%
	7	65.12±2.19 (100%)	52.37 ± 2.06 (80.44 %)	46.50±1.92 (71.40%)	42.03±1.78 (64.54%)
	15	62.09 ±1.89 (100%)	50.99±1.69 (82.12%)	44.28±1.43 (71.31%)	40.11±1.22 (64.59%)
Chelate muscle tissue	1	56.99±1.79 (100%)	50.86±1.65 (89.24%)	44.72±1.54 (78.43%)	38.25± 1.49 (67.11%)
	7	55.00±1.69 (100%)	48.28 ± 1.58 (87.78%)	42.99±1.48 (78.16%)	33.45 ±1.42 (60.81%)
	15	51.23 ± 1.63 (100%)	45.27±1.56 (88.36%)	40.33 ± 1.52 (78.72%)	31.96 ± 1.39 (62.385)
Hepatopancreas	1	28.10 ±1.35 (100%)	26.88 ±1.33 (95.65%)	21.69±1.32 (77.18%)	20.83 ±1.30 (74.12%)
	7	26.52±1.34 (100%)	24.10±1.29 (90.87%)	19.71 ± 1.25 (74.33%)	18.25±1.23 (68.81%)
	15	22.10 ±1.31 (100%)	20.36 ± 1.29 (92.12%)	17.44±1.27 (78.91%)	15.17±1.24 (71.08%)
Gills	1	18.90±1.26 (100%)	15.48±1.25 (81.90%)	13.08± 1.22 (69.2%)	12.00±1.20 (63.49%)
	7	14.70± 1.24 (100%)	13.98± 1.23 (95.10%)	11.50±1.21 (78.23%)	10.94±1.19 (74.42%)
	15	12.29±1.20 (100%)	11.00± 1.17 (90.15%)	9.44 ± 1.14 (77.37%)	8.00±1.12 (65.57%)

Table 9. Two way ANOVA table showing the significance of the effect of mercury on the total protein levels in the tissues of *Scylla serrata*.

	Variables	Model	df	F	P	Fcrit
Total Protein	Abdominal muscle tissue	Treatment (T)	3	519.71	< 0.05**	3.223
		Duration (D)	2	172.55	< 0.05**	2.223
		T x D	6	137.91	< 0.05**	6.224
		Error	132			
	Chelate muscle tissue	Treatment (T)	3	2731.44	< 0.05**	3.225
		Duration (D)	2	582.50	< 0.05**	2.223
		T x D	6	210.68	< 0.05**	6.224
		Error	132			
	Hepatopancreas	Treatment (T)	3	689.82	< 0.05**	3.225
		Duration (D)	2	282.56	< 0.05**	2.223
		T x D	6	359.82	< 0.05**	6.224
		Error	132			
	Gills	Treatment (T)	3	671.28	< 0.05**	3.225
		Duration (D)	2	31.95	< 0.05**	2.223
		T x D	6	238.95	< 0.05**	6.224
		Error	132			

df – degrees of freedom F – 'F' Value P – 'P' value

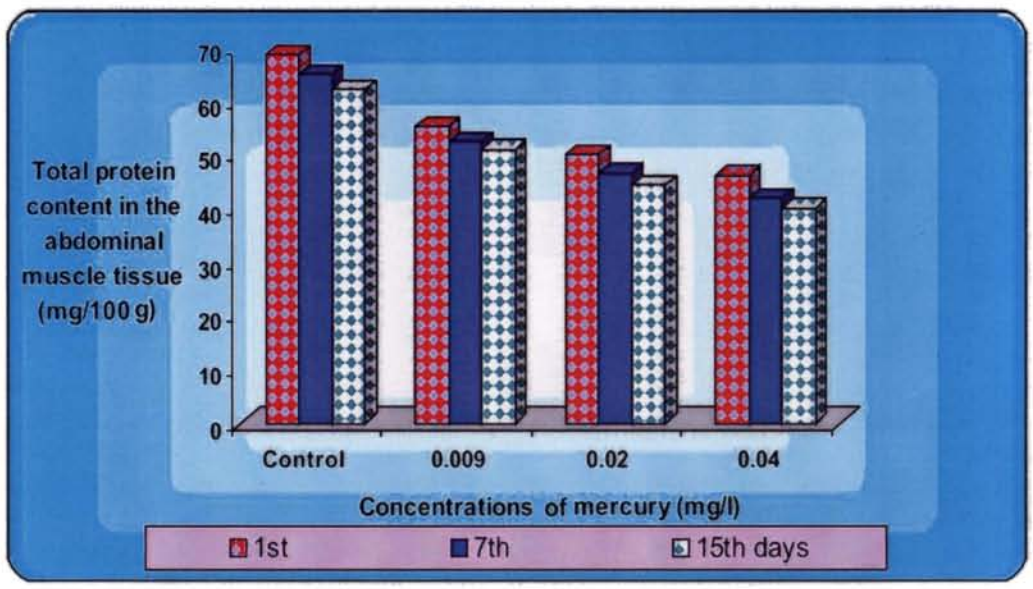


Figure 7. Total protein content in the abdominal muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

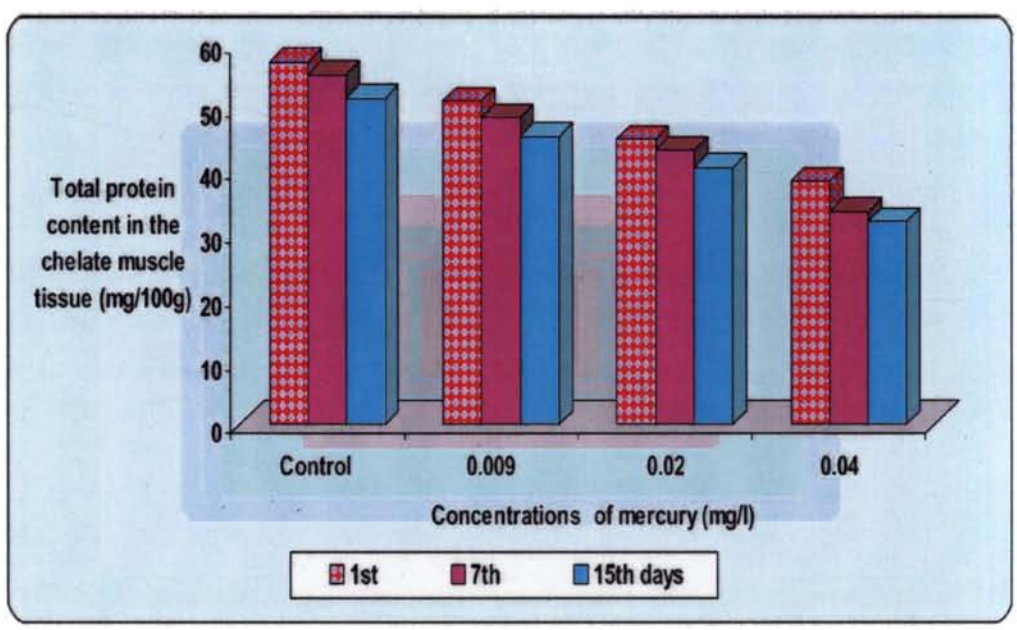


Figure 8. Total protein content in the chelate muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

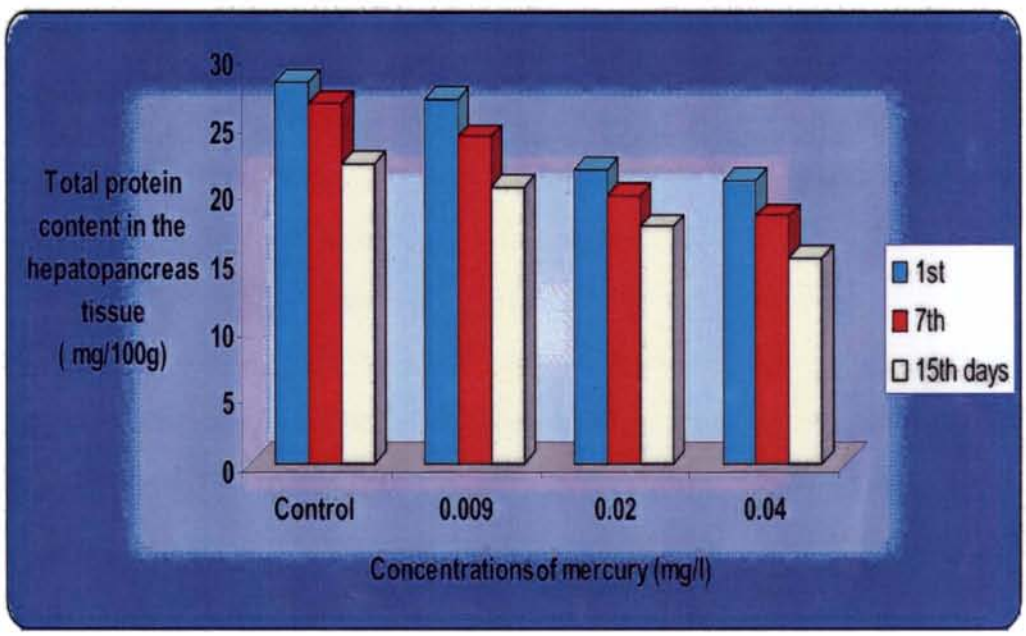


Figure 9. Total protein content in the hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

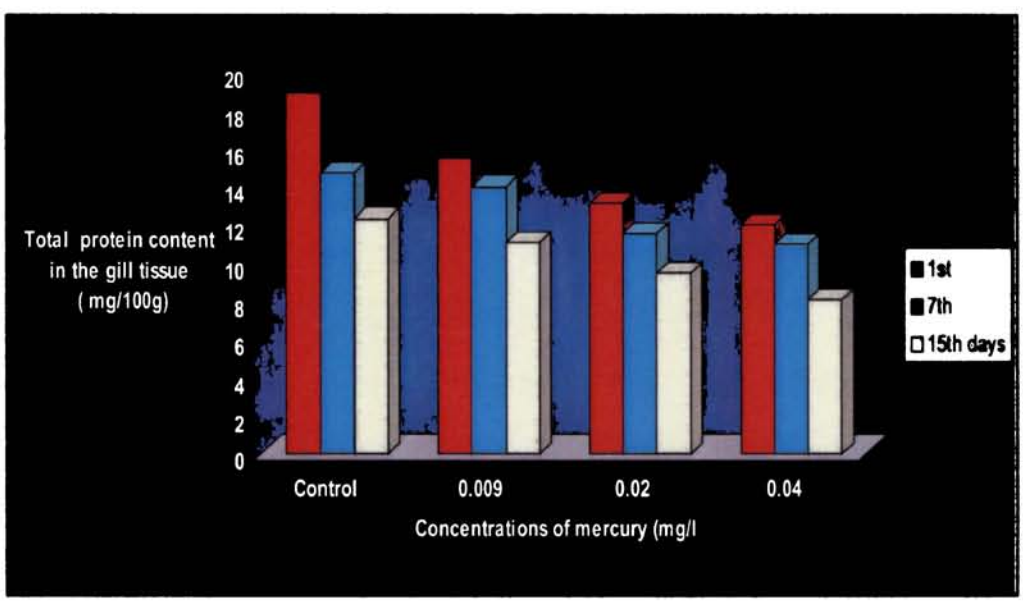


Figure 10. Total protein content in the gills of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

Carbohydrate

Abdominal muscle tissue:

In controls the total sugar content of the abdominal muscle tissue on the 1st day was 4.20± 0.33 mg/100 gm (Table 10, Fig. 11), where as in the experimentals the values on the 1st day were 3.42±0.23, 2.60+0.16, and 2.15±0.10 mg/100 gm (Table 10, Fig. 11). The control values on days 7 and 15 were 3.46 ± 0.15, and 2.16±0.12mg/100gm (Table 10, Fig. 11). Among the experimentals the values for the corresponding periods declined sharply. Significant changes in the total sugar content in the abdominal muscle tissue were observed in all the three treatments (F = 366.07, critical value 3.113, Table 11). Sub lethal concentrations of mercury in the medium produced significant changes in the levels of total sugar content during the different time periods also (F=450.20, critical value 2.223; Table 11). Treatment versus duration (TxD) showed an F value of 280.20 and critical value 6.553 (Table 11).

Chelate muscle tissue

In the controls total sugar content on the 1st day was 4.83± 1.02 mg/100gm (Table 10, Fig. 12). In the case of the experimentals the values on the first day were 3.86±0.10, 3.06±0.19, and 2.66±0.25 mg/100gm (Table 10, Fig. 12). Among the controls values on days 7 and 15 were 4.79± 0.33, and 3.96 ±0.15 mg/100gm (Table 10, Fig.12). Changes were observed in the 7th as well as 15 the day values in the experimentals (F= 1233.61, critical value as 3.113, Table 11). Significant changes were also observed during the different time periods (F=500.42, critical value 2.22, Table11). Treatment versus duration (TxD) gave an F value of 658.84 and critical value of 6.553(Table 11).

Hepatopancreas

The total sugar content in the hepatopancreas on the 1st day in the controls was 7.36 ± 0.32 mg/100gm (Table 10, Fig. 13). In the case of the experimentals the values on day 1 were 5.35± 0.09, 5.05± 0.01, and 2.56± 0.07 mg/100gm (Table 10, Fig. 13). Significant changes were observed in the values of the experimentals on 7th and 15th day of exposure (F=1357.38, critical value 3.11, Table 11), and also during the different time periods (F=58.56, critical value 2.22; Table 11). Treatment versus duration (TxD) gave an F value of 297.51, and critical value 6.55 (Table 11).

Gills

In the case of gill tissue, total sugar content in the controls on the 1st day was 6.15±0.28 mg/100gm (Table 10, Fig. 14), whereas in the experimentals the values for the same time period were 5.95±0.07, 4.80 ±0.15, and 3.67±0.12 mg/100gm (Table 10, Fig. 14). The values in the controls on days 7 and 15 were 5.15± 0.07, and 4.00±0.06 mg/100gm (Table 10, Fig. 14). Among the experimentals the values were significantly low as the concentrations (Table 10; F= 1721.93, critical value 3.11, Table 11) and time periods increased (F=585.79, critical value 2.22, Table.11). Treatment versus duration (TxD) gave an F value of 520.35, critical value 6.553, Table 11).

Table 10. Levels of total sugar in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values expressed as mg/ 100 gm dry weight of tissue. Each value is mean of six observations ± SD. Values in parenthesis are percentage change over control.

Tissues	Days	Control	0.009	0.02	0.04
Abdominal muscle tissue	1	4.20±0.33 (100%)	3.42±0.23 (84.79%)	2.60 ±0.16 (62.86%)	2.15±0.10 (51.19%)
	7	3.46±0.15 (100%)	2.75±0.07 (77.34%)	1.90±0.12 (54.91%)	1.20±0.33 (34.68%)
	15	2.16 ± 0.12 (100%)	1.77±0.28 (81.94%)	1.06 ±0.38 (49.07%)	0.82 ±0.05 (37.96%)
Chelate muscle tissue	1	4.83±1.02 (100%)	3.86 ±0.10 (81.98%)	3.06±0.19 (67.49%)	2.66±0.25 (55.07%)
	7	4.79 ±0.33 (100%)	3.56 ±0.13 (74.32 %)	2.61±0.13 (54.48%)	1.2 3±0.04 (24.04%)
	15	3.96 ± 0.15 (100%)	3.14 ± 0.25 (79.29%)	1.84 ±0.09 (43.93%)	1.05 ± 0.01 (26.51%)
Hepatopancreas	1	7.36 ±0.32 (100%)	5.35 ± 0.09 (34.78%)	5.05 ±0.01 (22.55%)	2.56 ±0.07 (63.17%)
	7	6.37±0.29 (100%)	4.65 ± 0.06 (55.41%)	2.60±0.03 (40.81%)	2.11±0.03 (33.12%)
	15	5.91 ± 0.25 (100%)	3.53 ± 0.18 (39.93%)	1.66 ± 0.01 (28.08%)	1.13 ± 0.02 (19.12%)
Gills	1	6.15±0.28 (100%)	5.95± 0.07 (96.74%)	4.80± 0.15 (78.04%)	3.67 ± 0.12 (59.67%)
	7	5.15±0.07 (100%)	2.97 ± 0.25 (57.66%)	2.52±0.07 (48.93%)	1.80± 0.15 (34.95%)
	15	4.00± 0.06 (100%)	2.98 ± 0.05 (58.75%)	2.10±0.09 (52.5%)	1.38 ± 0.06 (34.53%)

Table 11. Two way ANOVA table showing the significance of the effect of mercury on the total sugar levels in the tissues of *Scylla serrata*.

Variables		Model	df	F	Fcrit	P
Total Sugar	Abdominal muscle tissue	Treatment (T)	3	366.07	3.113	< 0.05**
		Duration (D)	2	450.20	2.223	< 0.05**
		T x D	6	280.20	6.553	< 0.05**
		Error	132			
	Chelate muscle tissue	Treatment (T)	3	1233.61	3.113	< 0.05**
		Duration (D)	2	500.42	2.223	< 0.05**
		T x D	6	658.84	6.553	< 0.05**
		Error	132			
	Hepatopancreas	Treatment (T)	3	1357.38	3.113	< 0.05**
		Duration (D)	2	58.56	2.223	< 0.05**
		T x D	6	297.51	6.553	< 0.05**
		Error	132			
	Gills	Treatment (T)	3	1721.93	3.113	< 0.05**
		Duration (D)	2	585.79	2.223	< 0.05**
		T x D	6	520.35	6.553	< 0.05**
		Error	132			

df – degrees of freedom F – 'F' Value P – 'P' value

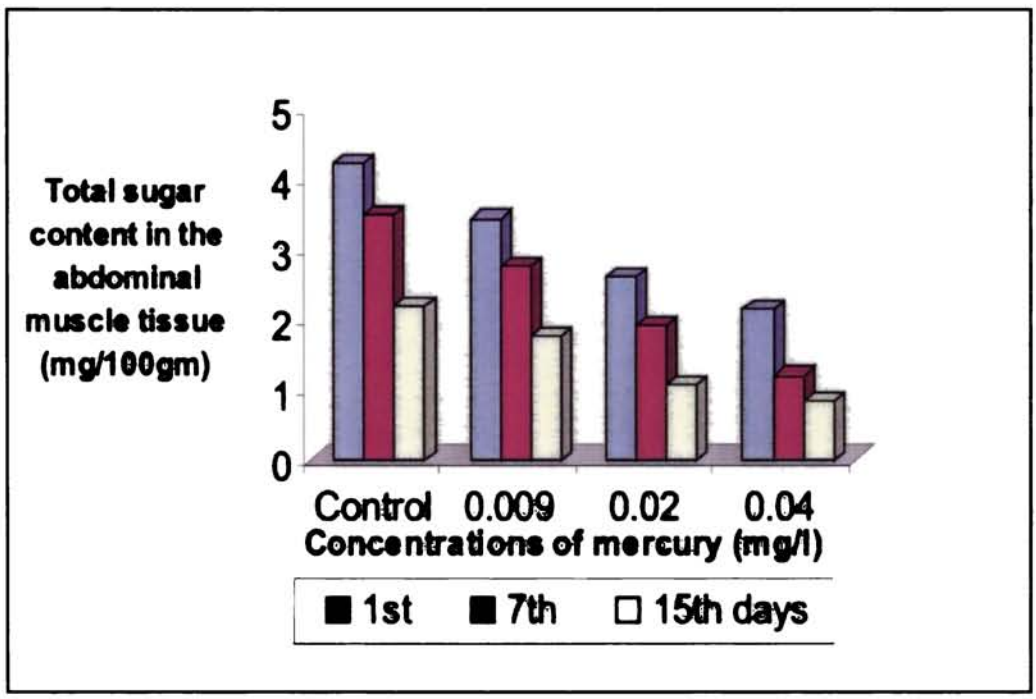


Figure 11. Total sugar content in the abdominal muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

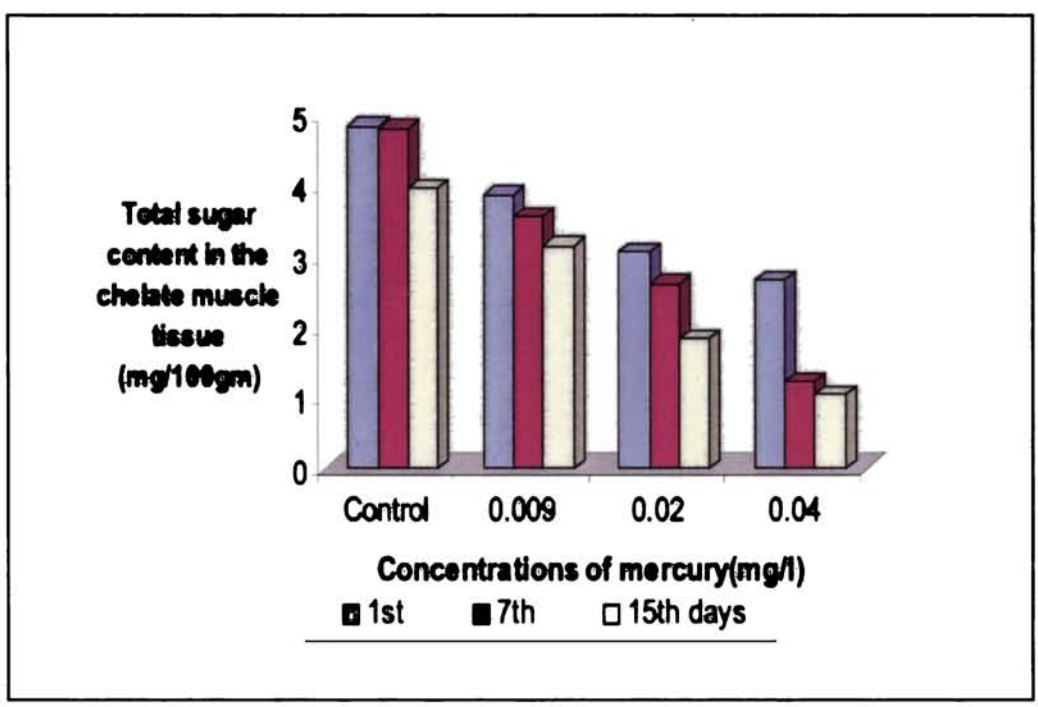


Figure12. Total sugar content in the chelate muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury (*n*-6)

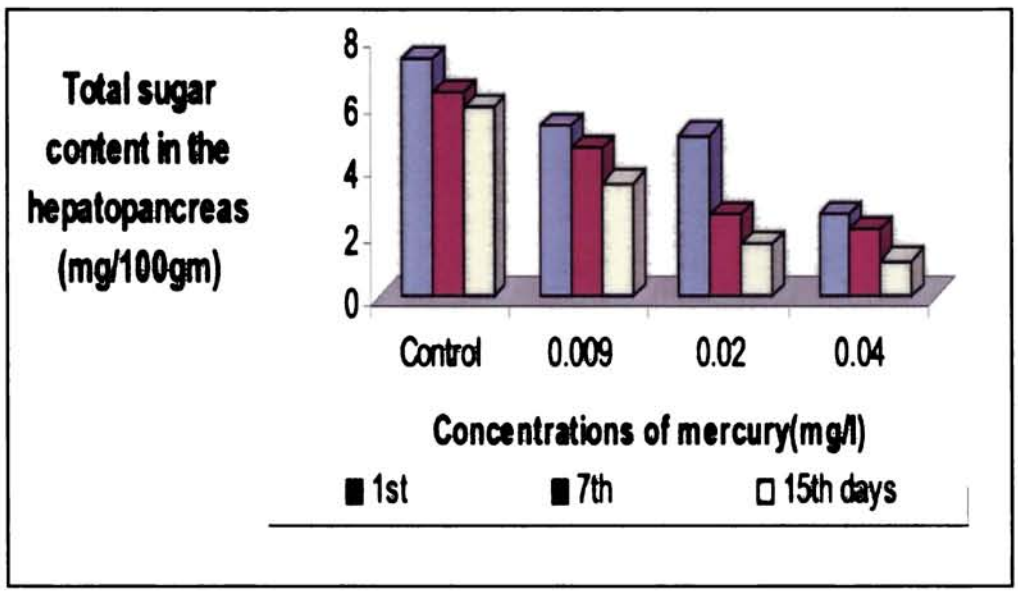


Figure 13. Total sugar content in the hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

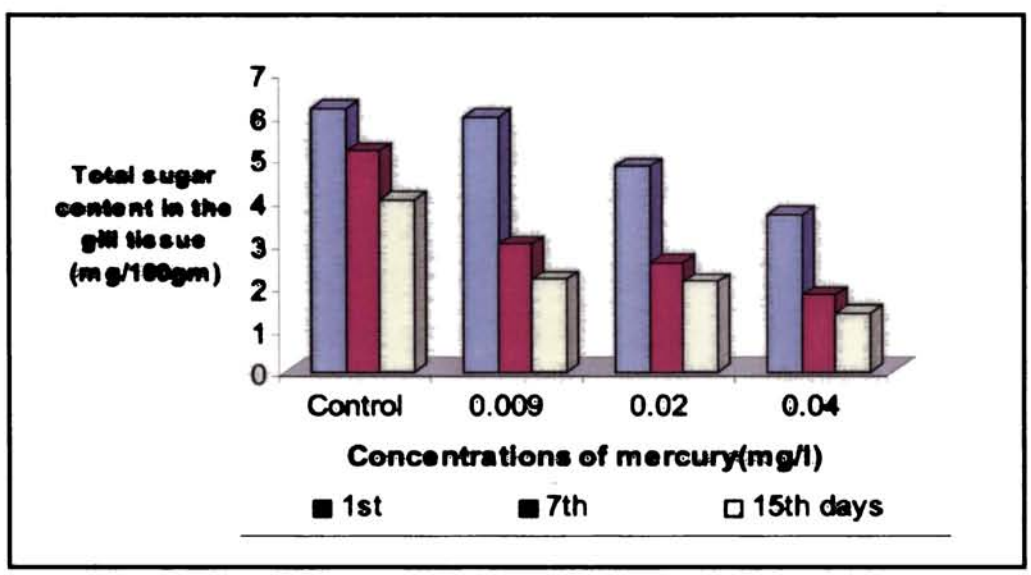


Figure 14. Total sugar content in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

Lipid

Abdominal muscle tissue

In controls total lipids in the abdominal muscle on the 1st day was 37.42± 1.43 mg /100 gm (Table 12, Fig.15), while in the experimentals for the same day the values were 27.18±1.93, 23.62±1.91, and 20.5 ±1.00mg/100 gm (Table 12, Fig. 15). The control values on days 7 and 15

were 29.53±1.61, and 26.33± 1.68 mg /100gm (Table 12, Fig. 15). Significant changes in lipid contents were observed on 7th and 15th day values in the experimentals (Table 12, Table 13- F=664.917; critical value 3.15) Significant changes in the total lipid content were also observed in all the experimentals (Table 12, Table 13-F =982.26, critical value 2.758). Concentration versus days (CxD) gave an F value of 10.114 and critical value 2.25 (Table 13).

Chelate muscle tissue

In the case of controls, the value on day 1 was 25.25 ±1.52 mg/100gm (Table 12, Fig. 16) where as in the experimentals for the same time period the values were 22.13 ±1.51, 20.53± 0.55, 18.68 ± 0.56 (Table 12, Fig. 16). The control values on days 7 and 15 were 20.22± 0.28, and 19.66 ± 0.21 mg/100 gm (Table 12, Fig. 16). In the experimentals significant changes in values were observed also on days 7 and 15 (Table 12, Table 13- F=836.90; critical value 3.15), so also in different concentrations (Table 12, Table 13- F= 530.586; critical values 2.758). Concentration versus days (CxD) gave an F value of 19.015, critical value 2.25 (Table 13).

Hepatopancreas

The total lipid content in the controls on days 1,7 and 15 were 108.83± 7.38 mg/100gm, 93.65±5.84, and 86.96±4.22mg 100gm (Table 12, Fig. 17). In the experimentals the values were found to be significantly low in all concentrations (Table 12, Table 13-F=209.575; critical value 2.758- Table 13), so also in different time- periods (Table 12, Table 13- F=304.086, critical value 3.150). Concentration versus days (CxD) gave an F value of 7. 484, and critical value 2.25 (Table13).

Gills

In the controls lipid values on days 1,7, and 15 were 97.82 ±5.16 mg/100 gm, 82.71±4.26, and 77.1 ±3.83 mg/100gm (Table 12, Fig.18)

while in the experimentals the values were significantly low in all concentrations (Table 12, Table 13-F= 467.752, critical value 2.75), as well as during different time periods (Table 12, Table 13- F = 243.66, critical value 3.15). Concentration versus days (CxD) gave an F value of 12.659, and critical value 2.254 (Table 13).

Table 12. Total lipid content in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values expressed as mg/100 gm dry weight of tissue. Each value is a mean of six observations ± SD. Values in parenthesis are percentage change over control.

Tissues	Days	Control	0.009	0.02	0.04
Abdominal muscle tissue	1	37.42±1.43 (100%)	27.18±1.9 3 (72.63%)	23.62±1.91 (63.12%)	20.5±1.00 (54.78%)
	7	29.53±1.61 (100%)	20.46±1.58 (69.29%)	18.53±0.43 (62.75%)	16.44±0.2 8 (55.67%)
	15	26.33±1.68 (100%)	18.41±1.53 (69.92%)	15.32±0.35 (58.18%)	13.18±0.4 9 (53.85%)
Chelate muscle tissue	1	25.25±1.52 (100%)	22.13±1.51 (87.64%)	20.53±0.55 (81.31%)	18.68±0.5 6 (73.66%)
	7	20.22±0.28 (100%)	18.4±0.53 (91%)	16.28±0.31 (75.57%)	14.17±0.73 (79.97%)
	15	19.66±0.2 1 (100%)	17.49±0.38 (88.96%)	13.82±0.66 (70.3%)	12.22±0.4 3 (62.16%)
Hepatopancreas	1	108.83±7.38 (100%)	97.31±5.85 (89.41%)	88.55±4.88 (81.37%)	82.77±3.32 (76.05%)
	7	93.65±5.84 (100%)	88.05±4.87 (94.02%)	77.31±4.78 (82.55%)	75.55±4.6 9 (84.94%)
	15	86.96±4.22 (100%)	77.27±4.27 (88.86%)	70.92±4.68 (81.55%)	60.98±4.29 (70.12%)
Gills	1	97.82±5.16 (100%)	87.78±5.98 (89.75%)	77.57±5.46 (79.31%)	62.17±2.46 (65.61%)
	7	82.71±4.26 (100%)	77.87±4.43 (94.16%)	68.11±2.18 (82.36%)	60.15±2.57 (72.73%)
	15	77.1±3.83 (100%)	73.5±3.38 (95.33%)	65.65±1.65 (85.15%)	57.91±1.94 (75.11%)

Table 13. Two way ANOVA table showing the significance of the effect of mercury on the total lipid content in the tissues of *Scylla serrata.*

Tissues	Source	Sum of Squares	df	Mean Square	F	F crit	P
Abdominal muscle tissue	Concentrations (C)	2070.487	3	690.162	982.260	2.758	< 0.05 **
	Days (D)	934.378	2	467.189	664.917	3.150	< 0.05 **
	C x D	42.639	6	7.107	10.114	2.254	< 0.05 **
	Error	42.158	60	.703			
	Total	3089.662	71	43.516			
Chelate muscle tissue	Source	Sum of Squares	df	Mean Square	F	F crit	P
	Concentrations (C)	409.512	3	136.504	530.586	2.758	< 0.05 **
	Days (D)	430.620	2	215.310	836.900	3.150	< 0.05 **
	C x D	29.353	6	4.892	19.015	2.254	< 0.05 **
	Error	15.436	60	.257			
	Total	884.921	71	12.464			
Hepatopancreas	Source	Sum of Squares	df	Mean Square	F	F crit	P
	Concentrations (C)	5131.362	3	1710.454	209.575	2.758	< 0.05 **
	Days (D)	4963.624	2	2481.812	304.086	3.150	< 0.05 **
	C x D	366.492	6	61.082	7.484	2.254	< 0.05 **
	Error	489.693	60	8.162			
	Total	10951.170	71	154.242			
Gill	Source	Sum of Squares	df	Mean Square	F	F crit	P
	Concentrations (C)	6512.448	3	2170.816	467.752	2.758	< 0.05 **
	Days (D)	2261.716	2	1130.858	243.669	3.150	< 0.05 **
	C x D	352.511	6	58.752	12.659	2.254	< 0.05 **
	Error	278.457	60	4.641			
	Total	9405.132	71	132.467			

df –degrees of freedom F- 'F' Value P-'P" value

** Significant at 1% level

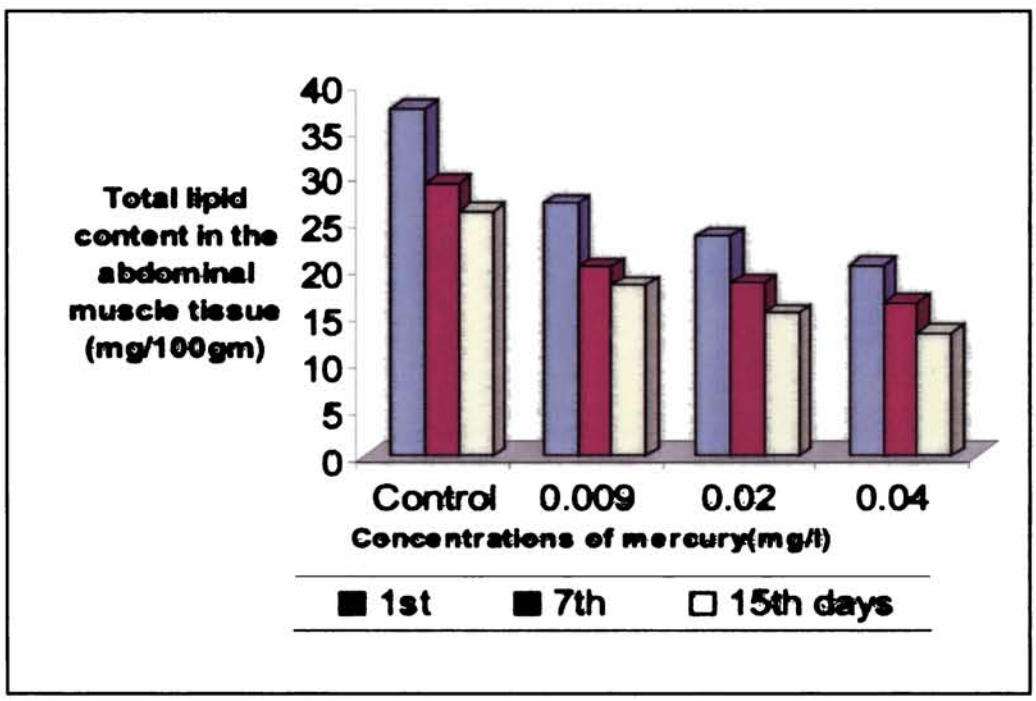


Figure 15. Total lipid content in the abdominal muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

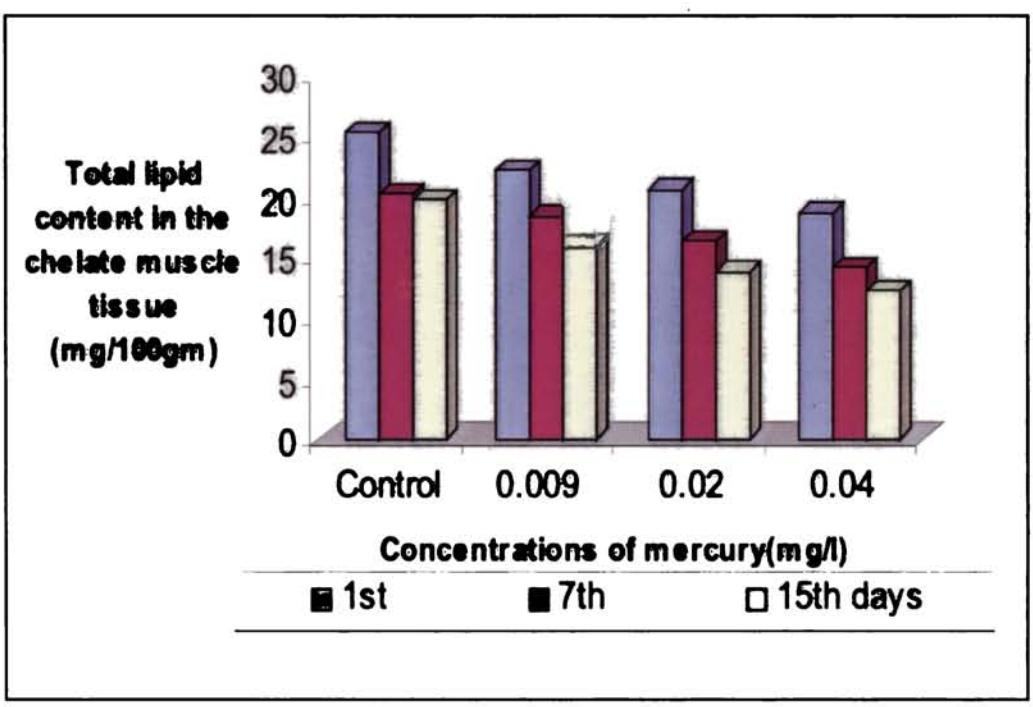


Figure 16. Total lipid content in the chelate muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

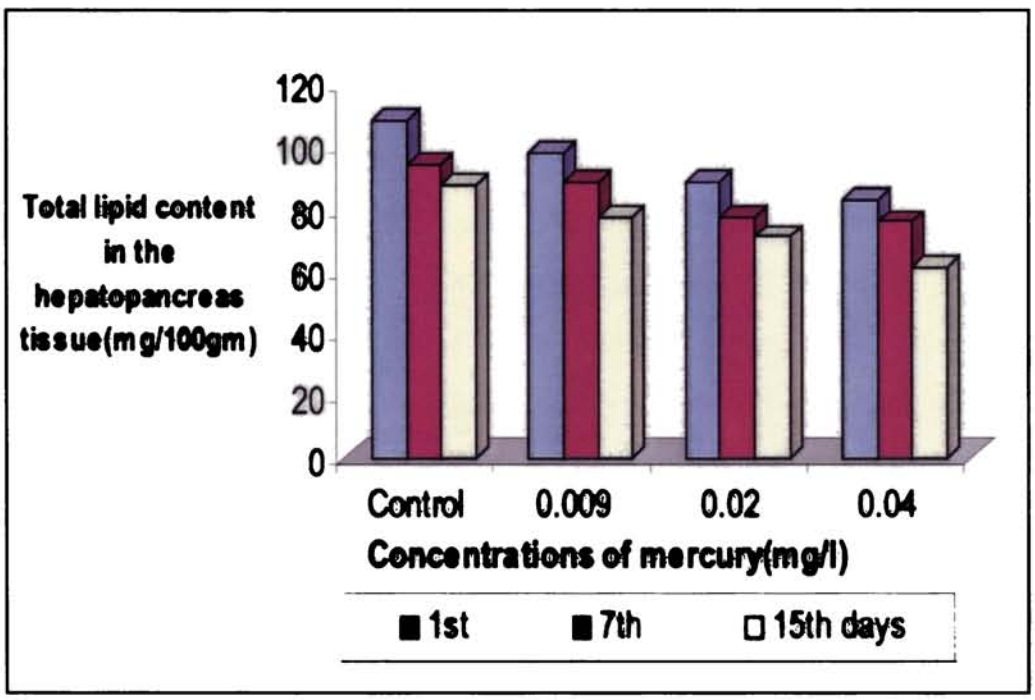


Figure. 17 Total lipid content in the hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

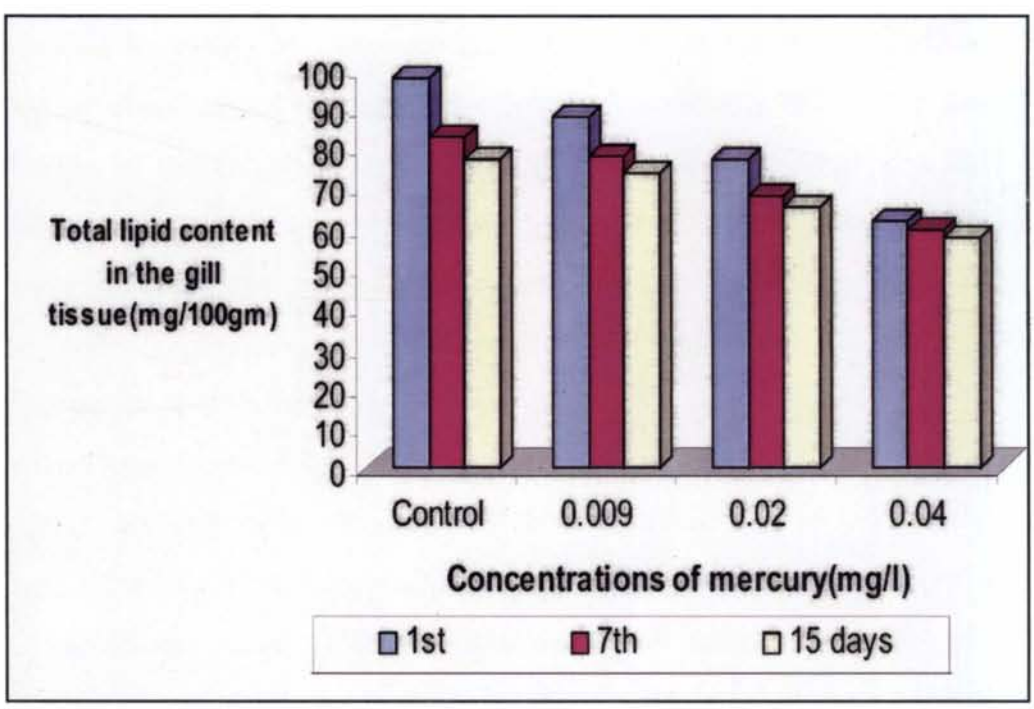


Figure 18. Total lipid content in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

3.5. Discussion

Protein

In the present study total protein contents in the tissues of the experimentals at most of the concentrations, and time periods showed significantly lower values. This decrease in protein content can be attributed to increased proteolytic activities. Similar observations of decreased protein content in *Lamellidens marginalis* were made by Ahmed et al. (1978). Rao et al. (1981) pointed out that ammonia formed during stress may be converted to glutamine and stored in the tissue to be utilized for amino acid and protein synthesis. Kulkarni et al. (1989) reported that increased levels of serum proteins in *Scylla serrata* suggest that proteins might have been released into the blood probably due to mobilization of protein from hepatopancreas and muscles under stressed conditions due to malathion exposure. They added that these proteins in the blood are probably utilized for production of additional energy required to meet the increased metabolic rate of the stressed animals. Similar observations were made by George Varghese (1992). The protein profiles of individual tissues such as gill, muscle, hepatopancreas, heart, and thoracic ganglion showed a loss in protein on the 15th day of exposure to cadmium chloride, with the maximum depletion in thoracic ganglion of the crab, *Barytelphusa cunicularis* (George Varghese, 1992). Increased concentration of protein in *Etroplus maculatus* after 48 hr exposure to copper was reported by Hilmy et al.(1985). Cyriac (1990) reported low protein content in the muscles of the fish *Oreochromis mossambicus* exposed to copper and mercury. In low concentration of copper there was no significant variation in the protein content compared to that of the controls. But in high concentrations of mercury significant decrease in protein level started at 120hr and continued up to 168 hr. A decrease in total protein content could possibly be due to protein breakdown with

increased amino acid pool in the tissue. It was also reported that decreased protein moiety suggests damage to hepatic tissue and an intensive proteolysis resulting in increased amounts of free amino acids to be fed into TCA cycle as ketoacids (Rao,et al., 1984). Loss of weight and elevation of nitrogenous compounds in tissues of fish exposed to benthiocarb were reported by Rao et al. (1987) in *Sarotherodon mossambicus*. Rao et al. (1987) also reported increased protease activity, increased free amino acid content, but decreased protein level in liver, muscle, brain and gills of *Sarotherodon mossambicus*. They have added that the decrease in protein following exposure to mercury and copper suggests their possible degradation by increased proteolysis. This increased proteolysis could be attributed to the damage caused to lysosomal membranes thus permitting the leakage or release of lysosomal enzymes into the cytosol. Radhaiah et al. (1987) observed that amino acids in the kidney increased along with a decrease in the protein values. This proves that intense proteolytic activity in tissues can increase amino acids in the liver. Such increases in amino acids after exposure to toxicants in different organisms were noticed by Girija and Rao (1984). Rath and Misra (1980) examined the changes in nucleic acids and protein content in the liver, muscle and brain of *Tilapia mossambica* exposed to an insecticide. Post-exposure studies revealed a significant decline in DNA, and RNA content of the liver, muscle and brain. They observed that liver exhibited a greater loss of protein than the muscle. It has been demonstrated that in the cells of metal exposed organisms, Cd, Hg and Cu were able to reduce protein synthesis not only by reducing the rate of RNA synthesis, but also influencing the attachment of polyribosomes to the rough endoplasmic reticulum, and probably damaging the ribosome themselves (Viarengo et al., 1980). Similar observations were also reported by Loganathan (1995) in *Macrobrachium malcolmsonii* exposed to sub lethal concentration to mercury and lead.

In the present study protein contents in the hepatopancreas and gill tissues of the controls were comparatively less than in the other two tissues. Statistically significant decrease in protein values was found in all the tissues during the three time-periods, and also in different sub-lethal concentrations of mercury. The decline in protein values may be due to the mobilization of protein to other parts of the body as indicated by Viarengo et al. (1982). Katticaran (1988) had reported that during detoxication of metals, the synthesis of protein declines, and a declining trend was observed during the days of exposure. Similar changes were reported by Mule et al. (1994) who noticed decreased whole body protein content during acute and chronic exposure of the gastropod, *Thiara tuberculata*. Cyril et al. (1993) examined *Cyprinus carpio* exposed to sub lethal concentrations of nickel and lead for 5,10,15 days, and reported that protein and carbohydrate contents in muscle and liver decreased as the concentration of exposure period increased. Decrease in protein level was observed in *Penaeus indicus* due to copper (Sreenivasan, 1988) exposure. The decline in protein values may be due to some amount of energy has to be spent for locomotion from the energy yielding sources, since the animals are not fed during the experimental period. This observations closely resembles the previous observation in *Cyprinus carpio* exposed pesticide (Neelamegam et al., 2007) without addition of food.

Decreased protein levels were noticed in hepatopancreas, gill, muscle and haemolymph of the crab *Thalamitta crenata and Scylla serrata* on exposure to copper and mercury (Villalan et al., 1988;Rajathy, 1991). Total protein contents showed decreasing trend over a period of 4 days in the fresh water field crab, *Paratelphusa Jacquemontii* (Parate et al.,2003). Damage to gill tissue on exposure to toxicant was reported by Doughtie and Rao (1984), and Sunilla and Lindstorm (1985). Chatterjee and Bhattachariya (1986) suggested that in low concentration degradation

of protein exceeds protein synthesis where as in higher concentration the synthesis exceeds degradation. Similar inverse relations between concentration and protein were reported by Bhakthavasthalam and Reddy (1984). They also reported that energy demand was higher in higher concentration of the toxicant. Similar depletion of protein and total sugar in Labeo rohita due to copper toxicity were reported by Dhankar and Sliukia (2004). Finally, the fall in protein level is indirectly increased in catabolism and decreased anabolism of protein (Neelamegam et al.,2007).

Carbohydrates

In the present investigation significantly lower sugar levels were noticed in all the tissues, during the three time periods of exposure of crabs to the sub lethal concentrations of mercury.

The heavy metals tend to have a longer residence time in the gut with low permeability coefficient of divalent cations across the lipid bilayer membranes. Profuse binding on to the negative charged sites on the mucosal side of the gut increases the concentrations of heavy metals in the lumen. This interferes with the normal process of nutrient absorption, which explains the need for catabolism of stored energy (Farman Farmain and Socci, 1984). Carbohydrate deposits in the form of glycogen in tissues such as liver and muscle, provide the immediate energy requirements in teleost fishes under different kinds of stress (Dange, 1986 a). Glycogen depletion in the liver is reported in response to lead in *Puntius conchonius,* and in response to mercury (Gill and Pant, 1981). In some fishes glycogen levels were not affect by mercury (Sastry and Rao, 1984). Tort and Torres (1988) exposed dogfish *Scyliorhinus canicula* to sub lethal concentrations of cadmium, and observed an increase in glucose and lactic acid concentrations. George Varghese (1992) observed elevation of blood sugar levels in *Barytelphusa cunicularis* exposed to mercury, copper and zinc and attributed this to the physiological stress

caused by the heavy metals. When crabs absorb very little oxygen from the environment the respiratory metabolism is depressed and therefore stored intracellular glycogen is utilized. The increased lipid content in hepatopancreas may be either channeled to derive energy due to decline in the levels of glucose and glycogen or utilized in the synthesis of structural components of cells under endosulfan toxic stress (Rajeswari, 1989). In *Macrobrachium kistensis* it was reported that there existed an inverse relationship between blood glucose level and mid gut glycogen levels on account of copper and zinc pollution, and it was suggested that an inter conversion operates between the two energy sources depending on the energy requirement of the organisms (Nagabhushanam and Kulkarni, 1981). The muscle and gills of the crabs presented a picture similar to that of the hepatopancreas as regards to their free sugar levels. Increase in free sugars has been reported in fishes and crustaceans exposed to pollutants (Sakundala, 1992). Ramamurthy et al. (1984) and Rajathy (1991) had reported that mercury intoxication leads to decrease in glycogen level, and there was an initial increase followed by a decrease in glycogen and glucose levels of the blood. These authors explained that the initial increase was due to glucogenolysis to generate more glucose to meet energy requirement demanded by the stressful situation, and the subsequent decrease was due to the inhibition of enzymes involved in carbohydrate metabolism. Shrivastava et al. (2003) studied glucose content in the muscle of *Heteropneustes fossilis*, and reported significant decrease in muscle glucose content from 1st week of treatment of carbaryl. Reddy et al. (1986) observed reduction in carbohydrates in the rice field crab *Oziotelphusa senex senex* exposed to malathion. There are various reports which indicate that the blood sugar levels are elevated on exposure to a variety of toxicants which caused several alterations and stressful conditions in the organism (Gluth and Hanke , 1984; Sastry and Subhadra, 1985; Tewari et al., 1987). Ramamurthy et al. (1984) had reported that

mercury toxication leads to a decrease in glycogen level, and there is an initial increase followed by a decrease in glycogen/ glucose level in blood. The authors have explained that the initial increase is due to glycogenolysis to generate more glucose to meet energy requirements demanded by the stressful situation, and subsequent decrease is due to the inhibition of enzymes involved in carbohydrate metabolism.

Lipids

In the present study the total lipid content in all the selected tissues of the crabs exposed to the thee sub lethal concentrations of mercury showed significantly decreased values at all the time periods. Similar depleted levels of lipid were also observed in *Thalamita crenata* exposed to copper (Villalan et al., 1988), and *Barytelphusa hydrodroma* exposed to lead and chromium (Sakundala, 1992). In view of the above facts it is suggested that lipid would be utilized during stressed situation when there might be demand for more energy to tide over the situation. Various authors also reported similar observations. A significant concentration dependent decrease in the lipid content was observed in *Cirrhinus mrigala* (Rao et al., 1984), and *Heterometrus fulvipes* (Rajyalakshmi and Reddy, 1988) when exposed to carbaryl. Ferrando and Andres –Moliner (1991) reported significant lowering of the lipid level in *Anguilla* exposed to lindane. The considerable decreases in total lipids might be a sequel to the efforts of the organism to replenish any glycogen deficiency caused by any extraneous factor, and or to mitigate the multi-furious toxicity of the xenobiotics present in the animals (Rao and Rao, 1981). Chronic exposure of the cod, *Gadus morhua* to crude oil resulted in an increase in fatty acids and phospholipids but decrease in the triglyceride concentration. These effects indicated an enhanced mobilization and utilization of stored triglycerides to meet the increased energy demands (Dey et al., 1983). The decrease in total phospholipids observed in

Oncorhynchus kistuch and *Oreochromis mossambicus* when exposed to PCB's and methyl parathion (Rao and Rao, 1984), respectively, could be attributed to their use of energy to resist a stress.

The sub lethal and median lethal doses of carbaryl on *Rana tigrina* for 96 hours to determine their toxic effect on lipid and cholesterol levels in the liver, muscle and intestine, and cholesterol level in blood at the same period showed decreases in cholestrol in the blood (Sampath et al. 1997). Loganathan (1995) also reported similar observations in the fresh water prawn *Macrobrachium malcolmsonii* exposed to mercury and lead. Vijay Kumar and Kannupandi (1989) also observed similar results in the mangrove crab, Sesarma brockii when exposed to the organophosphate insecticide phosphomidon. Decrease in total lipid content might be due to utilization of lipids during the toxic stress as suggested by Tantorpole et al. (2003). Agarwal and Srivastava (2004) were reported highest sensitivity of cholesterol to zinc exposure followed by glycogen and proteins in Channa punctatus .

Chapter 4

BIOCHEMICAL STUDIES-II: ENZYMES

4.1. Introducion

Enzymes are a major class of proteins in the living system, which function as catalysts directing and accelerating biochemical reactions. They offer a potential area for the monitoring of biological effects and have attracted a great deal of attention of scientists from various fields. Toxicant at each step in any metabolic process is dependent on a specific enzyme; inhibition (or) acceleration of one enzyme activity is bound to cause a series of metabolic disorders. The effects of toxicants on the key enzymes of metabolism have become a topic of common interest to toxicologists and biochemists.

Enzymes may affect the activities or functions of metals in several ways. They can bind to a number of sites on proteins including imidazol, histidyl , carboxyl and especially sulfhydryl side chains (Kench ,1972). Binding at or near enzyme active site may interfere with substrate binding. Binding at locations away from the active site can still cause conformational changes leading to alterations in enzymatic activity or loss of regulatory function (Ulmer, 1970)

Studies of Gould et al. (1976), and Thurberg et al. (1977) have demonstrated that structural and other properties of enzymes as well as specific activities can be affected by exposure of animals to pollutants possibly leading to loss of metabolic flexibility. Responses of enzymes also vary with the rate and magnitude of absorption of the toxicants. Thus, the

impact of xenobiotics at cellular and sub-cellular levels can be perceived by enzymological studies. Metals influence the rate of action of certain enzymes by activation, inactivation, or uncoupling reactions through mechanisms yet to be defined. Hence, a study of metabolic and enzyme activities of aquatic organisms is essential to provide a tangible basis for anticipating and understanding the ecological effects of an accelerated input of heavy metals into the freshwater or aquatic environment.

Many xenobiotics enter the environment through the activities of man, and are accumulated in different organisms. The existence and functioning of detoxification system have been considered to be of great significance recently, and changes in the levels of enzyme activities or in the total content of the enzymes are considered as specific indicators of stress (Bayne et al., 1979; Lee et al., 1980; Moore, 1985). It is also known that many xenobiotics cause cell injury by reacting primarily with biological membranes or membrane components (Moore, 1985).Metals can combine with enzymes in many ways. Binding of metals at remote location on the enzyme molecule will influence the activity, which could range from activation to complete inhibition (Jackim et al., 1970).

The principal reaction of mercury involves binding of thiols and formation of stable mercaptides (Hughes, 1957). Alteration in cell membranes brought about by binding of mercury to sulfhydryl groups could stimulate membrane turn over and production of hydrolytic enzyme (Arstila and Trump, 1968). Mercury is also shown to inhibit enzymes of Kreb's cyle (Yoshino et al., 1966), as reducing levels of ATP could decrease extrusion of hydrolytic enzymes from cells and thereby causing increased activity of acid phosphatase (ACP) in lysosomal mitochondrial fraction (Hinton and Koenig, 1975).

Lysosomes and cell membrane are the first target of xenobiotics as lysosomes are concerned with the disintegration of foreign bodies, and cell membrane is the first barrier to a pollutant. When the lysosomal membrane is made unstable, the enzymes will be released into the cytoplasm. Thus, monitoring of alterations in lysosomal latency, and acid hydrolase's activities are found to be two excellent indices of pollution (Moore and Stebbing, 1976). Phosphates are metal requiring enzymes whose activity may get modified by added cations.

Acid and Alkaline Phosphatases (ACP and ALP) are groups of enzymes that hydrolase phosphomonoesters in a relatively non-specific manner with optimum activity in the acidic and alkaline ranges, respectively. Acid phosphate is a lysosomal marker enzyme and is reported to be a good stress indicator in biological systems. Xenobiotics can cause injury to lysosome, which later release the hydrolytic enzyme leading to cell atrophy. This type of injury resulting in destabilization of lysosomal membrane bears a quantitative relationship to the magnitude of stress response (Bayne et al., 1979, 1982) Alkaline phosphatase is a plasma membrane bound enzyme involved in membrane transport and other intracellular functions.

The present investigation is to determine the feasibility of evaluating the enzyme activity profiles for diagnosing sub-lethal effects of mercury in crabs. The two enzymes chosen for this purpose were Acid and Alkaline phosphatases.

4.2. Review of Literature

Aquatic organisms, in general, are found to accumulate exceptionally high levels of heavy metals in tissues without showing any discernible biochemical stress. This clearly suggests that these organisms have evolved some kind of intrinsic mechanism to detoxify the impact of heavy metals.

Hinton et al. (1973) studied the effect of mercuric chloride intoxication in channel catfish and observed marked changes in ACP and ALP in liver. Lysosomes play an important role in the sequestration and detoxication of heavy metals and is well documented although it is not certain whether this occurs through organo-metallic complexes ,which are partially degraded ,or through direct uptake from cytoplasm followed by chelation with lipofuschins (George,1983).This has led to recognition of the importance of biochemical and cytochemical studies in evaluating the effect of a variety of pollutants (Moore,1980; Patel and Eapen,1989).

Acid and alkaline phosphatase activities in *Lamellidens marginalis* exposed to heavy metals, cadmium, copper and mercury were studied by Rajalakshmi (1992). Sridevi et al. (1998) studied the antioxidant enzyme activity in a fresh water field crab, *Barytelphusa guerinii* exposed to chromium. Acid phosphatase activity in the tissues of the crab *Oziotelphusa senex senex* on exposure to methyl parathion was examined by Reddy et al. (1986). ATPases activity in *Anabas scandens* due to lead nitrate toxicity was reported by Mary et al. (1994). Alkaline phophatase activity in *Scylla serrata* due to toxicity of dithothreitol or 2-mercaptoethanol was investigated by Rong et al.(2000). Heavy metal toxicity studies in two euryhaline crabs, *Callinectes sapidus* and *Carcinus maenas* with reference to carbonic anhydrase activity were carried out by Hollie et al. (2002).

Company et al. (2004) studied the toxic effects of cadmium, copper and mercury in antioxidant enzyme activities in the gills of *Bathymodiolus azoricus*. Acetyl cholinesterase and antioxidant enzyme activities in *Mytilus galloprovincinalis* and *Mullus barbatus* were reported by Lionetto et al.(2003). The response to environmental salinity and dopamine on alkaline phosphatase activity was studied in chelate muscle tissue of the euryhaline crab *Cyrtograpsus angulatus* by Pinoni and Lopez (2004). Effects of PH, temperature and digestive enzyme profiles in mud crab, *Scylla serrata* were

determined by Pavasovic et al. (2004). Sastry and Anuradha (2004) studied the effects of cadmium and copper on enzymological parameters in the freshwater fish, Cyprinus carpio. Biomarkers of heavy metals in marsh crab,Parasesarma erythodactyla were reported by Macfarlane et al. (2006).

4.3. Materials and Methods

Assay of Alkaline Phosphatase activity:

A total of 18 treated crabs and 6 controls was used for the 1st, 7th and 15th day study – periods. At the end of these time-periods, the animals were sacrificed and the hepatopancreas and gill tissues were removed and processed for enzyme studies. A sample of 20mg of each tissue was taken for enzyme analysis.

Alkaline phosphatase activity was determined according to the method described in Sigma Technical Bulletin No.104 with slight modifications (Anon, 1963). To study the enzyme activity, 0.05 M glycine-NaOH buffer of *p*H 9.0 and 9.4 was used for gill and hepatopancreas extracts, respectively. To 1 ml of frozen buffer, 0.1 ml of extract was added. To this buffer enzyme mixture, 0.1 ml of substrate (2.0 mg of *p*-nitrophenol phosphate sodium salt (Merck) in 0.1 ml distilled water was added and incubated for 60 minutes, at 37 $\pm$ 0.5^{0}C. After incubation the reaction was stopped by adding 2 ml of 0.25 N NaoH. The yellow color developed was read at 410 nm. For alkaline phosphatase also protein content of the extract was estimated by Lowry's method (1951). From this μ moles of *p*-nitrophenol liberated was calculated, and the enzyme activity is expressed as μ mol mg of *p*- nitrophenol liberated / hr/g protein.

Assay of Acid Phosphatase activity:

A total of 18 treated crabs and 6 controls was used for the 1st, 7th and 15th day study-periods. At the end of these days, the animals were sacrificed and the hepatopancreas and gill tissues were removed, and processed for

estimation of acid phosphatase activity. A sample of 20mg of each tissue was taken for enzyme studies.

Acid phosphatase activity was determined by employing the method described in Sigma Technical Bulletin No. 104 with slight modification (Anon, 1963) To 1 ml of (0.1 M) frozen citrate buffer of *p*H 4.2, 0.1ml of enzyme extract was added. To this buffer enzyme mixture, 0.1 ml of substrate containing 2.0 mg of *p* –nitrophenol phosphate sodium salt (Merck) in 0.1 ml of distilled water was added and incubated for 60 minutes at 37 $\pm$ 0.5 ^{0}C. After incubation, the reaction was stopped by adding 2 ml of 0.25 N NaoH. *p*-nitro-phenol phosphate was hydrolysed to *p*-nitrophenol by the enzyme during the incubation period. The yellow colour developed was red spectrophotometrically at 410 nm. The concentration of *p*-nitrophenol formed was calculated from the standard graph. Simultaneously, the protein content of the extract was also estimated by Lowry's method (1951). The enzyme activity is expressed as μ mol mg of *p*-nitrophenyl liberated /hr/g protein.

4.4. Results

Alkaline Phosphatase

Hepatopancreas

On the 1st day, alkaline phosphates activity in the hepatopancreas of the controls was 1.46$\pm$0.11 mg *p*NPP/hr/g protein (Table 14, Fig. 19). In the mercury treated experimentals on the 1st day at three sub lethal concentrations the activities were 2.11$\pm$ 0.26, 1.88 $\pm$0.16, and 1.65 $\pm$0.31 mg *p*NPP/hr/g protein, respectively (Table 14, Fig. 19). Declining trends in activities were observed on increasing the concentration/treatment (F=21.31;critical value; 3.12;Table 15). Increasing the duration of exposure time has also caused significant changes in all the three treatments (F=12.97;

critical value; 2.22; Table 15). Treatment versus duration (TxD) showed an F value of 47.24 and critical value 6.22 (Table 15).

Gill

On the 1st day alkaline phosphatase activity reading in gill tissues of the control organisms was 1.32±0.23 mg *p*NPP/hr/g protein (Table 14 , Fig. 20). In mercury treated organism on the 1st day at three sub lethal concentrations the activities were 1.57±0.08, 1.67±0.10, and 1.36±0.18 mg *p*NPP/hr/g protein (Table 14, Fig. 20). Decreasing trends were observed on the 7th as well as on the 15th day of exposure (F=15.28;critical value 2.22; Table 15). Increased concentrations resulted in significant changes in values of all the three treatments/concentrations (F=109.60; critical value; 3.12.Table 15). Treatment versus duration gave an F value of 242.95, and critical vale 6.22, Table 15).

Table 14. Levels of alkaline phosphatase activity in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values are expressed as mg *p*NPP/h/g protein. Each value is a mean of six observations ± SD. Values in parenthesis are percentage change over control.

Tissues	Days	Control	0.009	0.02	0.04
Hepato pancreas	1	1.46 ± 0.11 (100%)	2.11± 0.26 (150%)	1.88± 0.16 (134.28%)	1.65 ± 0.31 (114.28%)
	7	1.67 ± 0.21 (100%)	1.85+0.09 (112.5%)	1.17 ± 0.10 (73.12%)	1.09± 0.15 (68.12%)
	15	2.05 ± 0.09 (100%)	1.23± 0.05 (58.5%)	1.12 ± 0.16 (53.65%)	1.06± 0.14 (51.70%)
Gills	1	1.32 ± 0.23 (100%)	1.57 ± 0.08 (118.93%)	1.67± 0.10 (77.77%)	1.3 6 ± 0.18 (60.86%)
	7	1.8 4 ± 0.09 (100%)	2.02 ± 0.23 (140.27%)	2.26 ±1.09 (171.21%)	1.1 8 ± 0.16 (179.16%)
	15	2.07 ± 0.07 (100%)	2.43 ± 0.39 (165.70%)	1.27 ± 0.22 (227.08%)	1.14± 0.29 (257.57%)

Table 15. Two -way ANOVA table showing the significance of the effect of mercury on the alkaline phosphatase activity in the hepatopancreas and gill tissues of *Scylla serrata*.

	Variable	Model	df	F	F crit	P
Alkaline phosphatase	Hepato pancreas	Treatment (T)	3	21.31	3.125	< 0.05**
		Duration (D)	2	12.97	2.221	< 0.05**
		T x D	6	47.24	6.223	< 0.05**
		Error	132			
	Gill	Treatment (T)	3	109.60	3.125	< 0.05**
		Duration (D)	2	15.28	2.221	< 0.05**
		T x D	6	242.95	6.223	< 0.05**
		Error	132			

df – degrees of freedom F – 'F' Value P – 'P' value

** significant at 1% level

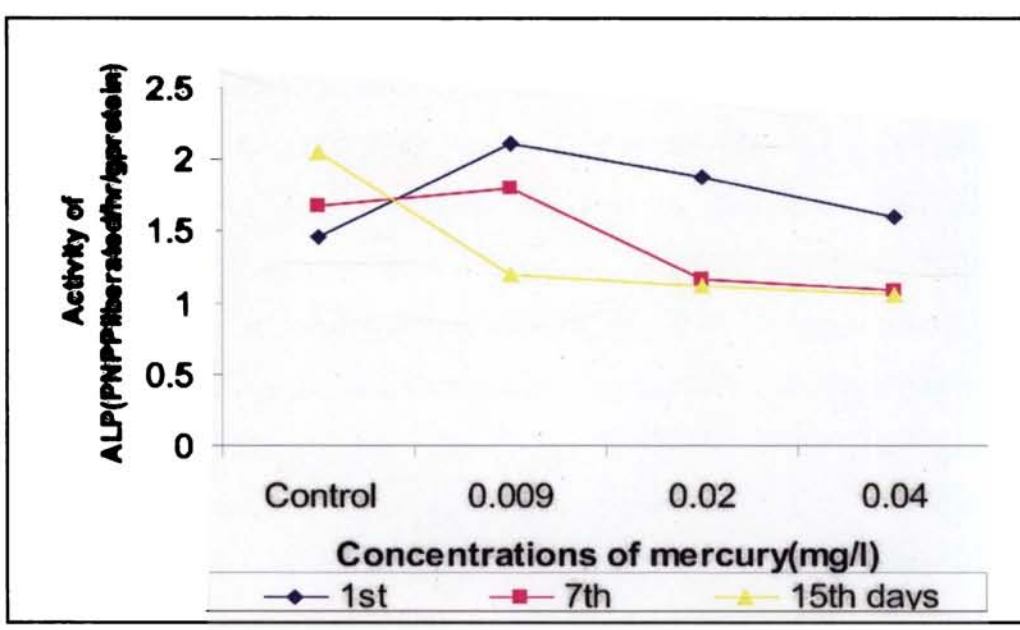


Figure 19. Alkaline phosphatase activity (p-nitrophenylphosphate liberated/hr/gprotein) in hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

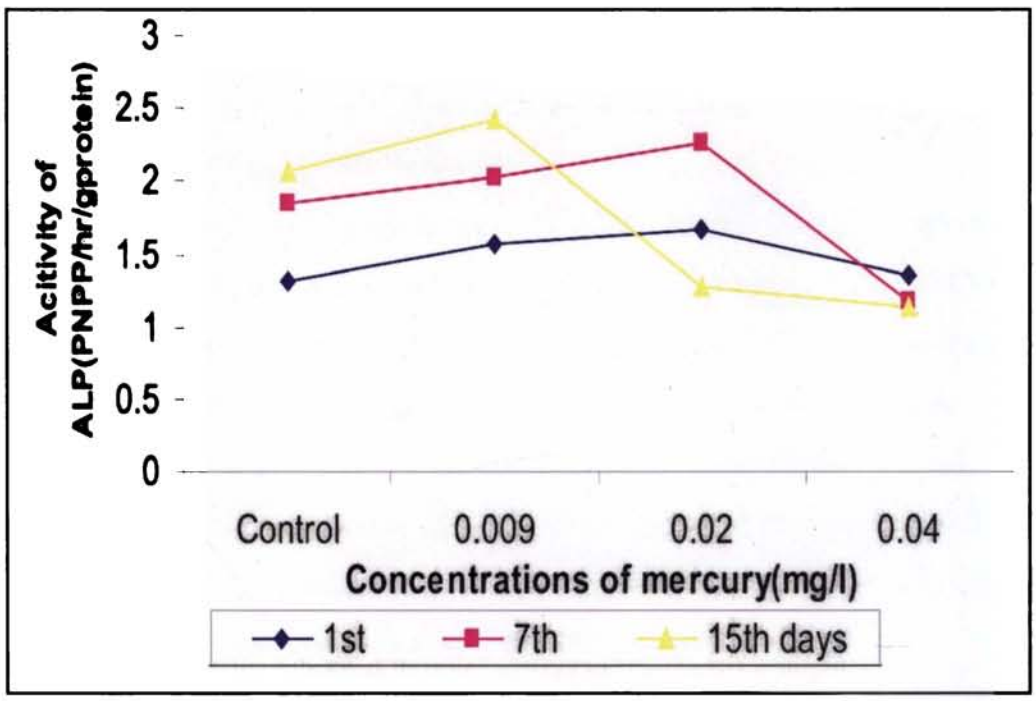


Figure 20. Alkaline phosphatase activity (p-nitrophenylphosphate liberated/hr/gprotein)in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n=6$)

Acid Phosphatase

Hepatopancreas

Acid phosphatase activity in the control organisms on the 1st day was 1.95±0.80 mg *p*NPP/hr/g protein (Table 16, Fig. 21). The enzyme values on the 1st day in crabs exposed to the three sub- lethal concentrations of mercury were 1.50±0.25, 1.70±0.55, and 1.63±0.18 mg *p*NPP/hr/g protein, respectively (Table 16, Fig. 21). Decreasing trends in enzyme activity levels with increasing concentrations of the metal were observed (F=45.85; critical value- 3.12, Table 17), (Table 16,Fig. 21). Increasing the duration of exposure also showed significant changes in enzyme activities in the hepatopancreas (F=8.39, critical value- 2.22, Table 17). Treatment versus duration (TxD) gave an F value of 36.70, and critical value 6.22 (Table 17).

Gill

Control organisms's showed acid phosphatase activity on the1st day as 1.54±0.34 mg *p*NPP/hr/g protein (Table 16, Fig. 22). In the case of mercury exposed ones, enzyme activity values on the 1st day at the three sub lethal concentrations were 2.07±1.99,1.91 ±0.02, and 1.85 ±0.35 mg *p*NPP/hr/g protein (Table 16 ,Fig. 22). On the 7th and 15th day of exposure, the general trend was towards decreased activity levels though with fluctuations (Table 16), (F=1249.06, critical value 2.22; Table 17). Increasing the concentrations also showed significant changes (F=615.08, critical value 3.12, Table 17) Treatment versus duration (TxD) gave an F value of 607.02 and critical value 6.22 (Table 17).

Table 16. Levels of acid phosphatase activity in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values are expressed as *p*NPP/hr/mg protein. Each value is a mean of six observations ± SD. Values in parenthesis are percentage change over control.

Tissues	Days	Control	0.009	0.02	0.04
Hepato pancreas	1	1.95 ± 0.80 (100%)	1.50 ± 0.25 (76.92%)	1.70 ± 0.55 (87.17%)	1.63 ± 0.18 (83.58%)
	7	1.31 ± 0.20 (100%)	1.36 ± 0.28 (103.81%)	1.27 ± 0.08 (96.94%)	1.02 ± 0.07 (77.86%)
	15	1.17 ± 0.02 (100%)	1.28 ± 0.05 (109.40%)	1.10 ± 0.01 (94.01%)	1.00 ± 0.06 (85.47%)
Gill	1	1.54 ± 0.34 (100%)	2.07 ± 1.99 (134.41%)	1.91± 0.02 (124.62%)	1.8 5± 0.35 (87.66%)
	7	1.39 ± 0.13 (100%)	1.15 ± 0.11 (82.73%)	1.63 ± 0.13 (117.26%)	1.24± 0.08 (89.20%)
	15	1.34 ± 0.07 (100%)	1.05 ± 0.03 (77.61%)	1.16 ± 0.04 (85.82%)	1.00 ± 0.06 (75.53%)

Table 17. Two-way ANOVA table showing the significance of the effect of mercury on acid phosphatase activity in the hepatopancreas and gills of *Scylla serrata*.

Variable	Model	df	F	Fcrit	P
Hepatopancreas	Treatment (T)	3	45.85	3.125	< 0.05**
	Duration (D)	2	8.39	2.221	< 0.05**
	T x D	6	36.70	6.223	< 0.05**
	Error	132			
Gill	Treatment (T)	3	615.08	3.125	< 0.05**
	Duration (D)	2	1249.06	2.221	< 0.05**
	T x D	6	607.02	6.223	< 0.05**
	Error	132			

df – degrees of freedom F – 'F' Value P – 'P' value

** = Significant at 1% level

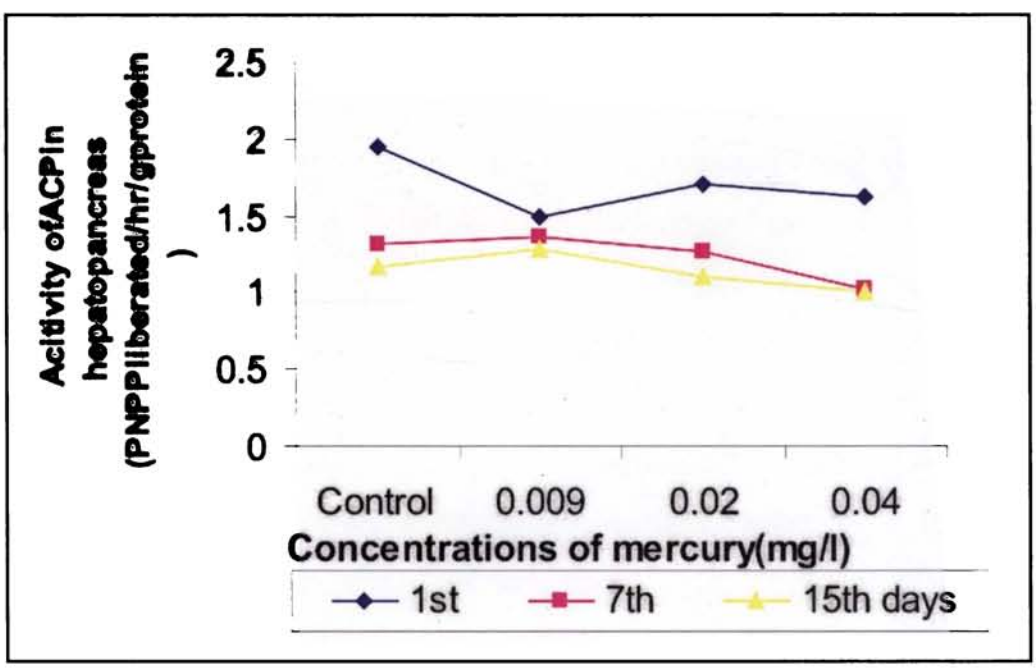


Figure 21. Acid phosphatase activity (p-nitrophenylphosphatase liberated/hr/gprotein) in hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

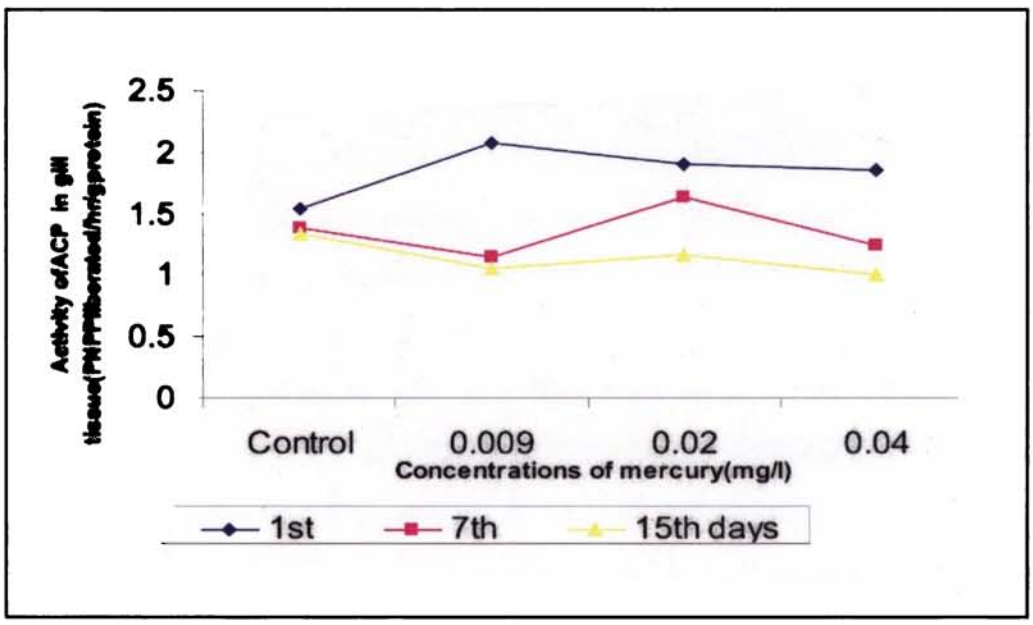


Figure 22. Acid phospatase activity (p-nitrophenylphosphate liberated/hr/gm protein) in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n=6$)

4.5. Discussion

Alkaline Phosphatase Activity

Significant fluctuations in enzyme activity levels were observed in the present study. It may be noted that metal ions are capable of activating, inactivating or neutralizing enzyme production and subsequent release to the system. Alterations brought about by different environmental conditions resulting in the release of enzyme were demonstrated in *Mytilus* (Moore et al., 1978). These types of alterations could possibly be resulting due to mobilization and detoxication of previously immobilized metal ions as suggested by Moore and Lowe (1979). This phenomenon explains the sudden elevation in enzyme activity as observed at certain time periods.

At low concentration of mercury (0.009 mg/l) hepatopancreas ALP activity was significantly higher compared to the control values on day 1 and day 7 but lower on day 15. At medium concentration (0.02mg/l) higher activity was observed only on day 1 but lower activities in the

remaining time period. At high concentration (0.04mg/l) enzyme activity was higher on day 1 but lower in the remaining time periods when compared with the control values. It is interesting to note that among the experimentals the enzyme activity levels were progressively decreasing with increasing concentrations of the metal on all days. With increase in the exposure time the enzyme activity levels were found to decrease, in general. Similar decline in ALP activities were reported by Verma et al. (1981) in liver and kidneys of *Mystus vittatus* following long term exposure to pesticides. Decrease in ALP activity was also reported in *F.heteroclitus* exposed to beryllium and silver (Jackim et al., 1970), in the liver of C. *punctatus* dosed with mercury (Rana and Sharma, 1982); in *S. mossambicus* exposed to mercuric chloride (Naidu et al., 1984), in *C. punctatus* exposed to cythion (Narayan Ram and Sathyanesan 1985), and in *H. fossilis* exposed to cadmium (Sastry and Subhadra, 1985). Decrease or fluctuations in enzyme activities have also been reported by many workers in different tissues of fishes treated with metals (Hilmy et al., 1985; Sastry and Subhadra; 1985). Similar observations were also reported by Thaker et al. (1993) in the shrimp *Callinassa tyrrhena* subsequent to mercury exposure. The generally low enzyme activity in the hepatopancreas can be due to the inhibition of protein synthesis. The binding affinity of heavy metal ion with protein is generally intense (Hilmy et al., 1981). The metal ion may also cause injury to mitochondrial system, which markedly blocks the action of enzyme. Decrease in ALP activity in some other tissues of fishes dosed with different heavy metals has also been reported (Saleem and Alikhan, 1973; Sastry and Agrawal, 1979 ,b; Koyama et al., 1985; Shaffi and Jeelani, 1985).

Mercury had also affected the gill enzyme activity profile. Gill alkaline phosphatase activity in mercury exposed animals was found to be affected when values of the experimentals were compared with those of

the controls during the exposure periods of 1, 7 and 15 days. In the gill tissue, increased ALP activity was seen in the experimentals exposed to low (0.009 mg/l), medium (0.02 mg/l) and high concentrations (0.04mg/l) on day 1 of exposure when compared with the control values, increased values at low and medium level of exposure, but decreased value at high concentration on day 7, and increased value at low concentration but decreased values at medium and high concentrations on day 15 Similar observations of increased ALP levels were made by Babu and Vasudev (1984) in the gill tissue of *Lamellidens marginalis*. Alkaline phosphatase is believed to be associated with the plasma membrane and endoplasmic reticulum (Davison and Gregson, 1965; Hart and Fouts, 1965). ALP is involved in membrane transport and carbohydrate metabolism. Verma et al. (1981) recorded decline in ALP activity in the gill tissue of *Mystus vittatus* following long term exposure to pesticides. Inhibition of ALP activity in gill tissue was also reported by Hilmy et al. (1987). The time dependant decrease in enzyme activities as observed in the present study may be due to increased inhibition of enzyme activity. According Hilmy et al.(1981) diminished enzyme activity occurs when the metal conjugates are formed with the enzyme. Sharp decline in activity can be attributed to the leakage of enzyme into the haemolymph following tissue damage. Cadmium is reported to cause inhibition of ALP activity in the gill tissues of fishes (Hilmy et al., 1981).

Acid Phosphatase Activity (ACP)

Lysosomes and the hydrolytic enzyme acid phosphatase present in the lysosome, play an important role in the detoxification process by compartmentalization and accumulation of metals that enter the cell (George, 1983). This process is effective in containing the metals in the lysosomes until the storage capacity of the lysosomes is overloaded or the lysosomes are damaged directly by the accumulated contaminant (Moore

et al., 1985). Similar observations were reported by Reddy et al. (1986). In crabs exposed to methyl parathion the ACP activity was reported to be higher in the early period but later the level decreased. The elevation in ACP levels was attributed to tissue damage and the subsequent release of the enzyme. Increase in ACP activity in response to heavy metals and other toxicants has been reported earlier also. Elevation of ACP activity in the liver, among the other tissues, has been reported in *Heteropneustes fossilis* in response to different organic pesticides (Thomas and Murthy, 1976) and in carp in response to PCB (Ito et al., 1980). There are also reports where increased ACP activity was recorded in other tissues on exposure to different metals (Hilmy et al., 1985; Sastry and Subhadra, 1985). Inhibition of ACP activity was reported in the muscle tissue of *Sarotherodon mossambicus* following exposure to phenol, and the pattern of decrease in activity showed a direct relationship.

In the present study, decreased hepatopancreas ACP activity was observed on day 1 in animals exposed to low, medium, and high concentrations when compared with the control values. Slight increase was observed on the 7^{th} day in low concentrations, but decreased activity in medium and high concentrations compared with control values. On the 15^{th} day the ACP activity was higher at low concentration but lower at medium and high concentrations when compared with the control values.

Nagarathnamma (1982) has made a similar observation in *Cyprinus carpio* exposed to organo phosphate pesticide. Stimulation of acid phosphatase activity in presence of sub lethal levels of mercury has been reported by Hossain and Dutta (1986). A gradual decline in acid phophatase activity with increasing concentration or extended exposure to the toxicant has also been observed (Saxena et al., 1982). Variations in the activity levels of acid phosphatase with concentration and duration of exposure to the toxicant metal present were reported by Hinton and

Koenig (1975). An increase in ACP activity in the liver of *H.fossilis* on exposure to mercury, and in *Cyprinus carpio* in response to cadmium exposure (Koyama et al.,1985) has also been recorded. Significant inhibition in hepatic ACP activity was reported by Saleem and Alikhan (1973), and Dalela et al.(1980). The general trend of increased inhibition of enzyme activity can be attributed to the failure of protein synthesis, decreased production of the enzyme or decreased release. Inhibition of protein synthesis on exposure to toxicants is widely reported (Murthy and Devi, 1982; Ramalingam and Ramalingam, 1982). The overall depression in phosphatase activity can also be attributed to injury elicited to the mitochondrial system.

In the present study, gill acid phosphatase activity in the experimentals was showing a decreasing trend, in general, except on day 1, in all the three concentrations. Increased activity was observed in the gills of the clam, *Protothaca stamina* following exposure to copper (Roesijadi, 1980). Presence of metal ion causes increased availability of lysosomal acid hydrolases to metabolise and sequester the metals in a non-toxic form (Chandy and Patel, 1985). Patil et al. (2004) reported the activities of ACP,ALP,GOT and GPT enzymes were increased with increase in cadmium level at 2 mg/l in the gill of oyster Crassostrea cuculata.

Chapter 5

HISTOPATHOLOGICAL STUDIES

5.1. Introduction

Owing to the dynamic nature of their environment, the aquatic organisms are subjected to a wide range of physical and chemical conditions through natural and anthropogenic influences, and are more or less always under some form of stress. The response of marine biota to a pollutant can be detected at different levels of organization and responses. Biological responses due to stress include morphological, physiological, biochemical, genetical, and behavioral changes (Widdows, 1985).

The effect of a toxic compound on an aquatic species would be direct or indirect. A direct effect is caused by direct action of the toxic substance on the aquatic organism. The most obvious direct effect is acute, and consists of irreversible damage to vital organ functions resulting in rapid morbidity and death. A chronic direct effect differs from an acute one in that the toxicant causes a sub lethal change in the animal, which may or may not be the eventual cause of death. Sub lethal changes can occur from a single encounter or from continuous exposure to a toxicant over a long period of time. Direct and induced chronic effects of toxicant exposure are of particular importance to those concerned with the aquatic environment. Many toxicant induced tissue pathological changes have been non-specific and this is not surprising as aquatic toxi-pathology is in its infancy. As science matures and more descriptive studies are made by combining histopathological results with those of biochemical and physiological studies, the complete reaction of an aquatic organism to a toxicant can be defined for diagnostic purposes in future.

The study of structural damage of organs and/or tissues is an integral part of pollution toxicology. Organisms in general and aquatic ones in particular are easily susceptible to the toxic effects of environment contaminants. A clear understanding of the cause and effect of such toxic reactions could be identified only with the help of histopathology. Histopathological techniques are rapid, sensitive, reliable and comparatively inexpensive tools for the assessment of stress response to pollutants. Depending on the nature of the toxicant, some of the cellular changes could be specific whereas some could be of a general nature (Moore, 1993).

The cell has a great capacity to adaptation and is able to respond to changes in the internal and external environments by alteration in both cellular structure and function. Cells, which have reached their limit of adaptability, begin to show structural changes, which indicate their failure to withstand the changed environment. If advance conditions persist or if the initial pathology stimulus is severe, then these processes continue and progress into a sequence of events leading to cell death (cell necrosis) (Varanasi, 1989; Menon, 1999). Thus, histological approaches and techniques are useful for studying changes in the structure of tissues and their composite cells, thus giving indications of the degree of stress and of the adaptive capability of the organism. Cell damage is a result of persistent or irreversible biochemical and sub cellular dysfunction caused by stress. Often stressed cells undergo irreversible structural and biochemical changes, which result in alterations in the physiology of the animal. Assessment of histopathological manifestation provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism. The route that the toxicant takes during its metabolism, often dictates the choice of organs for examining the effect of xenobiotics.

The aim of this histopathological investigation was to assess the extent of external and internal pathological lesions caused by mercury on the gills, hepatopancreas, abdominal muscle tissue, and chelate muscle tissue of *Scylla serrata.*

5.2. Review of Literature

Cellular responses offer a prompt, ideal, and useful tool for estimating the extent of damage caused by pollutants on living organisms (Moore, 1986). Physiological, histological and ultra- structural studies have shown that heavy metal ions interfere with respiration and osmoregulation by disrupting the structure of the gills in fishes and crustaceans (Soegianto et al., 1999b). Singh et al. (1996) studied the bioassay and histopathological changes of the gills of *Channa punctatus* exposed to copper and mercury. Cadmium and zinc induced changes in the structure and function of gills in different species of shrimps and crabs were reported by a number of researchers (Chassard-Bouchad, 1993;Victor, 1994; Soegianto et al., 1999a.) Studying the effect of heavy metals on the mud crab *Scylla serrata* many reports have pointed out pathological manifestations (Narayanan et al., 1990; Jayakumari et al., 1999). Manisseri and Menon (1995) had observed histopathological changes in the penaeid shrimp *Metapenaeus dobsoni.* Sarojini et al. (1990) conducted histopathological studies to find out the adverse effects of metal exposure to ovarian development in the fresh water crab *Barytelphusa guerini* . Victor et al. (1985) observed cytopathological effects of cadmium in the ovaries of the fresh water prawn *Macrobrachium idea.*

Lawson et al. (1995) studied the ultra structural changes in the gill epithelium of *Carcinus maenas* after exposure to copper. Gupta et al. (1996) had examined the histopathological changes in the snail, *Pila globosa* treated with two different doses of lead acetate viz.,0.1mg/l and 10mg/l for 60 days. Domouthsidou et al. (2000) had reported the ultra structural localization of

heavy metals (Hg,Ag,Pb and Cu) in the gills and digestive gland of *Mytilus galloprovincinalis*. Muller et al. (1991) examined the histological changes on the gill tissue of *Salvelinus fontinalis* exposed to aluminium. Rodriguez (1994) reported pathological lesions in the larvae of *Chasmagnathus granulatus* on exposure to cadmium. Kalita et al. (2002) studied the behavioral and hispathological changes in the gill tissue of the fresh water crab *Paratelphusa spinigera*. Palaniappan et al. (2003) studied the histopathological effects of two sub lethal concentrations of nickel on Cirrhinus mrigala.Sarita and Sudha(2002)studied the pathological lesions in the gill tissue of freshwater fish Nandus nandus after exposure to copper sulphate and lead nitrate.

Heavy metals have a long biological half-life period that pose a major threat to all aquatic organisms. Therefore, it was thought worthwhile to investigate the structural changes in the tissue of *Scylla serrata* following exposure to mercury.

5.3. Materials and Methods

A batch of 12 crabs was introduced separately to each sub lethal concentration- low (0.009mg/l), medium (0.02mg/l), and high (0.04 mg/l) of mercury. A group of 12 crabs maintained simultaneously in metal- free water served as the controls. After 30 days of exposure, the animals were sacrificed, and gills, hepatopancreas, abdominal muscle tissue and chelate muscle tissue were dissected out, and fixed in Zenker's solution for 6 hours. After the specific time period, the samples were washed in running tap water for 24 h, and stored in 70% alcohol. After treatment with graded alcohol, acetone and xylol, the samples were embedded in paraffin wax and blocks prepared. Serial sections of 8-10μ were cut in a rotary microtome, processed and stained with Hematoxylin and Eosin (Bell and Lightner, 1988).

5.4. Results

The gills of *Scylla serrata* are formed of a number of lamellae or broad flattened plates arranged serially in pairs along a control gill stem. A thin layer of chitin covers the entire outer surface of the gill. Underlying the chitin is a continuous layer of epithelial cells. At irregular intervals pillar cells join the lamellae. The distal part of the lamella is expanded. The epithelial cells of the lamellae are continued as the lining of the gill stem and large connective tissue cells compose the chief support of the gill stem (Fig. 1).

In low concentration (0.009mg/l) the changes were perceptible enlargement of intralamellar space densely packed with granular material, and loss of gill structure (Figs.1a,1b). In the case of medium (0.02mg/l) concentration the following changes were seen: haemocoel with coarse amorphous to fibrous materials, thickened gill lamellae, and massive haemocytic infiltration (Figs.1c,1d,1e). In high concentration (0.04mg/l) the cytoplasm of phagocytes were found to be free from any engulfed material, and gills developed bulbular swelling at the tip (Figs. 1f ,1g).In hepatopancreas, the following histological changes were observed:

Hepatopancreas of the control crab (Fig.2): The yellowish –brown tissue of the hepatopancreas occupied much of the cephalothoracic cavity. Histologically, the tubules consisted of an epithelium composed of four cell types E-cells (embryo nalenzellen), the F-cells(fibrillenzellen), the B-cells (blazen zellen), and the R-cells(rest zellen).The E-cells, which were generally among the smallest of the hepatopancreatic cell types were undifferentiated polyhedral cells. They had high nucleo-cytoplasmic ratio, and were concentrated in the distal tip of the tubules, which is the area of proliferation. The F-cells, which appeared striated due to extensively developed rough endoplasmic reticulum, were tall columnar epithelial

cells with basally situated nuclei. They are secretary in function and present in the mediodistal, and medioproximal portions of the tubules. The B-cells, which are secretary and excretory in function having a single large vacuole and compressed basal nuclei were the largest of hepatopancreatic cell types seen mainly in the proximal areas of the tubules. The R-cells, the most abundant of the four cell types, had multi vacuolated cytoplasm and are storage in nature. They were seen in the mediodistal and proximal areas of the tubules.

In low concentration slight changes were observed in B- secretary cells (blazen zellen), and F- fibrillar cells (Fig. 2a). In medium concentration, the cells were disfigured; clumped and intercellular spaces could not be observed (Fig. 2b). In high concentration, general degeneration of the tubular and intertubular tissues was observed, so also extensive vacuolation and complete loss of tubular structures and necrosis (Fig. 2c, 2d, 2e).

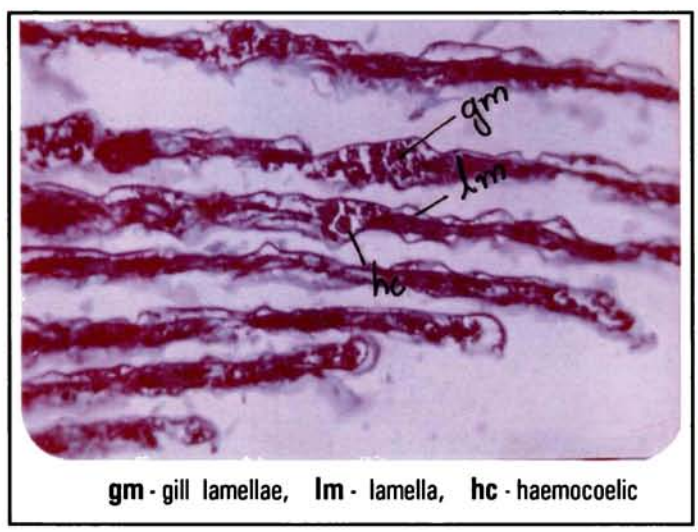
Abdominal muscle tissue of the control crab was made up of muscle cells containing contractile filaments that move each other and change the size of the cell. Muscle tissue derived from mesoderm contains protein, and myosin filament (thread-like) form multi nucleate cells that assemble into fibers called myofibrils (Fig.3).

In low concentration, the changes were necrosis and appearance of granular material in between the muscle fibers (Fig.3a). In medium concentration, atrophy and wavy appearance of the muscle fibers, fragmentation of the muscle fibers, and intermuscluar areas with granular exudates were observed (Figs. 3b, 3c, 3d). In high concentration wavy appearance of basophilic deposits, and atrophy and focal disappearance of the muscle fibers were marked (Figs.3e, 3f).

In the chelate muscle tissue the muscle cells contained contractile filaments that move each other and change the size of the cell. Muscle

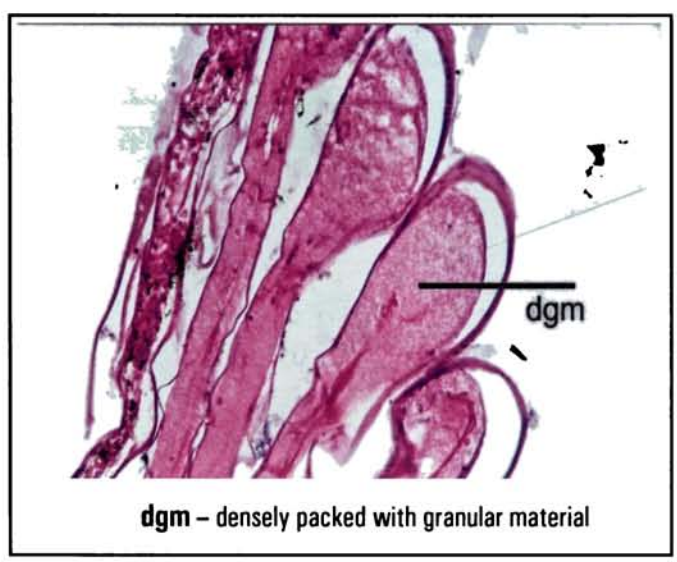
tissue derived from mesoderm contained protein, and myosin filaments (thread- like) formed multi nucleate cells that assembled into fibers called myofibrils (Fig. 4).

In low and medium concentrations, atrophy and necrosis (Fig. 4a), loss of muscle structure, and necrosis (Fig.4b) were observed. In high concentration, complete loss of muscle structure and basophilic granules were visible (Fig.4c).



gm - gill lamellae, **lm** - lamella, **hc** - haemocoelic

Figure 1. Normal structure of the gill of *Scylla serrata* showing thin gill lamellae with haemocoelic space. X 20



dgm – densely packed with granular material

Figure1a. Gills of *Scylla serrata* exposed to low concentration of mercury showing enlargement of intralamellar space densely packed with granular material (Dgm). x 20

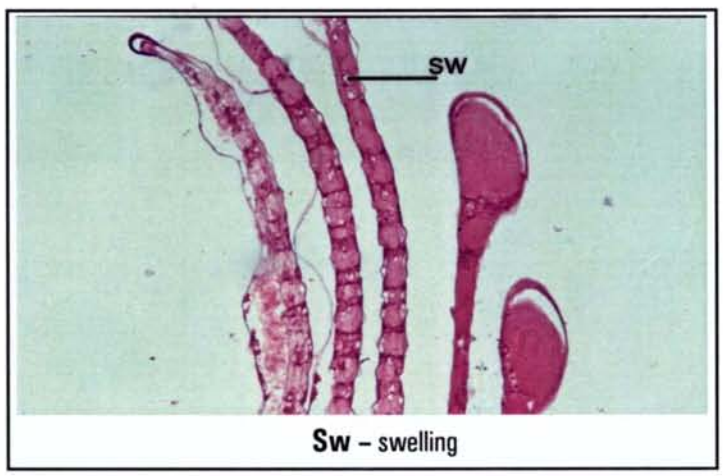


Figure 1b. Gills of *Scylla serrata* exposed to low concentration of mercury showing loss of gill structure. x 20

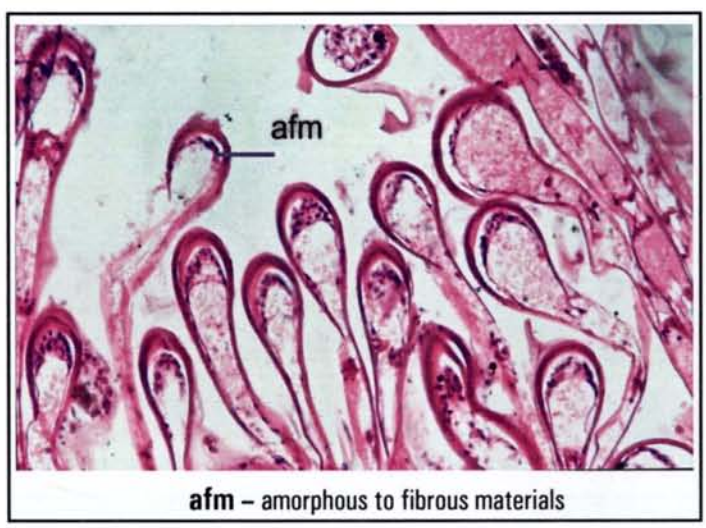


Figure 1c. Gills of *Scylla serrata* exposed to medium concentration of mercury showing coarse amorphous to fibrous materials, and bulb like swelling. x 20

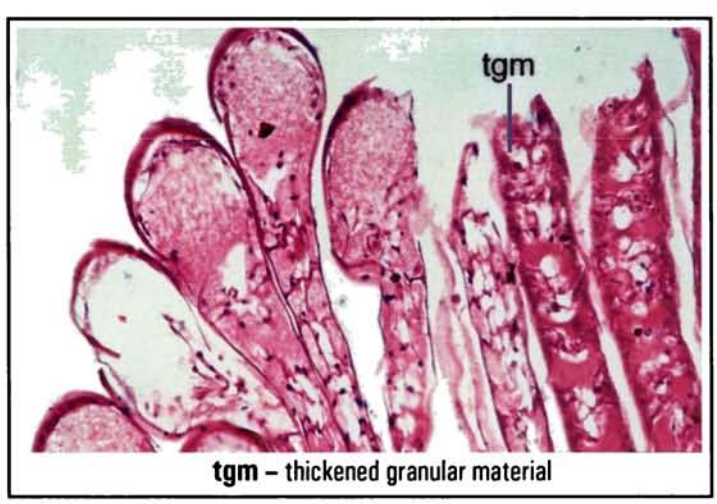


Figure1d. Gills of *Scylla serrata* exposed to medium concentration of mercury showing thickened gill lamellae. x 20

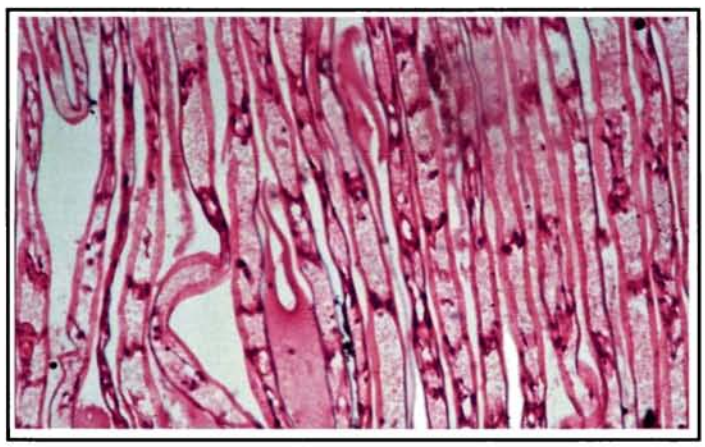


Figure 1e. Gills of *Scylla serrata* exposed to medium concentration of mercury showing haemocytic infiltration.x 20

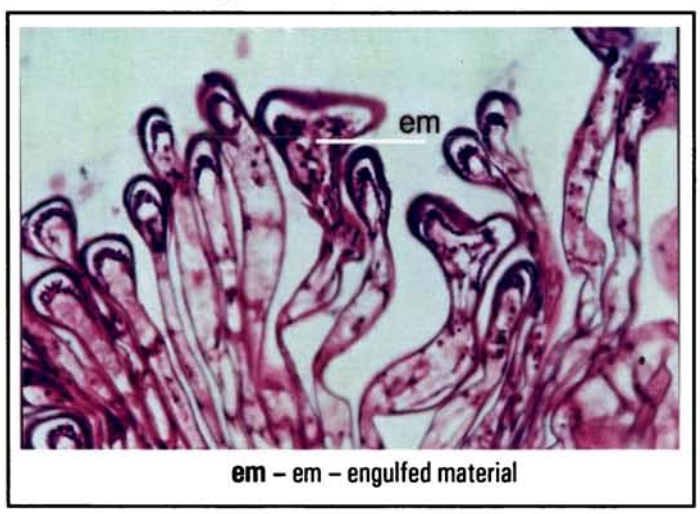


Figure 1f. Gills of *Scylla serrata* exposed to high concentration of mercury showing phagocytes with cytoplasm free from engulfed material. x 20

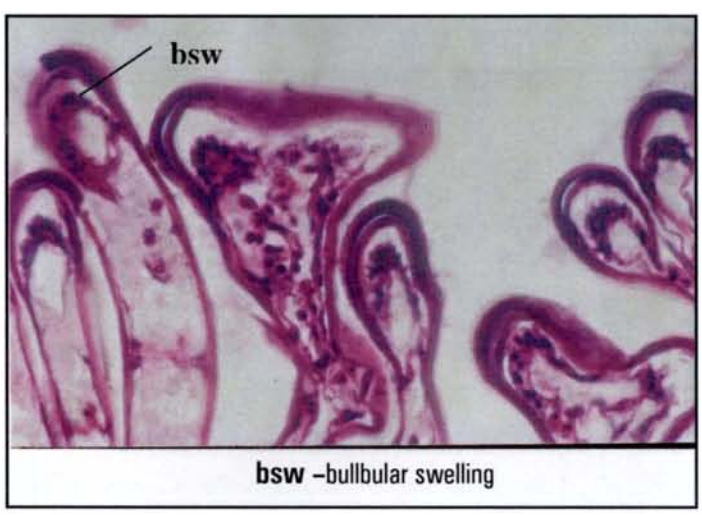


Figure 1g. Gills of *Scylla serrata* exposed to high concentration of mercury showing bulbular swelling at the tip of the gills, and necrosis. x 20

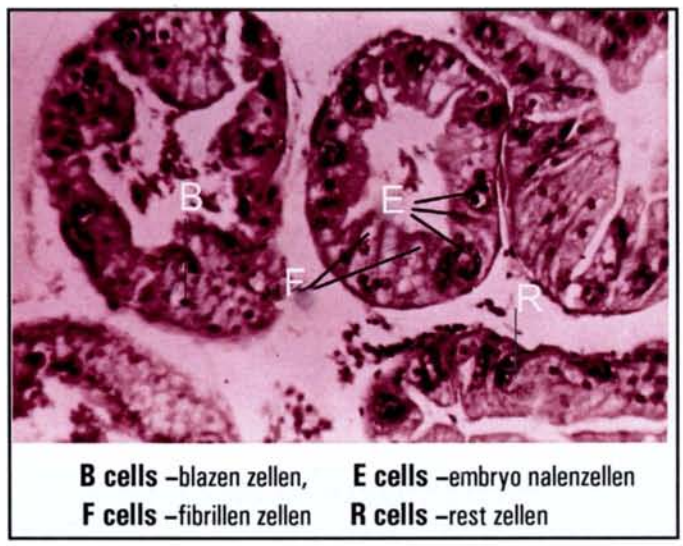


Figure 2. Normal structure of Hepatopancreas of *Scylla serrata* showing the B, E, F & R cells. x 20

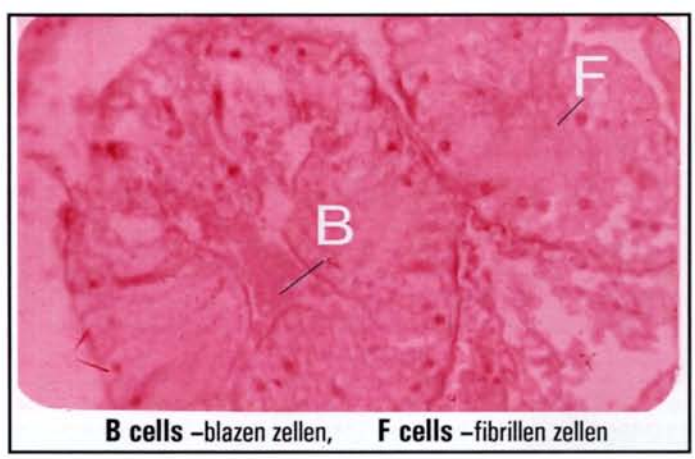


Figure 2a. Hepatopancreas of *Scylla serrata* exposed to low concentration of mercury showing changes in the secretary cells(B) and fibrillar cells(F). x 20

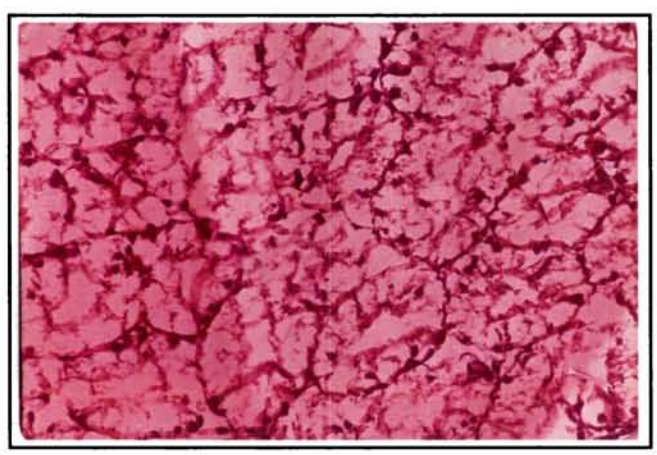


Figure 2b. Hepatopancreas of *Scylla serrata* exposed to medium concentration of mercury showing clumped cells without intercellular spaces. x 20

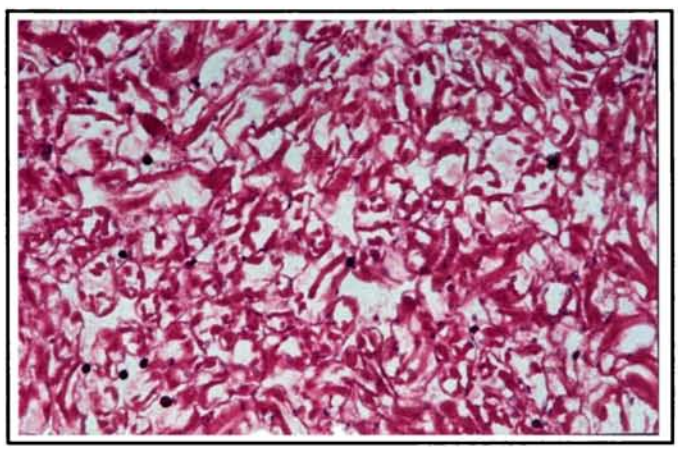


Figure 2c. Hepatopancreas of *Scylla serrata* exposed to high concentration of mercury showing general degeneration of the tubules, and inter tubular tissues. X 20

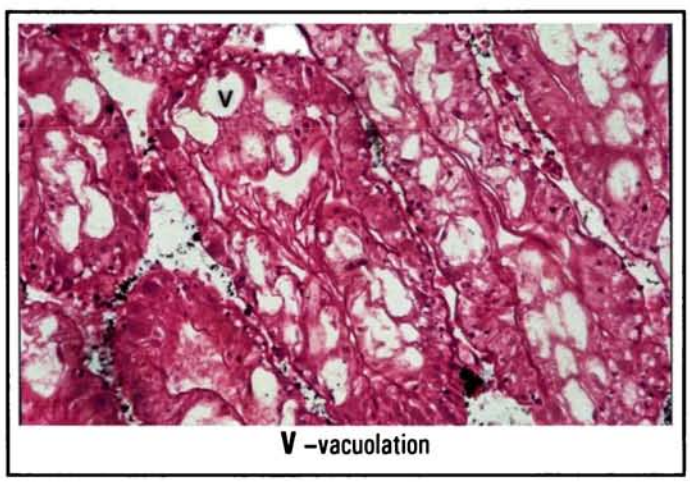


Figure 2d. Hepatopancreas of *Scylla serrata* exposed to high concentration of mercury showing extensive vacuolation. x 20

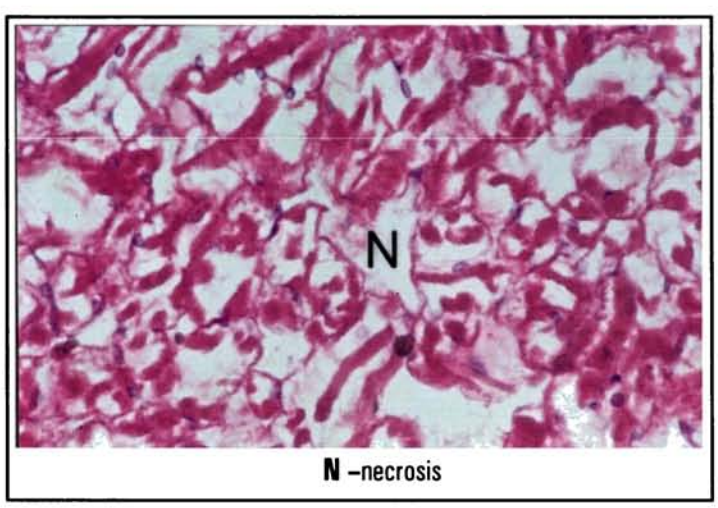


Figure 2e. Hepatopancreas of *Scylla serrata* exposed to high concentration of mercury showing complete loss of tubules structure, and necrosis. x 20

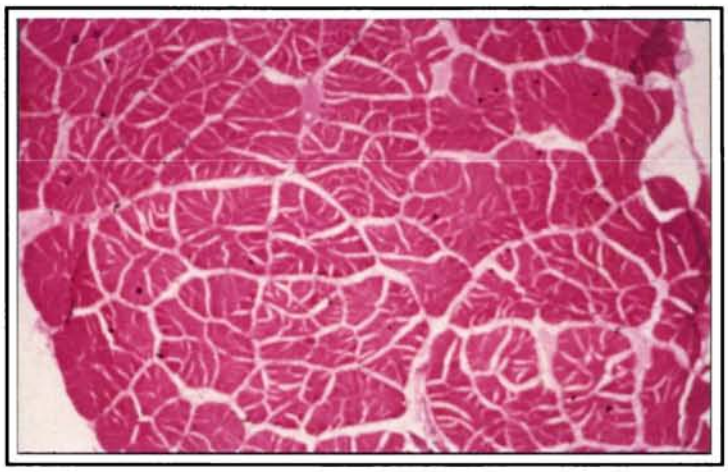


Figure 3. Normal structure of Abdominal muscle tissue of *Scylla serrata* showing the muscle fibres. x 20

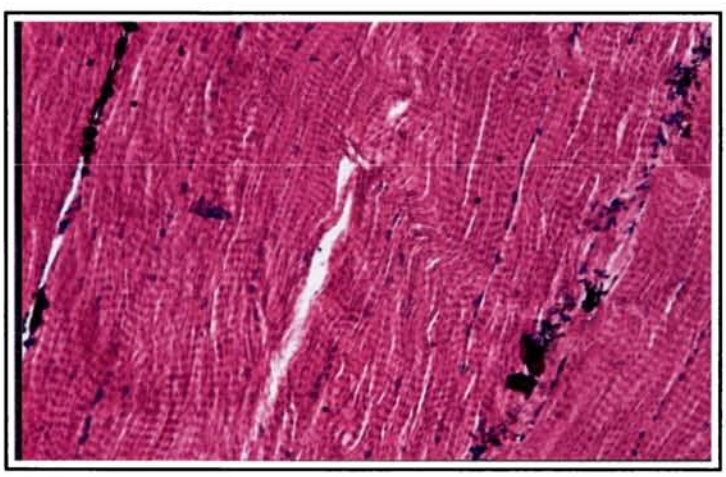


Figure 3a. Abdominal muscle tissue of *Scylla serrata* exposed to low concentration of mercury showing necrosis and appearance of granular material in between the muscle fibers. x 20

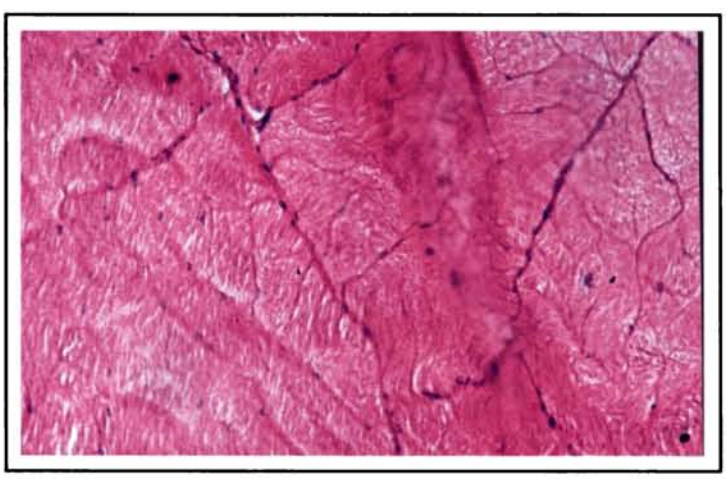


Figure 3b. Abdominal muscle tissue of *Scylla serrata* exposed to medium concentration of mercury showing atrophy of the muscle fibers. x 20

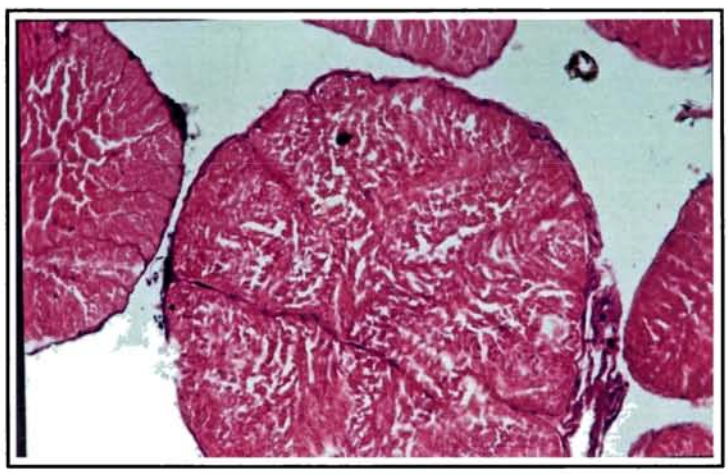


Figure 3c. Abdominal muscle tissue of *Scylla serrata* exposed to medium concentration of mercury showing fragmentation of muscle fibers. x 20

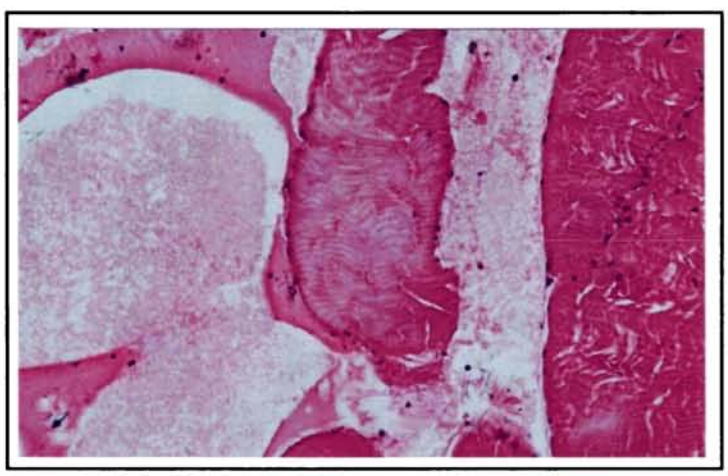


Figure 3d. Abdominal muscle tissue of *Scylla serrata* exposed to medium concentration of mercury showing granular exudates in Inter muscular areas. x 20

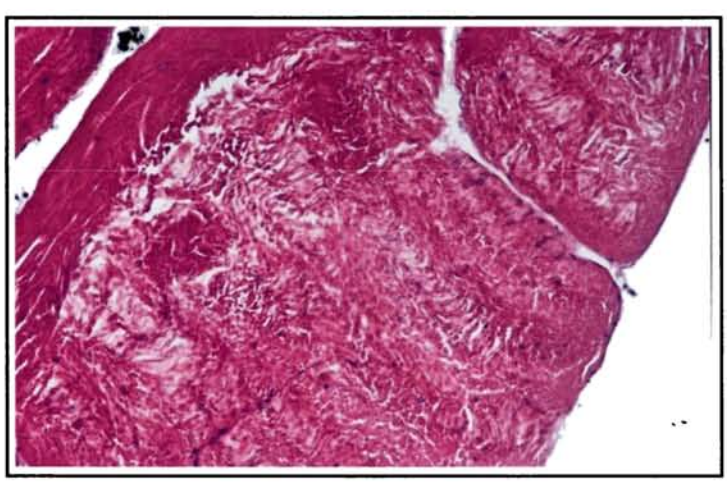


Figure 3e. Abdominal muscle tissue of *Scylla serrata* exposed to high concentration of mercury showing atrophy and wavy appearance of basophilic deposits. x 20

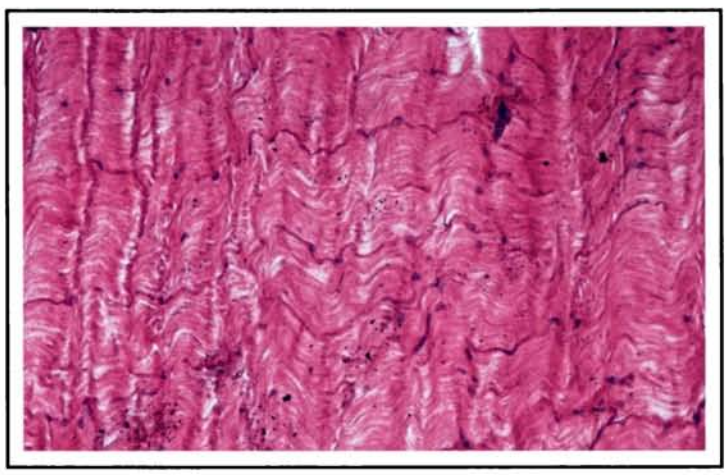


Figure 3f. Abdominal muscle tissue of *Scylla serrata* exposed to higher concentration of mercury showing, atrophy and focal disappearance of muscle fibers. x 20

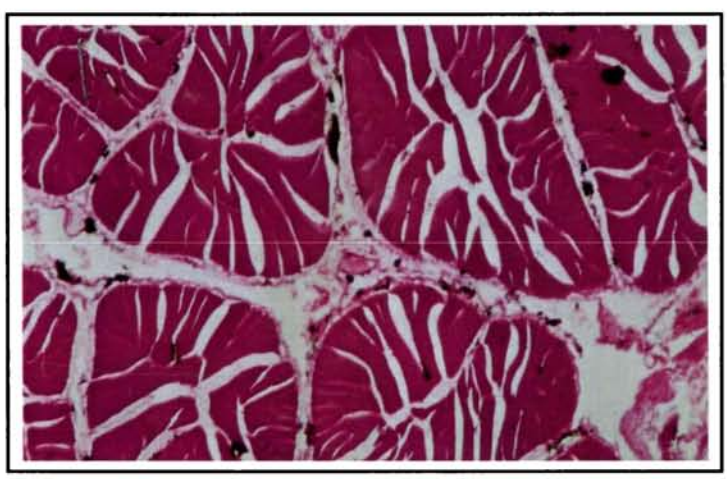


Figure 4. Normal Chelate muscle tissue of *Scylla serrata* showing muscle fibers. x 20

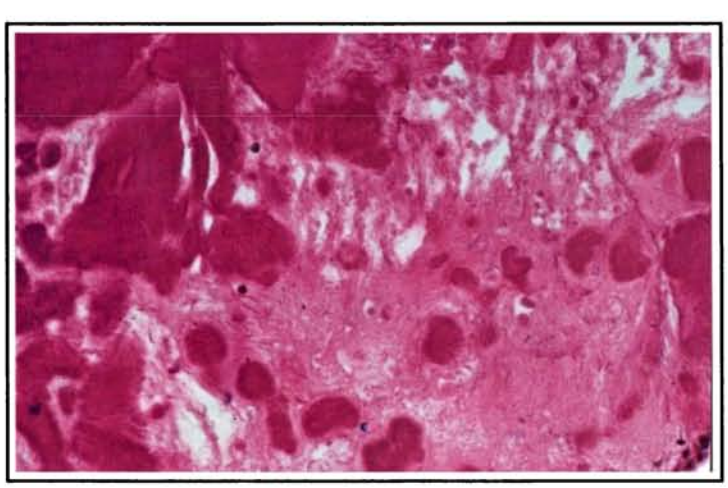


Figure 4a. Chelate muscle tissue of *Scylla serrata* exposed to low concentration of mercury showing atrophy and necrosis. x 20

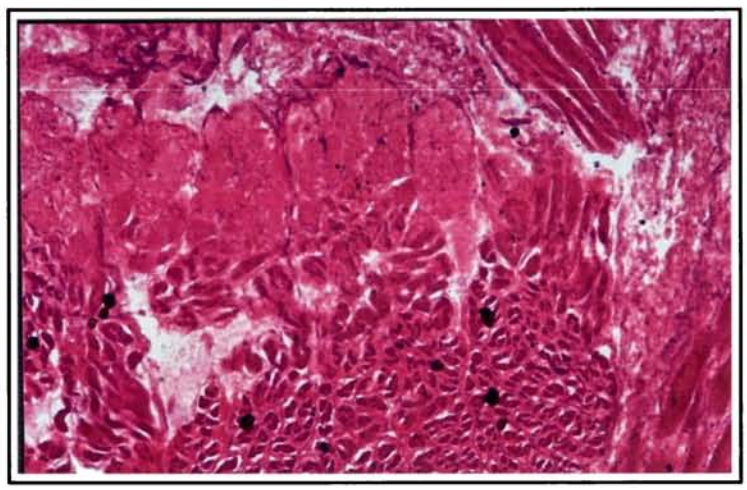


Figure 4b. Chelate muscle tissue of *Scylla serrata* exposed to medium concentration of mercury showing loss of muscle structure and necrosis. x 20

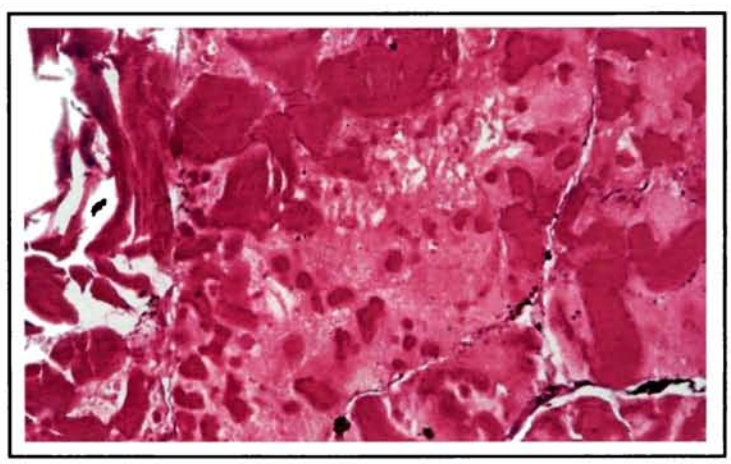


Figure 4c. Chelate muscle tissue of *Scylla serrata* exposed to high concentration of mercury showing the complete loss of muscle structure and basophilic granules. x 20

5.5. Discussion

The gills, having large surface area and being directly exposed to environmental changes, are the prime target of pollution. The accumulation of zinc, cadmium and mercury by the gills results in morphological changes within the cells, which suggest effects on the functions of the organelles. The gills have been reported to be the main sites of absorption of heavy metals present in the medium (Bryan, 1964,1968). Nodular gill disease, hemocytic hyperplasia, and sloughing of walls of haemocytes were the other indications of cadmium toxicity

(Victor,1994).Considerable thickening of the gill epithelium and reduction of haemolymph space resulted in restriction of respiratory gas exchange as shown by a marked hypoxemia (Nonnotte,1993).The magnitude of alterations in the structure highlights the permeability of the surface of gills to pollutants, which in turn imply perturbations in the physiological functioning of the animal as a whole. In the present investigation, the histopathological changes observed in the low (0.009mg/l), medium (0.02mg/l), and high (0.04mg/l) concentrations of mercury were enlargement of intralamellar space, loss of gill structure, necrosis, and massive haemocytic infiltration. Similar observations were made by Victor et al. (1985) in gill pathology and haemocyte responses in *Macrobrachium idea* exposed to mercuric chloride. Patil and Kaliwal (1989) also observed that the degree of damage to gill tissue increases according to the concentration and period of exposure of zinc in *Macrobrachium hendersodyanum.*

Mallatt (1985) has observed similar observations in fish exposed to metals and other irritants. The changes included necrosis of lamellar epithelium, vacuolation, epithelial cells swelling, proliferation and epithelial lifting. The changes reported in the gills of *Labeo rohita* exposed to methyl mercury for 60 days included swelling at the tip of the secondary lamellae and the intralamellar space being filled with hyperplastic epithelium. Darmono et al. (1990) and Lawson et al. (1995) reported similar structural changes in gills exposed to cadmium in the banana shrimp, *Penaeus merguiensis* , and copper in *Carcinus maenas.* They discussed alteration to the gill epithelial cells, including a decrease in the number of plasma membrane infolding, extensive vacuolation, and a change in ribosomal distribution of microtubular network. Singh et al. (1996) reported similar observations in *Channa punctatus* . They discussed loss of micro ridges, fusion and thickening of gill lamellae, and

increased production of mucus on the gills of the fish. Pandey et al. (1991) reported the same observations in the estuarine mullet, *Liza parsia*. They observed that the intralamellar space was filled with hyperplastic epithelium by day 15 of lead exposure. Muller et al.(1991) noticed desquamation of the gill epithelium and lamellar fusion of the gill tissue in the fish *Salvelinus fontinalis*. Extensive vacuolation of epithelial cells following exposure to zinc and cadmium could be inferred to acute damage of gill cells possibly caused by osmotic imbalance of the cells (Hebel et al., 1999). Similar observations were made by Usharani (2003) in *Scylla serrata* exposed to zinc and cadmium.

In the present study, the hepatopancreas showed changes in the F- and B cells in low concentration of mercury, and cells were found clumped, and intercellular spaces invisible in the medium concentration, and a general degeneration, loss of tubules structures, vacuolation, and necrosis of cells in the high concentrations of mercury exposed *Scylla serrata*. Similar observations were observed in the *Scylla serrata* exposed to zinc and cadmium, lead, arsenic, and selenium as reported by Krishnaja et al. (1987) and Usharani (2003). Krishnamoorthy et al. (1996) also reported changes such as elongation of hepatopancreatic cells, and shrunken cells in *Macrobrachium lamarrei* exposed to low (0.0065ppm), and high (0.0215ppm) concentrations of copper. Destructive and deteriorative changes in the hepatopancreas and gills were observed in *Penaeus indicus* exposed to Zn at a low concentration of 100 ppb (Viswanathan and Manisseri ,1995)

The structural changes noticed in the abdominal muscle tissue and chelate muscle tissue as atrophy, necrosis, wavy appearance and granular material in between in the muscle fibers, fragmentation, loss of muscle structure, appearance of basophilic deposits of the muscle fibers were caused as a result of exposure of crabs to the three sub lethal

concentrations of mercury for 30 days. There were no marked changes in the muscle tissues. Extensive damages were seen in the gills followed by hepatopancreas, abdominal muscle tissue and chelate muscle tissue. The extent of damage is dependent on the duration of period of exposure, and concentration of the toxicant.

Chapter 6

IONIC REGULATION

6.1. Introduction

The internal homeostasis in aquatic organism is maintained through influx and efflux of ions, which exist in bound and free forms, and both forms of ions are important in cellular metabolism. Crustaceans occupy a variety of habitats such as sea, brackish and fresh water systems and they have the ability to maintain their intracellular osmotic and ionic balance, which has been documented in shrimps, and crabs (Mantel and Farmer, 1983). Studies on ionic regulation are as valid as any other tool in discerning the physiological changes in an organism due to a pollutant. It was well documented that metals disturb the water electrolyte status and these could serve as sensitive physiological index (Christensen et al, 1977)

Many physiological processes are regulated by the movement of ions into and out of organs, tissues and cells. During the past decade a variety of new techniques and approaches have contributed to a deeper understanding of the myriad influences ions have on the function and structure of organisms. From respiration and excretion to neurological control and metabolic processing, ions and their regulation occupy a central role in the physiology of fish as well as other animals. But regulation of the osmotic concentration of their body fluids remains unclear despite many excellent studies on tissues and cell function. Aquatic environment is well known for its dynamic and homogenous nature. A notable feature of this habitat is the stability of environmental

conditions over vast areas, which allow the biota to enjoy wider distributions. On the contrary the coastal zones are subjected to continuous biological, chemical, physical, geological, and meteorological interactions. The littoral habitat known for its abundance and variety of life is prone to greater interactions of the above environmental parameters and is colonized by species, which can tolerate wider fluctuations in ambient environmental conditions.

Anisosmotic extra cellular, and isosmotic intracellular regulations are the two major physiological tactics for salinity (Matushima et al., 1987). Osmoregulation, the ability to actively maintain osmotic concentrations in extra cellular fluids that can be significantly higher than those in the surrounding environment, is a necessary and fundamental physiological adaptation that allows marine and estuarine animals to invade the most dilute areas of the aquatic habitat (Henry, 2001).

Biosphere renders two major habitats for supporting life, namely, the aquatic and the terrestrial. Among these, the former predominates in area and volume and the available evidences indicate that life originated in this habitat. Like other environments, the successful colonization of aquatic environment demands environmental tolerance. Studies on the physiological convulsions for combating environmental oscillation are considered together as a subdivision of comparative physiology, the state of the art environmental or ecological physiology, which addresses nature's enigmatic phenomenon of adaptation. Aquatic environment can broadly be divided into fresh and marine environments. Typical freshwater and oceanic environments are comparatively stable over vast areas, which allow biota to enjoy wider distributions, whereas the neritic and estuarine environments are highly dynamic in nature. In addition to nutritional uncertainties, inhabitants of coastal marine and estuarine realm face considerable diurnal and seasonal variations in salinity, which alter

their rates of metabolism and activity. In the estuarine realm, where the saltwater and fresh water meet, changes in salinity are more pronounced. Major problems that an animal has to face in this realm are osmotic and ionic changes in the medium. Osmotic and ionic regulation is a result of the active regulation of ions and water in the body either by active uptake from the medium or by removal from the body (Cameron, 1978).

Intertidal organisms in tropical regions are frequently subjected to rapid changes in salinity and temperature (Moore, 1972; Brosnan, 1992; Coates, 1992). Temperature and salinity stresses are important environmental influences affecting the survival of intertidal organisms (Gilles and Pequeux, 1983; Hawkins, 1995; Stillman and Somero, 1996). In aquatic animals, acid-base status and ion exchanges are related at least on two distinct ways, either actively controlled ion movements may act as acid-base imbalances (for example, transfer of acid-base equivalents coupled with strong ion movements across the gills ensures active compensation of respiratory acid-base disturbances induced in fishes and crustaceans by environmental hypercapnia), or hyperoxia (review, Truchot, 1987a; Mc Donald et al., 1989). A similar coupling could also operate with passive diffusional ion fluxes when the capacity of ion regulatory mechanisms is exceeded by altered ionic gradients.

Terrestrial crabs were defined by Burggren and Mc Mahon (1988) as those having behavioral, morphological, and physiological or biochemical adaptations allowing activity out of water. They may be found in a wide range of environments, from those frequently flooded by the tide to those, which are quite dry. In the great majority of crustaceans, ionic and osmotic regulation, as well as acid-base balance, are carried out by the gills, specialized to work in the aquatic environment. With the occupation of the mesolitoral and the area above high tide, adaptations concerning these aspects were made necessary. The

gills of aquatic organisms are multifunctional organs that play important roles in respiration, osmoregulation, and in volume and acid-base regulation. Although the significance of the gills in ion transport functions in crustaceans is evidently recognized (Towle, 1990; Lucu, 1990), the relationship between their respiratory and ion-regulatory functions has been emphasized by only a few, that too not very recent studies (King, 1966; Quinn and Lane , 1966; Engel et al., 1975).

Decapods that have successfully adapted to terrestrial life confront a progressive loss of water which can be mitigated either by behavioral mechanisms or by other strategies, such as retention of water in the branchial chamber in an attempt to maintain the original habitat medium, development of mechanisms for water aspiration from the substrate, or a reduction of the rate of urine production (Kormanik and Harris, 1981; Taylor-Jones and Taylor, 1986). All these processes contribute to equilibrate the loss of salts and water in terrestrial crabs. Adjustments for air breathing and acid-base balance are also involved with the osmotic and ionic regulation; compensatory changes in one process may require modification in the other. Imbalance in body water and ionic levels may cause metabolic regulation resulting in the production of acidic end products, which could also exert a considerable influence over hemolymph oxygen and carbon dioxide transport (Burggren and McMahon, 1981; DeFur and McMahon, 1984)

With these objectives, the present work was carried out to find out the ionic levels of sodium, potassium, calcium and magnesium in mercury-exposed *Scylla serrata.* Heavy metals and pesticides have been found to alter the ionic regulation in penaeid shrimps and crabs, the grass shrimp *Palaemonetes pugio* (Roesijadi et al., 1976), *Gammarus marinus* (Wright, 1986), and *Barytelphusa guerini* (Tulasi et al., 1990).

6.2. Review of Literature

Hemolymph osmotic and ionic (Na^+, K^+,Ca^{++} and Mg^{++}) concentrations were determined for both sexes of mangrove crabs *Ucides cordatus* and *Goniopsis cruentata* as reported by Maria et al.(1987). Haemolymph osmolality and ion (Cl^-) concentrations were studied by Warburg et al.(1987) in two intertidal crabs, *Pachygrapsus marmoratus* and *Pachytransversus*. Transfer of acid-base equivalents coupled with strong ion movements across the gills ensure active compensation to respiratory acid-base disturbances in fishes and crustaceans by environmental hypercapnia or hyperoxia (Truchot,1987a; McDonald et al.,1989). The effects of seawater concentration, ion substitution, and ion transport inhibitors on oxygen consumption were studied by Cedomil lucu and Dijana (1995) in the gills of shore crab *Carcinus mediterraneus*. Effect of lead on Na+ balance in freshwater cray fish Cherax destructor were studied by Mark et al.(1998).

Effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab, *Chasmagnathus granulatus* were evaluated by Vitale et al.(1999). Osmotic and ionic regulation in *Chasmagnathus granulatus* were studied by Bromberg et al. (1995). Osmotic and ionic regulation during hyposaline exposure, and in response to emersion in air breathing crab, *Leptograpsus variegates* were examined by Ashley and Stephen (1997). Toxicity of microcystin on ion regulation and antioxidant system in gills of *Chasmagnathus granulatus* were determined by Vinagre et al. (2002). Stephen and Mike (2004) studied the survival and physiological responses in low salinity in two intertidal crabs *Clibanarius taeniatus* and *Clibanarius virescens*. Gill (Na^+,K^+)_ATPase and phosphatase activities by potassium and ammonium ions were analyzed by Masui et al.(2003). Kennedy et al. (2005) were studied sodium sulfate toxicity in freshwater crustacean Ceriodaphnia dubia.

6.3. Materials and Methods

Eighteen crabs each were introduced into low (0.009mg/l), medium (0.02mg/l), and high (0.04mg/l) sub lethal concentrations of mercury for 1, 7, and 15 days. An equal number of crabs served as the controls. First set of 18 crabs (6 from each concentration) was killed on 1st day, the second set of 18 crabs on the 7th day , and the third set of 18 crabs on the 15th day. Simultaneously, an equal number of controls was also sacrificed at the specific time-periods of 1, 7 and 15 days post- exposure. The tissues such as chelate muscle, abdominal muscle, hepatopancreas, and gills were dissected out, weighed and ionic composition of the tissues determined. The pooled samples of each tissue, weighing 250mg each, from the crabs, were placed in a beaker containing 25ml of Analar grade nitric acid. The contents were heated to 60°c to digest the tissues (Agemian and Chau 1976). The tissue digest was suitably diluted before estimation of various metals, and the dilution factor was taken into account while doing the calculations. Sodium and potassium levels were estimated using a flame photometer (Systronics Flame Photometer, 121), and the levels of calcium and magnesium were determined using a Varian Techtron Atomic Absorption spectrophotometer at wavelengths of 422.7nm, and 285.2nm, respectively. The ionic concentrations were expressed as mEq /Kg wet weight of tissue.

6.4. Results

Sodium

In the present study it was noticed the amount of sodium increased in all the four tissues viz., abdominal, chelate muscle tissue, hepatopancreas, and gills. It was recorded that the maximum amount of increase of sodium was observed in the gills and hepatopancreas, followed by abdominal muscle tissue and chelate muscle tissue (Table 18, Figs. 23, 24, 25, 26). There was a significant difference ($P<0.05$) in the sodium levels between the sub lethal concentrations, and days of exposure in all the four tissues tested.

In the case of abdominal muscle tissue the duration of exposures and concentrations also increased sodium levels as (F= 29.279, critical value 2.25,Table 19). In chelate muscle tissue all the three sub lethal concentrations showed significant increase in sodium level (F=569.869: critical value 2.758, Table 19). The days of exposures also significantly increased the sodium level (F=471.760, critical value 3.15, Table 19)

In the case of gill tissue also, the three sub lethal concentrations caused significant increase in the sodium levels ($P<0.05$). Days of exposure also significantly increased sodium levels (F=393.73, critical value 3.15, Table 19). As in the case of hepatopancreas tissue, days of exposure and concentrations also remarkably increased sodium levels (F=73.794: critical value 2.254, Table 19)

Potassium

Mercury reduced the amount of potassium in the abdominal tissue, hepaotopancreas, gill, and muscle tissue, and the values were significantly low in all the three sub lethal concentrations ($P<0.05$ Table 20, 21; Figs. 27,28,29,30.). The level of potassium significantly decreased as the concentrations increased ($P<0.05$ Table. 20,21; Figs.27, 28,29,30). The sub lethal concentrations of mercury decreased the potassium level in the tissues as days increased, and the decrease was statistically significant ($P<0.05$; Table 21)

Calcium

The amount of calcium decreased in all the four tissues studied (Table 22; Figs. 31,32,33,34). The level of calcium content decreased significantly as days increased ($P<0.05$; Table 23). The sub lethal concentrations of mercury also caused decrease in the calcium level in all the tissues as concentrations were increased ($P<0.05$, Table 23)

Magnesium

Magnesium levels increased in all the tissues of the crabs exposed to mercury. Chelate muscles showed the maximum increase (percentage-wise) in magnesium levels followed by other tissues (Table 24, Figs. 35,36,37,38). The levels increased significantly in all the tissues as duration, and concentrations of exposure were increased. (Table 25).

Table 18. Levels of sodium in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values expressed as mEq/kg wet weight of tissues. Each value is mean of six observations ± SD.Values in parenthesis are percentage change over control.

Tissues	Days		Control	0.009	0.02	0.04
Abdominal muscle tissue	1st day	Mean±S.D % change	113.46±1.58 (100%)	118.06±1.73 (104.05%)	121.82±2.57 (107.37%)	126.89±2.37 (111.84%)
	7th day	Mean±S.D % change	112.37±1.55 (100%)	124.15±2.62 (110.48%)	131.79±2.92 (117.28%)	137.99±2.98 (122.8%)
	15th day	Mean±S.D % change	117.81±1.63 (100%)	132.38±2.93 (112.37%)	143.4±3.88 (121.72%)	147.6±3.00 (125.29%)
Chelate muscle tissue	1st day	Mean±S.D % change	103.54±1.22 (100%)	115.41±1.93 (111.46%)	122.82±2.07 (118.62%)	131.36±3.61 (126.87%)
	7th day	Mean±S.D % change	116.32±1.52 (100%)	124.8±2.65 (107.29%)	131.69±0.03 (113.21%)	137.98±3.45 (118.62%)
	15th day	Mean±S.D % change	122.97±2.29 (100%)	131.51±2.71 (106.94%)	137.85±2.53 (112.1%)	141.61±3.79 (115.16%)
Hepatopancreas	1st day	Mean±S.D % change	152.54±3.76 (100%)	163.15±3.78 (106.96%)	171.45±3.95 (112.4%)	181.85±4.85 (119.21%)
	7th day	Mean±S.D % change	150.44±3.35 (100%)	173.26±3.37 (115.17%)	183.41±3.97 (121.92%)	197.15±4.33 (131.05%)
	15th day	Mean±S.D % change	148.05±3.92 (100%)	184.87±3.51 (124.87%)	196.03±3.98 (132.41%)	214.91±4.12 (145.16%)
Gill	1st day	Mean±S.D % change	185.88±3.24 (100%)	213.04±3.25 (114.61%)	223.47±3.92 (120.22%)	233.34±4.26 (125.53%)
	7th day	Mean±S.D % change	188.61±3.27 (100%)	223.94±4.19 (118.73%)	238.16±5.87 (126.27%)	243.15±2.12 (128.92%)
	15th day	Mean±S.D % change	198.13±3.63 (100%)	245.64±4.46 (123.98%)	255.43±4.14 (128.92%)	264.82±3.26 (133.66%)

Table 19. ANOVA for sodium levels in the tissues of *Scylla serrata* at three sub lethal concentrations of mercury.

Tissues	Source	Sum of Squares	df	Mean Square	F	F crit	P
Abdominal muscle tissue	Concentrations (C)	5361.116	3	1787.039	521.465	2.758	< 0.05 **
	Days (D)	2806.438	2	1403.219	409.465	3.150	< 0.05 **
	C x D	602.036	6	100.339	29.279	2.254	< 0.05 **
	Error	205.618	60	3.427			
	Total	8975.207	71	126.411			
Chelate muscle tissue	Concentrations(C)	5120.098	3	1706.699	569.869	2.758	< 0.05 **
	Days(D)	2825.747	2	1412.874	471.760	3.150	< 0.05 **
	C x D	137.989	6	22.998	7.679	2.254	< 0.05 **
	Error	179.694	60	2.995			
	Total	8263.528	71	116.388			
Hepatopancreas	Concentrations (C)	33319.734	3	11106.578	1256.456	2.758	< 0.05 **
	Days (D)	9049.739	2	4524.869	511.886	3.150	< 0.05 **
	C x D	934.432	6	155.739	17.618	2.254	< 0.05 **
	Error	530.376	60	8.840			
	Total	43834.281	71	617.384			
Gill	Concentrations(C)	21657.867	3	7219.289	1350.716	2.758	< 0.05 **
	Days (D)	4208.832	2	2104.416	393.733	3.150	< 0.05 **
	C x D	2366.464	6	394.411	73.794	2.254	< 0.05 **
	Error	320.687	60	5.345			
	Total	28553.850	71	402.167			

Df=degrees of freedom

P=0.05**=1%level

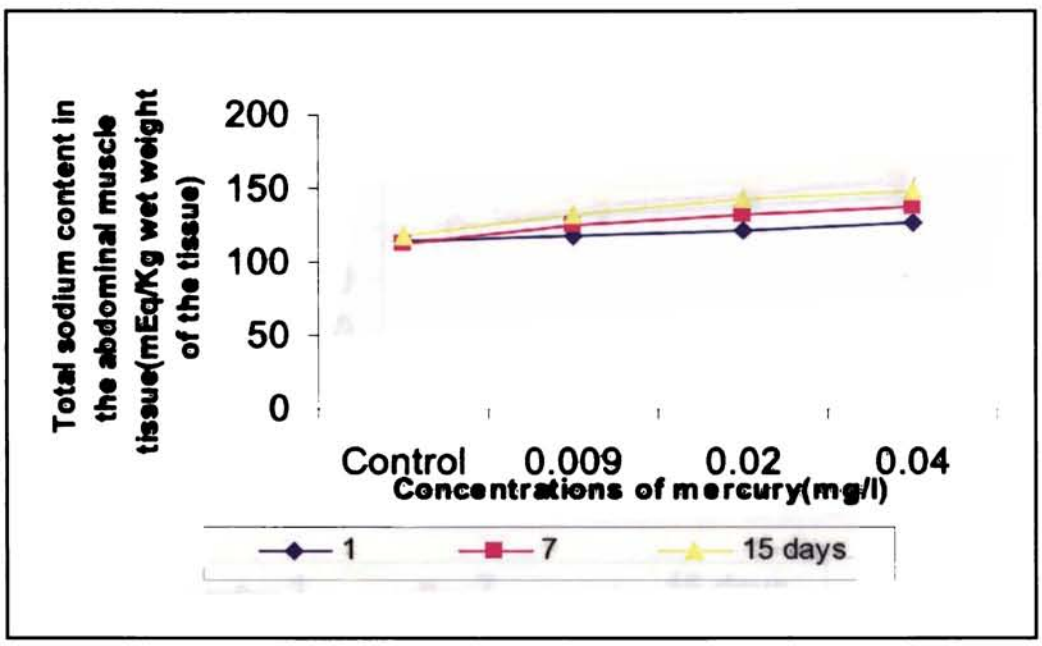


Figure 23. Total sodium content in the abdominal muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury (*n* = 6)

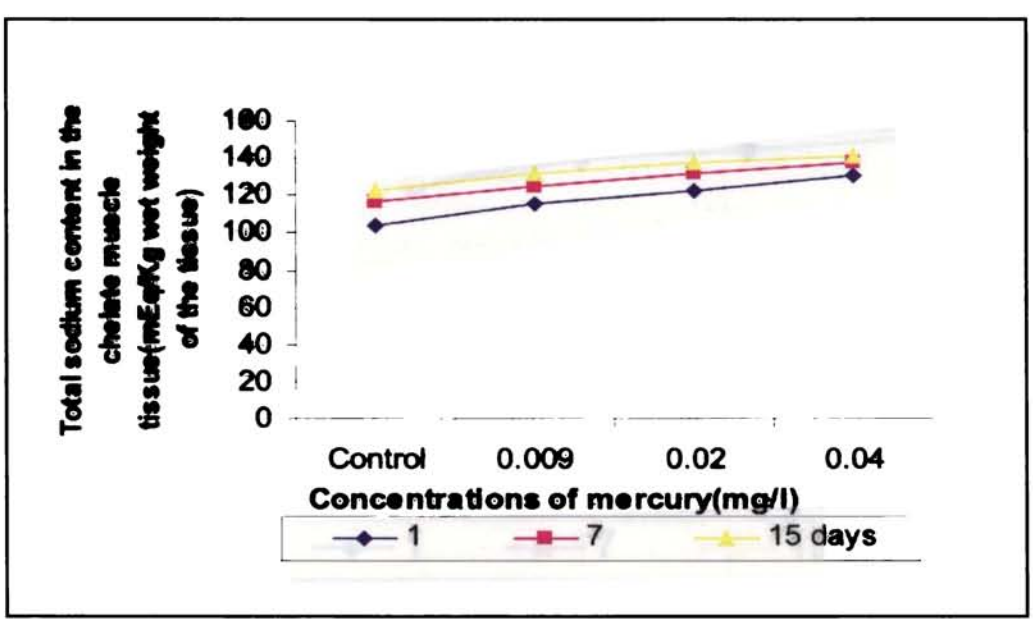


Figure 24. Total sodium content in the chelate muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury (n = 6)

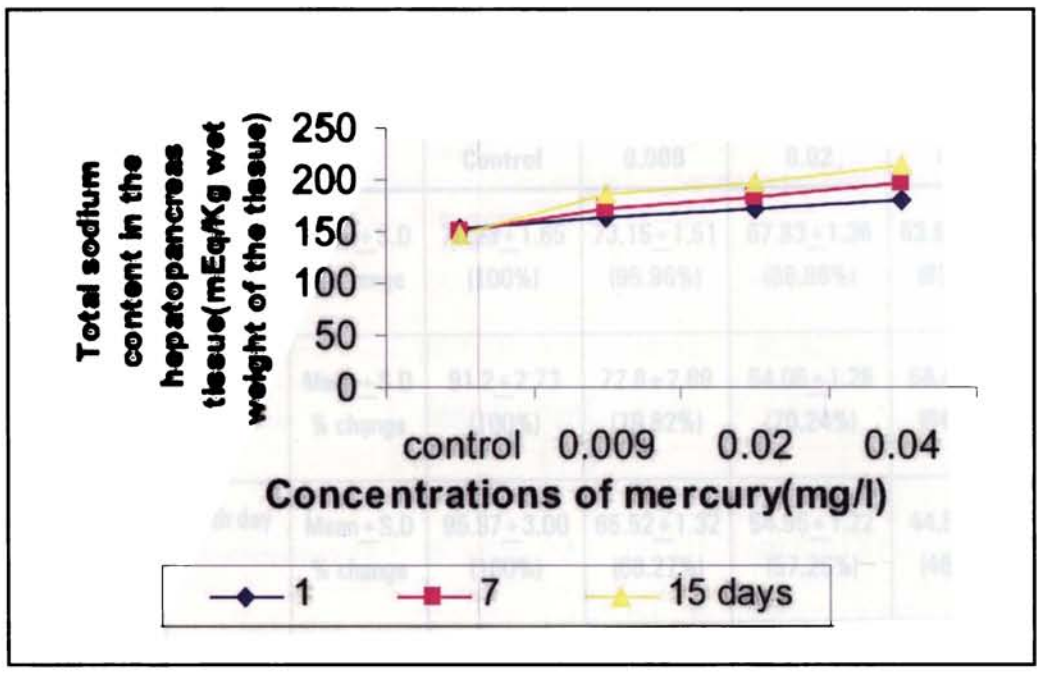


Figure 25. Total sodium content in the hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

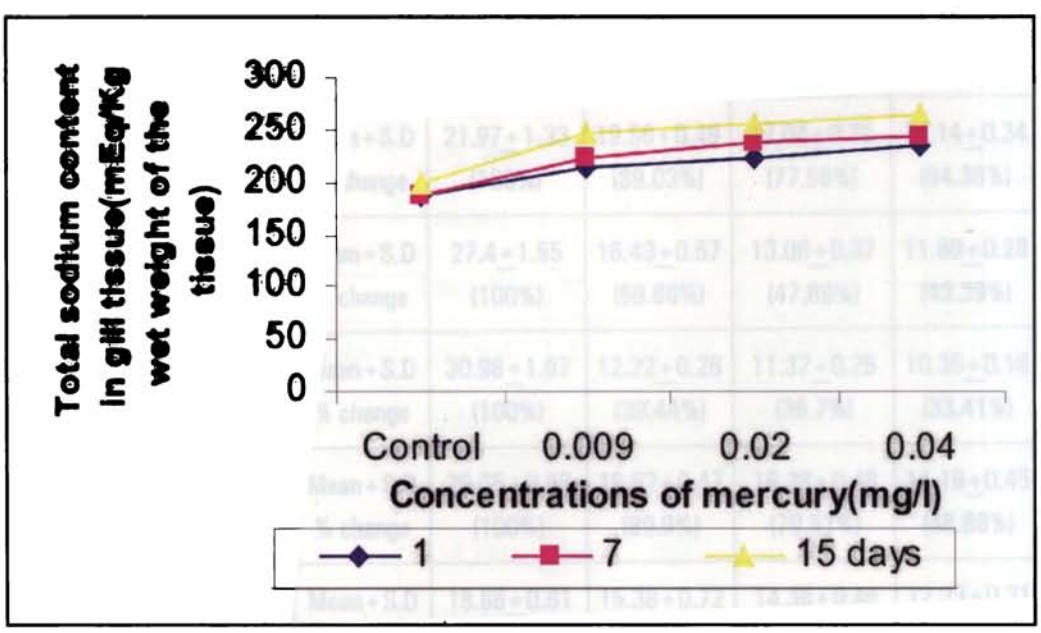


Figure 26. Total sodium content in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

Table 20. Levels of potassium in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values expressed as m Eq/kg wet weight of tissue. Each value is a mean of six observations ± SD.Values in parenthesis are percentage change over control

Tissues	Days		Control	0.009	0.02	0.04
Abdominal muscle tissue	1st day	Mean±S.D % change	76.23±1.65 (100%)	73.15±1.51 (95.96%)	67.83±1.36 (88.98%)	63.82±1.29 (83.72%)
	7th day	Mean±S.D % change	91.2±2.73 (100%)	72.8±2.69 (79.82%)	64.06±1.26 (70.24%)	58.4±1.17 (64.04%)
	15th day	Mean±S.D % change	95.97±3.00 (100%)	65.52±1.32 (68.27%)	54.95±1.22 (57.26%)	44.5±1.12 (46.37%)
Chelate muscle tissue	1st day	Mean±S.D % change	66.78±1.18 (100%)	55.89±1.07 (83.69%)	52.54±1.23 (78.68%)	48.02±1.16 (71.91%)
	7^{th} day	Mean±S.D % change	60.57±1.44 (100%)	50.54±1.57 (83.44%)	47.55±1.29 (78.5%)	42.89±1.19 (70.81%)
	15th day	Mean±S.D % change	54.83±1.63 (100%)	47.14±1.15 (85.97 %)	45.13±1.06 (82.30)	42.13±0.03 (76.83)
Hepatopancreas	1st day	Mean±S.D % change	21.97±1.33 (100%)	19.56±0.49 (89.03%)	17.04±0.35 (77.56%)	14.14±0.34 (64.36%)
	7th day	Mean±S.D % change	27.4±1.55 (100%)	16.43±0.57 (59.96%)	13.06±0.37 (47.66%)	11.89±0.28 (43.39%)
	15th day	Mean±S.D % change	30.98±1.87 (100%)	12.22±0.26 (39.44%)	11.37±0.25 (36.7%)	10.35±0.16 (33.41%)
Gill	1st day	Mean±S.D % change	20.65±0.85 (100%)	18.52±0.47 (89.9%)	16.38±0.48 (79.51%)	14.19±0.45 (68.88%)
	7th day	Mean±S.D % change	18.88±0.61 (100%)	15.38±0.72 (86.76%)	14.38±0.46 (76.17%)	12.23±0.31 (64.78%)
	15th day	Mean±S.D % change	16.79±0.33 (100%)	14.12±0.22 (84.1%)	12.2±0.14 (72.66%)	10.83±0.06 (64.5%)

Table 21. ANOVA for potassium levels in the tissues of *Scylla serrata* at three sub lethal concentrations of mercury.

Tissues	Source	Sum of Squares	df	Mean Square	F	F crit	P
Abdominal muscle tissue	Concentrations (C)	10461.388	3	3487.129	776.857	2.758	< 0.05 **
	Days(D)	542.130	2	271.065	60.387	3.150	< 0.05 **
	C x D	2671.232	6	445.205	99.182	2.254	< 0.05 **
	Error	269.326	60	4.489			
	Total	13944.076	71	196.395			
Chelate muscle tissue	Concentrations(C)	2619.681	3	873.227	594.570	2.758	< 0.05 **
	Days (D)	888.330	2	444.165	302.427	3.150	< 0.05 **
	C x D	67.611	6	11.268	7.673	2.254	< 0.05 **
	Error	88.120	60	1.469			
	Total	3663.742	71	51.602			
Hepatopancreas	Concentrations (C)	2344.290	3	781.430	1050.307	2.758	< 0.05 **
	Days(D)	45.572	2	22.786	30.627	3.150	< 0.05 **
	C x D	509.246	6	84.874	114.078	2.254	< 0.05 **
	Error	44.640	60	.744			
	Total	2943.749	71	41.461			
Gill	Concentrations(C)	399.991	3	133.330	427.029	2.758	< 0.05 **
	Days (D)	185.971	2	92.985	297.812	3.150	< 0.05 **
	C x D	2.358	6	.393	1.259	2.254	> 0.05**
	Error	18.734	60	.312			
	Total	607.054	71	8.550			

Df=degrees of freedom

P=0.05**=1%level

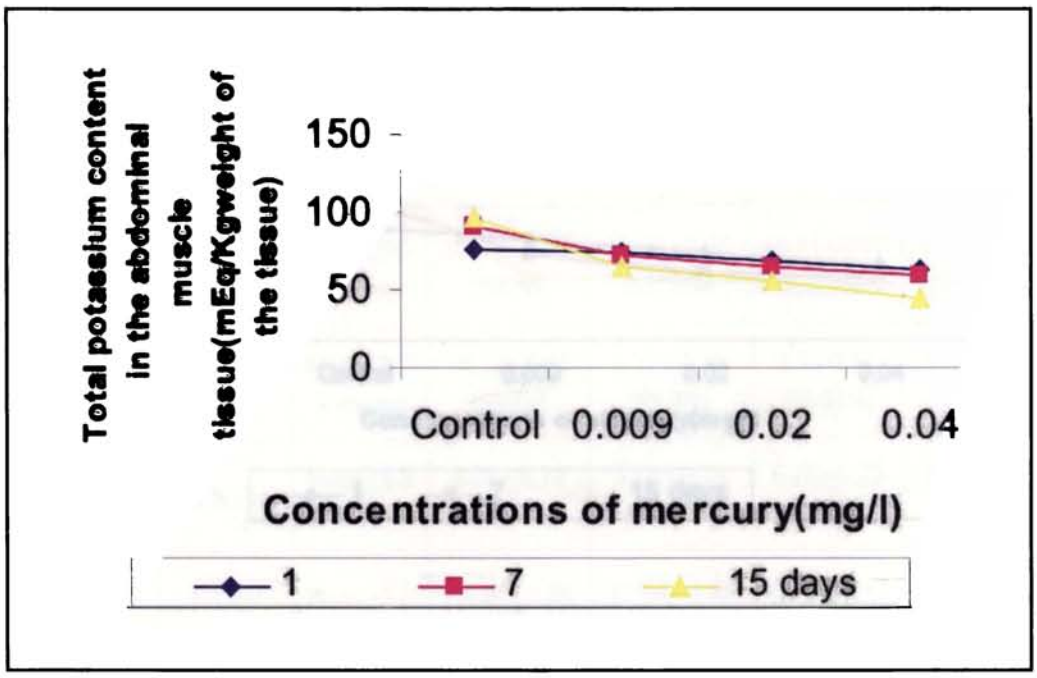


Figure 27. Total potassium content in the abdominal muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

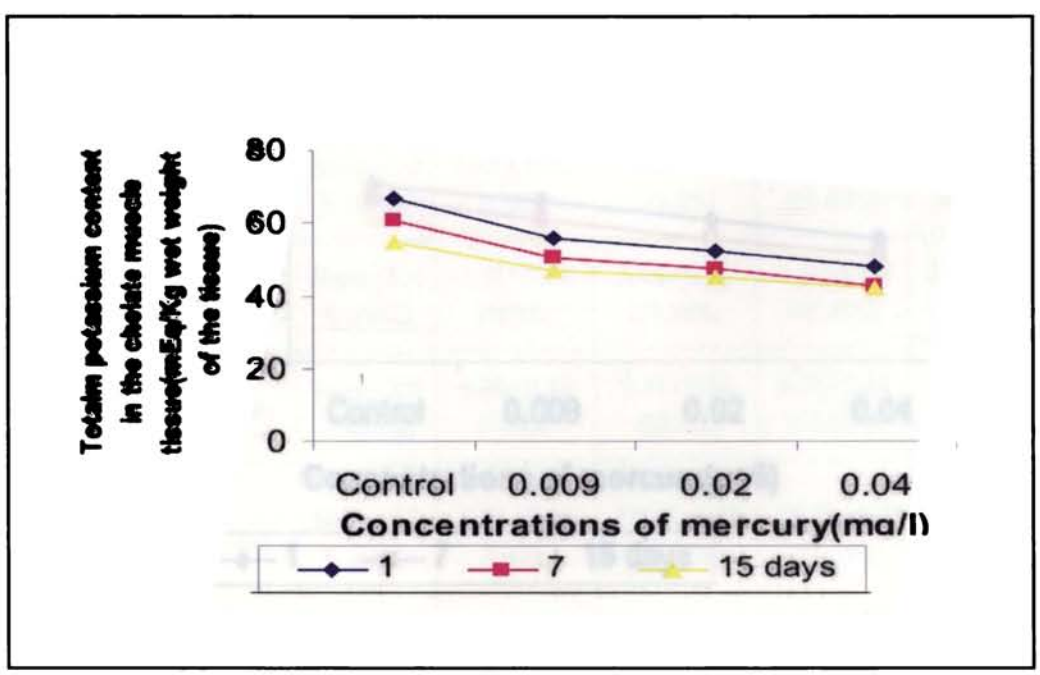


Figure 28. Total potassium content in the chelate muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

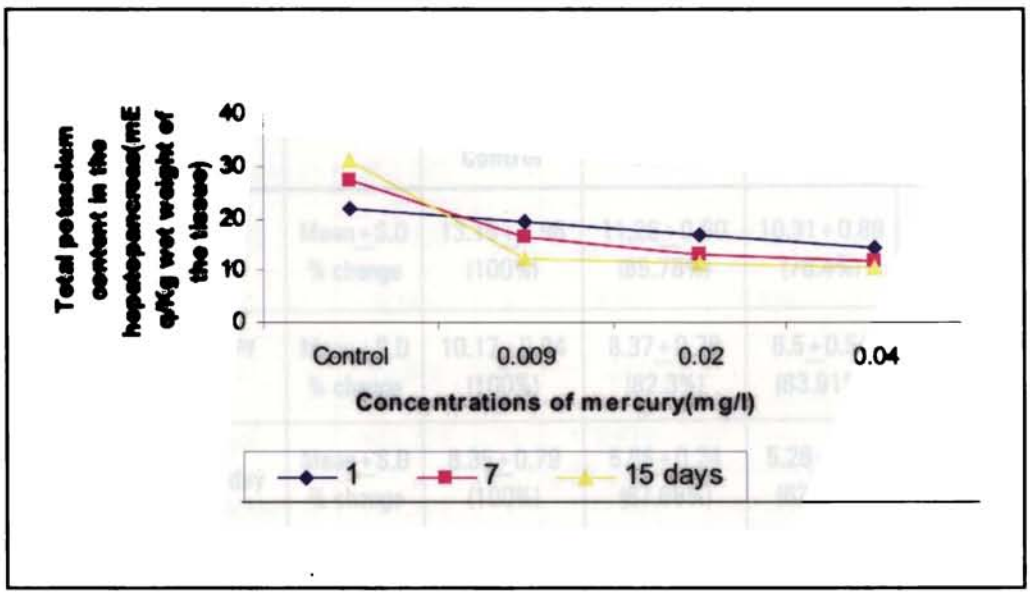


Figure 29. Total potassium content in the hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury (*n* = 6)

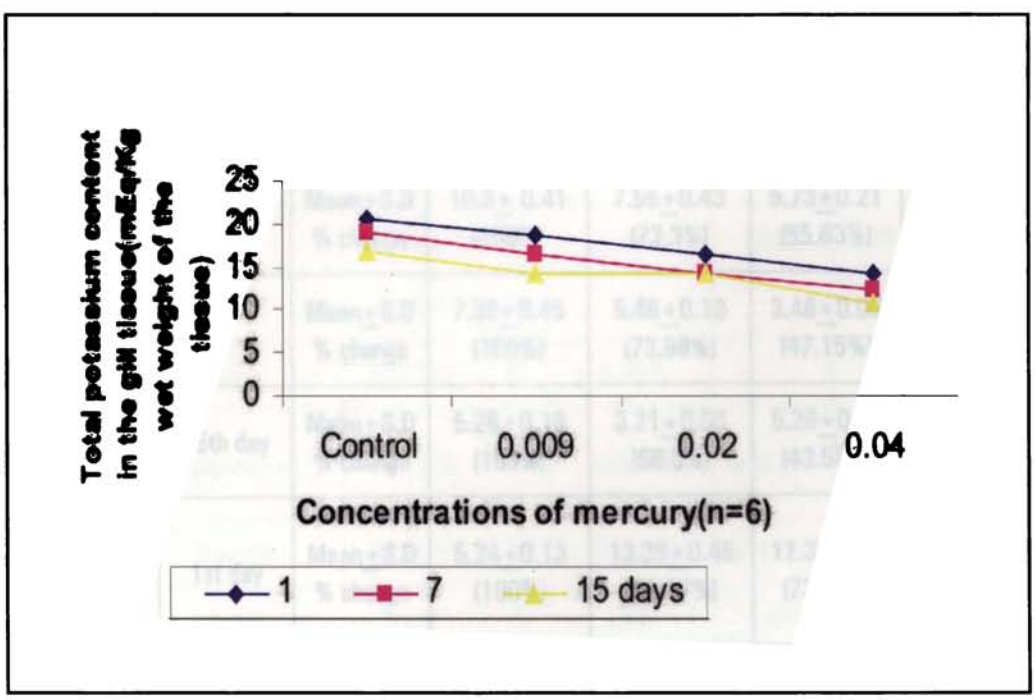


Figure 30. Total potassium content in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury (*n* = 6)

Table 22. Levels of calcium in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values expressed as mEq/kg wet weight of tissues. Each value is a mean of six observations ±SD. Values in parenthesis are percentage change over control.

Tissues	Days		Control	0.009	0.02	0.04
Abdominal muscle tissue	1st day	Mean±S.D % change	13.15±0.98 (100%)	11.28±0.90 (85.78%)	10.31±0.89 (78.4%)	8.78±0.83 (66.77%)
Chelate muscle tissue	7th day	Mean±S.D % change	10.17±0.84 (100%)	8.37±0.78 (82.3%)	6.5±0.54 (63.91%)	4.26±0.33 (41.89%)
	15th day	Mean±S.D % change	8.35±0.79 (100%)	5.65±0.24 (67.66%)	5.26±0.22 (62.99%)	2.19±0.08 (26.23%)
	1st day	Mean±S.D % change	14.26±0.99 (100%)	12.16±0.49 (85.27%)	10.46±0.51 (73.35%)	69.3±0.52 (65.22%)
Hepatopancreas	7th day	Mean±S.D % change	12.08±0.42 (100%)	10.19±0.23 (84.35%)	9.29±0.48 (76.9%)	7.4±0.24 (61.26%)
	15th day	Mean±S.D % change	10.3±0.45 (100%)	8.64±0.19 (83.88%)	7.28±0.23 (70.68%)	5.27±0.22 (51.17%)
	1st day	Mean±S.D % change	10.3± 0.41 (100%)	7.55±0.43 (73.3%)	5.73±0.21 (55.63%)	4.23±0.12 (41.07%)
	7th day	Mean±S.D % change	7.38±0.45 (100%)	5.46±0.18 (73.98%)	3.48±0.06 (47.15%)	2.43±0.04 (32.93%)
Gill	15th day	Mean±S.D % change	5.28±0.19 (100%)	3.21±0.08 (60.8%)	5.26±0.24 (43.56%)	1.67±0.02 (31.63%)
	1st day	Mean±S.D % change	5.24±0.13 (100%)	13.38±0.45 (85.27%)	11.37±0.43 (73.35%)	9.55± 0.22 (65.22%)
	7th day	Mean±S.D % change	12.95±0.51 (100%)	11.17±0.36 (84.35%)	9.2±0.27 (76.9%)	7.15±0.25 (61.26%)
	15th day	Mean±S.D % change	10.12±0.16 (100%)	9.51±0.14 (83.88%)	7.18±0.15 (70.68%)	5.1±0.13 (51.17%)

Table 23. ANOVA for Calcium levels in the tissues of *Scylla serrata* at three sub lethal concentrations of mercury.

Tissues	Source	Sum of Squares	df	Mean Square	F	F crit	P
Abdominal muscle tissue	Concentrations(C)	280.898	3	93.633	269.477	2.758	< 0.05 **
	Days(D)	375.789	2	187.895	540.764	3.150	< 0.05 **
	C x D	9.641	6	1.607	4.624	2.254	< 0.05 **
	Error	20.848	60	.347			
Chelate muscle tissue	Total	687.175	71	9.679			
	Concentrations(C)	231.099	3	77.033	497.215	2.758	< 0.05 **
	Days (D)	162.122	2	81.061	523.214	3.150	< 0.05 **
	C x D	2.516	6	.419	2.706	2.254	< 0.05 **
	Error	9.296	60	.155			
	Total	405.033	71	5.705			
Hepatopancreas	Concentrations (C)	242.471	3	80.824	407.505	2.758	< 0.05 **
	Days (D)	178.481	2	89.240	449.941	3.150	< 0.05 **
	C x D	11.410	6	1.902	9.588	2.254	< 0.05 **
	Error	11.900	60	.198			
	Total	444.263	71	6.257			
Gill	Concentrations(C)	313.960	3	104.653	870.804	2.758	< 0.05 **
	Days(D)	233.620	2	116.810	971.957	3.150	< 0.05 **
	C x D	3.226	6	.538	4.474	2.254	< 0.05 **
	Error	7.211	60	.120			
	Total	558.016	71	7.859			

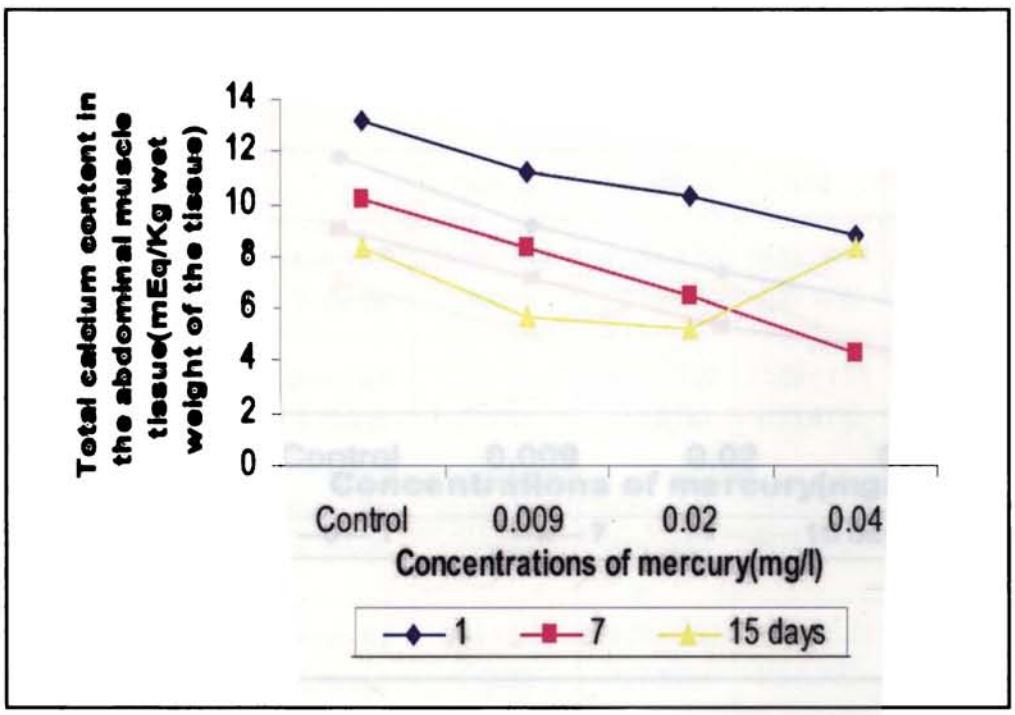


Figure 31. Total calcium content in the abdominal muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

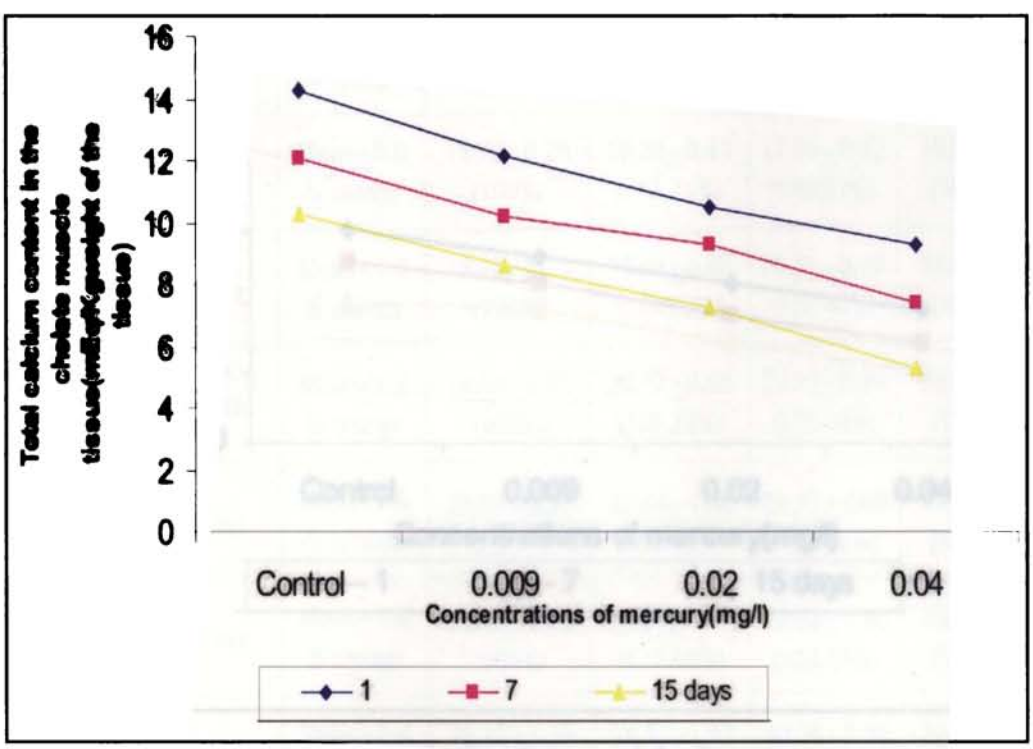


Figure 32. Total calcium content in the chelate muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

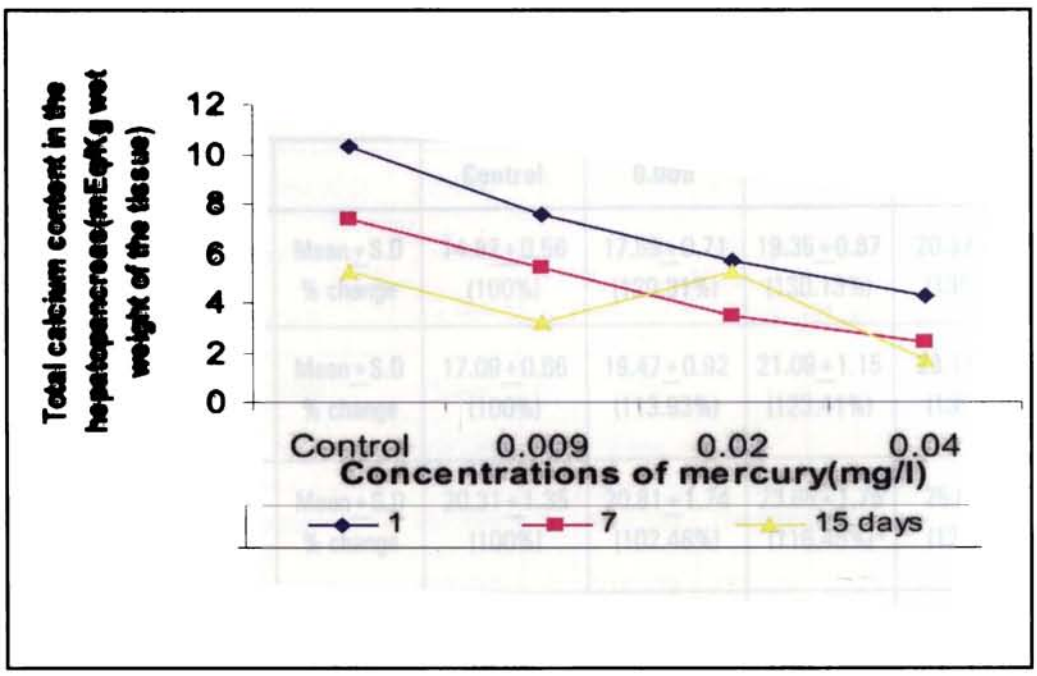


Figure 33. Total calcium content in the hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury (*n* = 6)

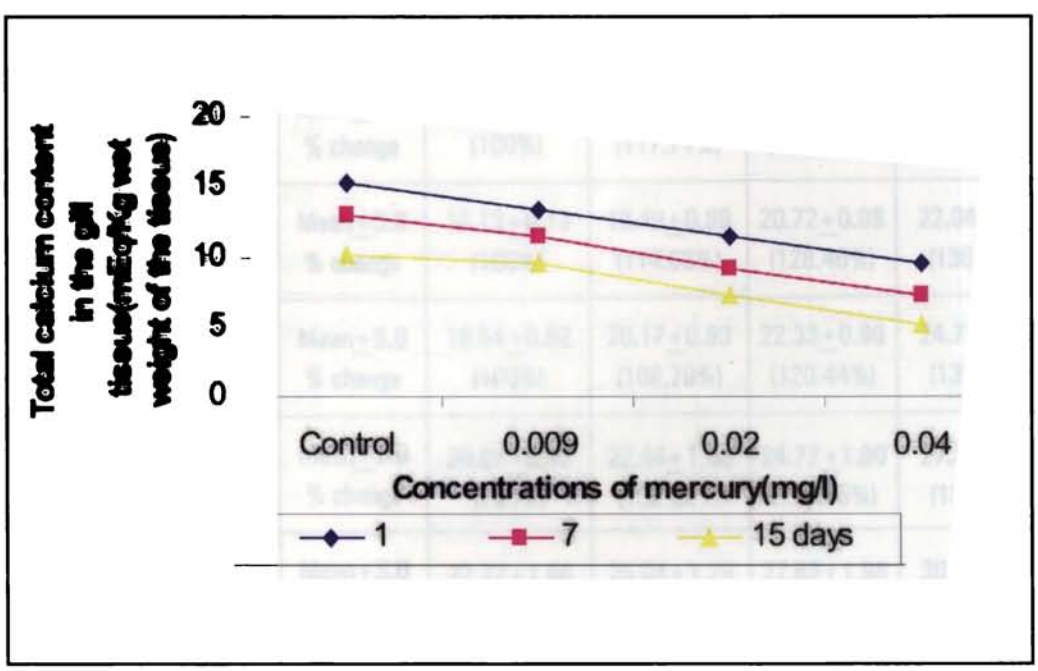


Figure 34. Total calcium content in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury (*n* = 6)

Table 24. Levels of magnesium in the tissues of *Scylla serrata* exposed to three sub lethal concentrations of mercury. Values expressed as mEq/Kg wet weight of tissue. Each value is a mean of six observations ±SD.Values in parenthesis are percentage change over control.

Tissues	Days		Control	0.009	0.02	0.04
Abdominal muscle tissue	1st day	Mean±S.D % change	14.87±0.56 (100%)	17.89±0.71 (120.31%)	19.35±0.87 (130.13%)	20.37±0.89 (136.99%)
	7th day	Mean±S.D % change	17.09±0.66 (100%)	19.47±0.92 (113.93%)	21.09±1.15 (123.41%)	23.17±1.43 (135.58%)
	15th day	Mean±S.D % change	20.31±1.35 (100%)	20.81±1.74 (102.46%)	23.65±1.75 (116.45%)	25.8±1.78 (127.03%)
Chelate muscle tissue	1st day	Mean±S.D % change	8.04±0.34 (100%)	10.29±0.37 (127.99%)	12.24±0.41 (152.24%)	14.32±0.45 (178.11%)
	7th day	Mean±S.D % change	10.26±0.39 (100%)	13.93±0.45 (135.77%)	16.33±0.49 (159.16%)	18.98±0.69 (184.99%)
	15th day	Mean±S.D % change	12.4±0.33 (100%)	16.02±0.43 (129.19%)	18.02±0.94 (145.32%)	22.24±1.42 (179.35%)
Hepatopancreas	1st day	Mean±S.D % change	13.78±0.24 (100%)	16.22±0.42 (117.71%)	17.75±0.42 (128.81%)	19.69±0.54 (142.89%)
	7th day	Mean±S.D % change	16.13±0.13 (100%)	18.49±0.89 (114.63%)	20.72±0.98 (128.46%)	22.04±0.97 (136.64%)
	15th day	Mean±S.D % change	18.54±0.82 (100%)	20.17±0.93 (108.79%)	22.33±0.96 (120.44%)	24.73±0.99 (133.39%)
Gill	1st day	Mean±S.D % change	20.66±0.92 (100%)	22.44±1.68 (108.62%)	24.72±1.90 (119.55%)	27.38±2.00 (132.53%)
	7th day	Mean±S.D % change	22.22±1.66 (100%)	25.04±1.76 (112.69%)	27.63±1.98 (124.35%)	30.05±2.01 (135.24%)
	15th day	Mean±S.D % change	25.56±1.45 (100%)	28.24±1.97 (110.49%)	30.25±2.39 (118.35%)	32.91±2.65 (128.76%)

Table 25. ANOVA for magnesium levels in the tissues of *Scylla serrata* at three sub lethal concentrations of mercury.

Tissues	Source	Sum of Squares	df	Mean Square	F	F crit	P
Abdominal muscle tissue	Concentrations(C)	326.357	3	108.786	334.344	2.758	<0.05 **
	Days(D)	246.107	2	123.054	378.195	3.150	<0.05 **
	C x D	14.101	6	2.350	7.223	2.254	<0.05 **
	Error	19.522	60	.325			
	Total	606.087	71	8.536			
Chelate muscle tissue	Concentrations (C)	657.898	3	219.299	788.931	2.758	<0.05 **
	Days(D)	432.046	2	216.023	777.145	3.150	<0.05 **
	C x D	22.268	6	3.711	13.352	2.254	<0.05 **
	Error	16.678	60	.278			
	Total	1128.890	71	15.900			
Hepatopancreas	Concentrations(C)	359.637	3	119.879	371.118	2.758	<0.05 **
	Days(D)	252.461	2	126.231	390.781	3.150	<0.05 **
	C x D	3.624	6	.604	1.870	2.254	>0.05 **
	Error	19.381	60	.323			
	Total	635.103	71	8.945			
Gill	Concentrations(C)	526.420	3	175.473	437.867	2.758	<0.05 **
	Days(D)	356.783	2	178.391	445.149	3.150	<0.05 **
	C x D	3.690	6	.615	1.535	2.254	>0.05 **
	Error	24.045	60	.401			
	Total	910.938	71	12.830			

df=degrees of freedom

P=0.05**=1%level

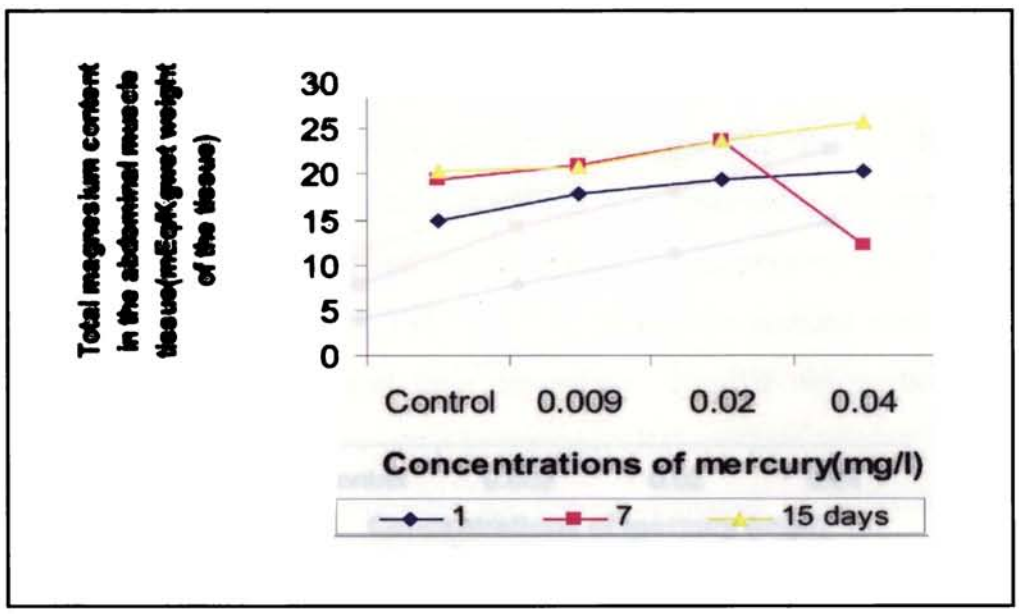


Figure 35. Total magnesium content in the abdominal muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

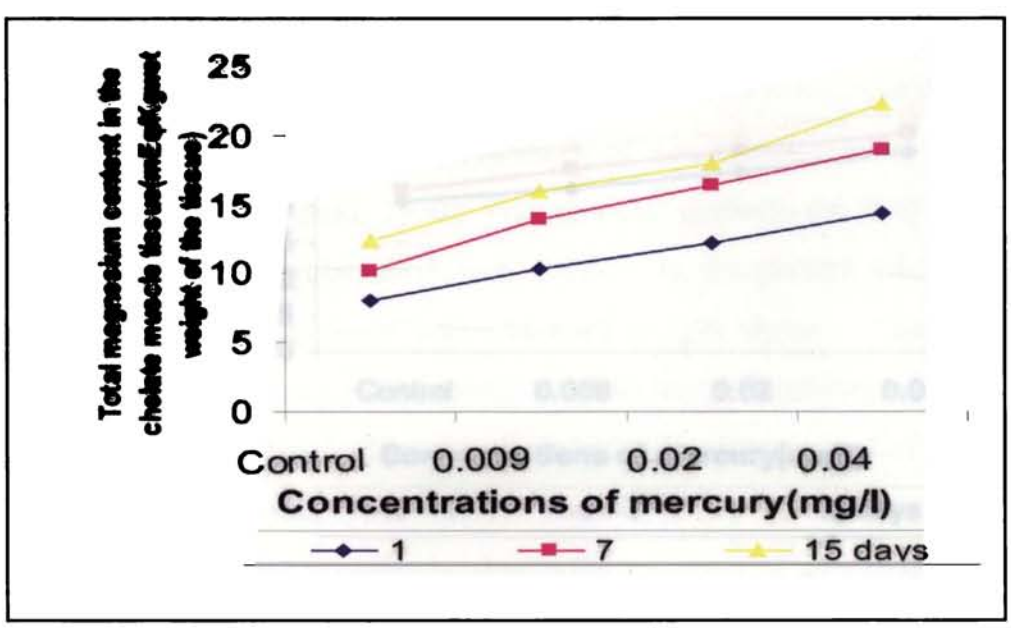


Figure 36. Total magnesium content in the chelate muscle tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

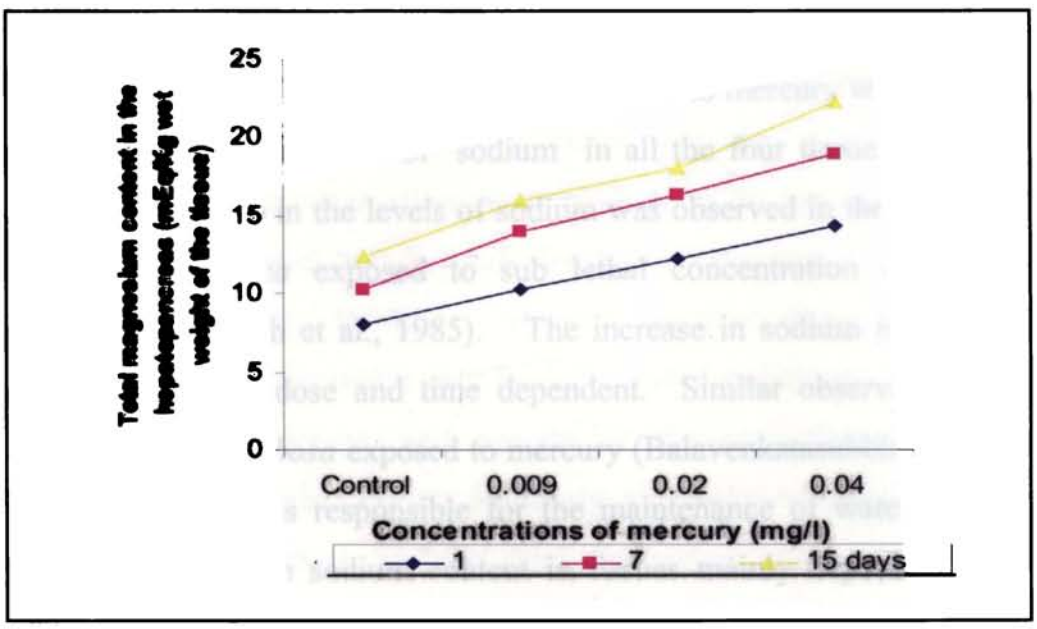


Figure 37. Total magnesium content in the hepatopancreas of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

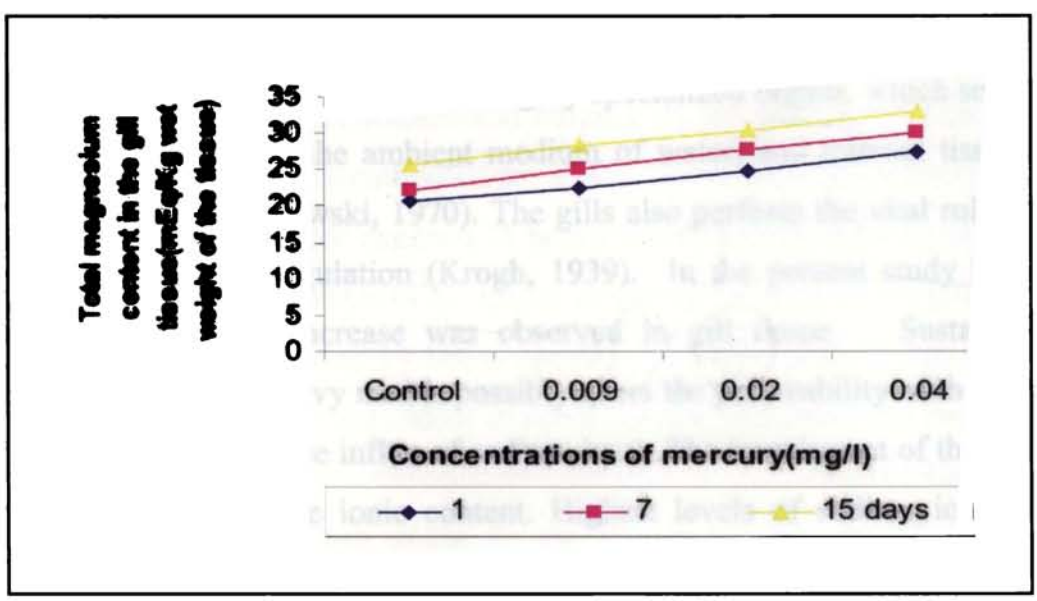


Figure 38. Total magnesium content in the gill tissue of *Scylla serrata* exposed to three sub lethal concentrations of mercury ($n = 6$)

6.5 Discussion

Sodium

In the present study, exposure of organism to mercury was found to cause increase in the levels of sodium in all the four tissues. Similar significant increase in the levels of sodium was observed in the tissues of *Tilapia mossambica* exposed to sub lethal concentration of copper (Balavenkatasubbiah et al., 1985). The increase in sodium ions in the present study was dose and time dependent. Similar observation was reported in *Pila globosa* exposed to mercury (Balavenkatasubbiah, 1984). In tissues, sodium is responsible for the maintenance of water balance (Bryan, 1960). The sodium content in tissues mainly depends on the efficient permeability of the biomemebrane, and also on the functional role of sodium pumps, which regulate the ionic content of the tissue. Heavy metals interact with lipids present in the lamellar membrane resulting in conformational changes in the lamellar structures, and consequent active and passive movement of the ions (Tulasi et al., 1990).

The gills in crustaceans are highly specialized organs, which serve as a barrier between the ambient medium of water, and internal tissues (Bergmiller and Bielawski, 1970). The gills also perform the vital role in osmotic and ionic regulation (Krogh, 1939). In the present study high amount of sodium increase was observed in gill tissue. Sustained exposure of gill to heavy metals possibly alters the permeability of the gill lamellae leading to free influx of sodium ions. The impairment of the gill tissues could alter the ionic content. Highest levels of sodium ions in hepatopancreas might possibly be due to the transport of ions from gill to this tissue. The sodium, potassium and magnesium dependant ATPases have a vital role in the active transport of these ions (Lockwood, 1977). Similar observations were also reported by Loganathan (1995).

Potassium

A significant decrease in the content of potassium was observed in all the four tissues of crabs exposed to mercury. It was observed that the decrease of potassium in the tissue was also dose and time dependent. Similar observations were reported in the tissues of *Tilapia mossambica* exposed to the sub lethal concentrations of copper (Balavenkitasubbiah, 1985). It was reported that sub lethal concentrations of chromium and lead decreased potassium content in the gill, hepatopancreas, and muscle of the crab *Spirolatelphusa hydrodroma* (Sakundala, 1992). Similarly, sub lethal levels of mercury and copper increased sodium and potassium contents in the haemolymph and digestive gland of *Pila globasa* (Balavenkatasubbiah, 1984). Potassium is an important constituent in tissues having a great influence on muscular activity. In addition, potassium is the main intracellular cation involved in several physiological functions, such as functioning of nerves and muscles, acid-base balance, and osmotic pressure (Satoskar, 1986). Potassium plays an important role in maintenance of the electrochemical balance of the tissues. A number of reasons have been documented for a decrease in the concentration of K^+ in animals that were exposed to heavy metals (Balavenkatasubbiah, 1984). The altered levels of K^+ might be due to injuries of the gill and intestinal mucosa caused by heavy metals. It was observed that there was cellular damage/impairment in renal tubules and intestinal mucosa of *Fundulas heteroclitus* exposed to cadmium. It was believed that the renal tubule dysfunction might be the reason for the change in K+ content in tissues (Gardner and Yevich, 1970). Similar observations were made in the activities of fishes and crabs by Sakundala, (1992).

Calcium

In the present study the levels of calcium decreased in all the four tissues. Similar observations were also reported in *Tilapia mossambica.* It was observes that copper brought down the calcium content in tissues, and liver recorded the maximum decrease (35%) followed by muscle, gill and brain (Balavenkatasubbiah et al., 1985). In calcium hypocalcemic condition observed in fish that were exposed to cadmium was believed to be caused by defect in the intestinal absorption of calcium, or by an impaired reabsorption of calcium by the renal tubules, or by a combination of these two mechanisms. Hypocalcemic condition is characterized by micro muscular irritability and tetany (Larrsson et al., 1981; Satoskar 1986; Sakundala, 1992). Similar decreased calcium levels were also reported in *Spiralatelphusa hydrodroma* exposed to chromium and lead (Sakundala, 1992). Similar decreased of potassium and calcium levels were also reported by Logaswamy et al. (2007) due to the exposure of dimethoate in the liver tissue of Cyprinus carpio.

Magnesium

In the present investigation the levels of magnesium were found increased in all the tissue samples. Similar increase in magnesium was reported in gill, hepatopancreas and muscle *of Spiralatelphusa hydrodroma* exposed to sub lethal concentration of chromium and lead for 30 days (Sakundala, 1992). Increase in magnesium level was also recorded in *Platichythys flesus* (Larsson et al., 1981), *Cyprinus carpio* (Muramoto, 1981), and *Barytelphusa guerini* (Tulasi et al., 1990) exposed to heavy metals. The ability of Pd exposed cray fish to activate Na transport was also reduced by 70% as determined by Mark et al. (1998).Thus the accumulation of large lead burdens in the gills and reductions in Na flux rates will effectively restrict the distribution of Cherax destructor into soft water bodies.

Chapter 7

ELECTROPHORETIC STUDIES

7.1 Introduction

An attempt has been made to assay qualitatively the total protein and metal binding proteins in the muscle , hepatopancreas and gill tissues of *Scylla serrata* , to analyze the protein composition of these tissues, and also to monitor the changes in the electrophoretic pattern subsequent to exposure of the organism to the three sub lethal concentrations of mercury. Proteins, by virtue of their genetically controlled variation, have been subjected to extensive research in recent years. Studies involving protein biochemistry have often led to formulation of new concepts and techniques, which could be utilized to unravel the taxonomic and evolutionary relationships. The primary structure of protein molecule with its amino acid sequence is genetically determined. The properties of protein can indicate the ultimate biochemical makeup and relationships among different organisms.

Electrophoresis is the process of movement of charged particles through an electrolyte when subjected to an electric field. If the particles are charged differently, they will move in opposite directions; positively charged particles migrating to the cathode, and the negatively charged to the anode. The rate of migration of particles to like charge will depend, among other things, on the net charge each carries. A complex mixture of tissue proteins can thus be separated into a number of fractions following electrophoresis due to this differential rate of migration of the component fractions. The sharpness of resolution depends upon the extent to which

each fraction is homogenous on its mobility. Protein synthesis is different in living organisms, and occurs as a measure of genetic differences between species. Such differences can be employed as criteria for species classification as well as its population differences.

Electrophoresis has become the common and main technique, as it is reasonably easy and inexpensive. In spite of the many physical arrangements for the apparatus and regardless of the medium through which molecules are allowed to migrate, all electrophoretic separations depend upon the charge distribution of the molecules being separated. One-dimensional electrophoresis is used for most routine protein and nucleic acid separations.

7.2 Materials and Methods

SDS – PAGE was done following the method of Laemmli (1970). Eighteen crabs as experimentals were exposed to each of the three sub-lethal concentrations of mercury as mentioned earlier, for three- time periods of 1, 7 and 15 days. Six crabs served as the controls. At the end of each time period six crabs were sacrificed and the tissues -abdominal muscle tissue, chelate muscle tissue, hepatopancreas and gill tissue- separated out and processed for further studies. The tissues samples from the controls were also processed simultaneously.

A sample of 200mg of each tissue was homogenized in phosphate buffered saline and the homogenate was passed through a clean cotton cloth to remove unbroken cell debris. The homogenates were then centrifuged at 15,000*g* for 15 min. and the supernatant was collected for gel electrophoresis (SDS-PAGE) studies:

0.625M Tris Hcl buffer, pH=6.8	=	20ml
20% SDS (W/V)	=	20ml

50% 2 – mercaptoethanol V/V	=	20ml
Glycerol	=	20ml
0.02% Bromophenol blue W/V	=	20ml
Total	=	100ml

Then the samples were quantified (Lowry et al., 1951). Equal quantity of protein from each sample was mixed with equal quantity of sample buffer and the mixture was heated in a water bath at 100^0C for 3min. The mixture was cooled to room temperature before applying on to the gels.

Stock Solution

a. Monomer (Acrylamide – Bis acrylamide 30:0.8) 30g of acrylamide and 0.80g of methylene bis acrylamide were dissolved in 100ml of distilled water

b. Resolving gel buffer (Tris Hcl pH 8.8), 36.3 g of Tris was dissolved in 48ml of I N Hcl and it was made up to 100ml using distilled water.

c. Catalyst N, N, N',N'- Tetramethylene diamine (TEMED).

d. Initiator: 0.15g of ammonium per sulphate was dissolved in 10ml of distilled water. This solution was prepared just before use.

e. SDS 10% W/V

f. Reservoir buffer (Tris – glycine, pH 8.3) 3g of Tris and 14.4g of glycine were dissolved in 1000ml of distilled water 0.1% SDS (W/V) was also added.

Preparation of gels: The rectangular glass plates (20 x 15 x 0.4 cm) of the gel electrophoresis were fixed to a stand. The resolving gel mixture was poured to a height of 12cm and over layered with a few

drops of distilled water. After polymerization (20 to 30 min), distilled water was removed completely and stacking gel mixture was poured to a height of 3cm. immediately a comb plate with 8 teeth was inserted. The assembly was left undisturbed for polymerization. After 30 min the comb was removed and assembly was attached to an electrophretic chamber.

The upper and lower reservoirs of the electrophoretic chamber were each filled with 500ml of reservoir buffer and care was taken to avoid air bubbles. Samples were loaded carefully on to the wells using lambda pipette fitted with disposable tips. The apparatus was connected to the power pack and a constant current of 60 v was supplied. When the samples entered the resolving gel, the current supply was increased to 120 v. After 12 hr, electrophoresis was stopped. The gel was separated by prying apart the glass plates gently.

Fixation, staining and destaining

Proteins in the gel were fixed and stained in 0.2% Coomassive brilliant blue. The gels were later destained with 30% methanol, and 70% acetic acid, and finally photographed.

7.3. Results

The results presented indicate the protein complexes that are resident in the various tissues of the treated and untreated crabs. These protein complexes were identified using polyacrylamide gel electrophoresis (PAGE).

SDS – PAGE revealed three major factions in the abdominal muscle tissue, and chelate muscle tissue of the control and treated organisms. These fractions fell in to three distinct categories: (1) 70KD, (2) 66KD, and (3)38 KD. The protein profiles of both the

muscle tissues (Figs.39-44) did not reveal any apparent change in the control and treated organisms, whereas in hepatopancreas and gill tissues these fractions fell in to four distinct categories: (1)150 KD, (2) 90-80KD, (3)55.2KD to 48 KD, and (4) 16.5 KD . A significant finding in these tissues was the disappearance of 150KD molecular weight protein fraction in the hepatopancreas and gill on the 1st 7th and 15th day of exposure period (Figs. 45-50). The fractions 2&3 with molecular weight ranging from 92-80KD appeared as faint bands in hepatopancreas and gill tissues in metal treated organisms. Fraction number four, 55.2KD to 48KD had an electrophoretic profile very different in the control and metal treated animals and its appearance, disappearance and reappearance did not follow any specific pattern, and may be related to the general metabolism of the proteins in the animals.

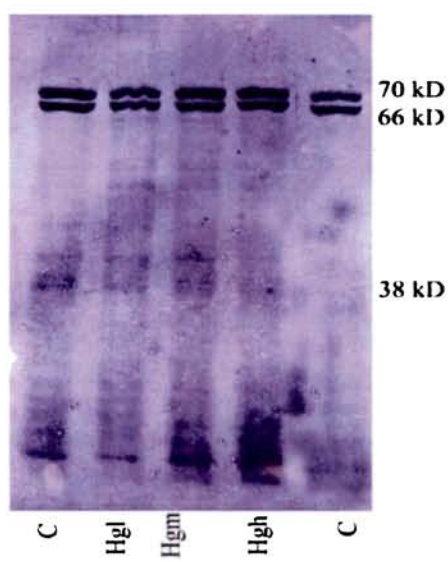


Figure 39. SDS – PAGE of abdominal muscle tissue on the 1st day of the crab *Scylla serrata* exposed to low, medium and high sub lethal concentrations of mercury.

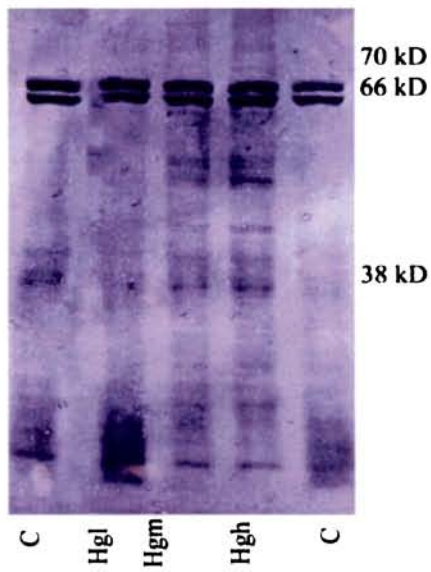


Figure 40. SDS – PAGE of abdominal muscle tissue on the 7th day of the crab *Scylla serrata* exposed to low, medium and high sub lethal concentrations of mercury.

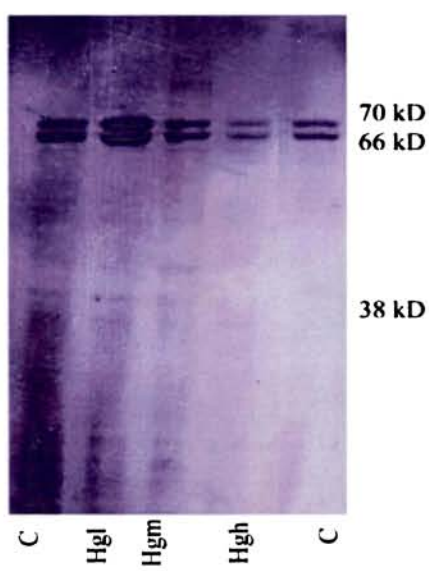


Figure 41. SDS – PAGE of abdominal muscle tissue on the 15th day of the crab *Scylla serrata* exposed to low, medium and high sub lethal concentrations of mercury.

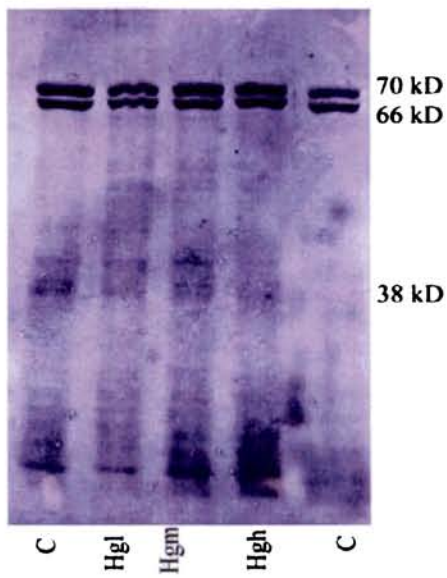


Figure 42. SDS – PAGE of chelate muscle tissue on the 1st day of the crab *Scylla serrata* exposed to low, medium and high sub lethal concentrations of mercury.

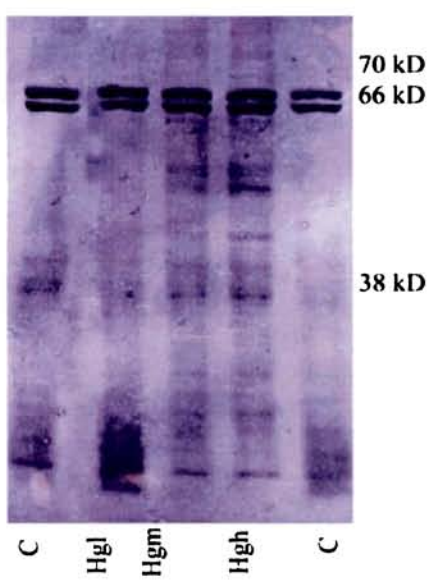


Figure 43. SDS – PAGE of chelate muscle tissue on the 7th day of the crab *Scylla serrata* exposed to low, medium and high sub lethal concentrations of mercury.

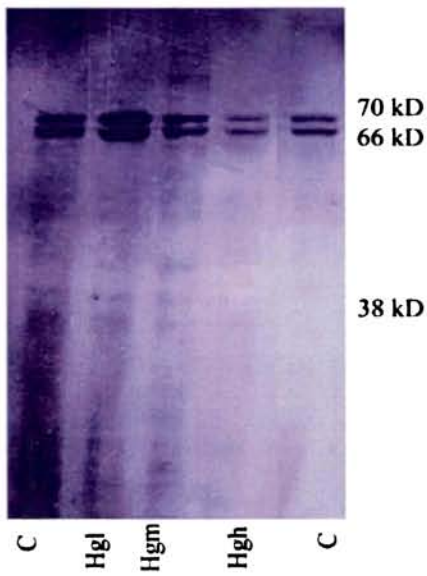


Figure 44. SDS – PAGE of chelate muscle tissue on the 15th day of the crab *Scylla serrata* exposed to low, medium and high sub lethal concentrations of mercury.

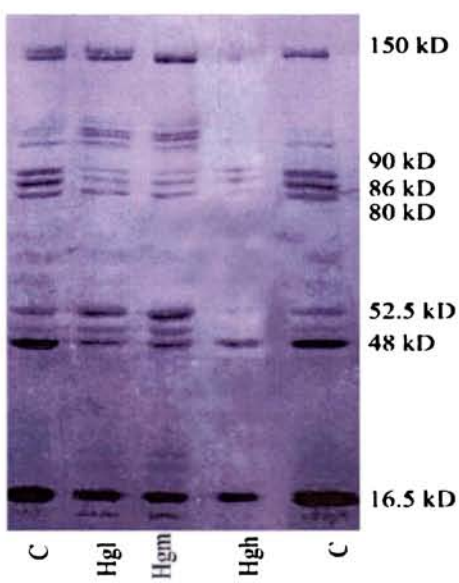


Figure 45.SDS-PAGE of hepatopancreas on the 1st day of the crab *Scylla serrata* exposed to low,medium,high sub lethal concentrations of mercury.

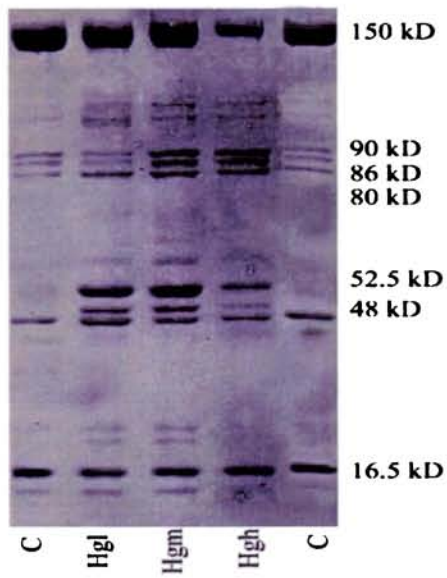


Figure 46. SDS-PAGE of hepatopancreas on the 7th day of the crab *Scylla serrata* exposed to low,medium and high sub lethal concentrations of mercury.

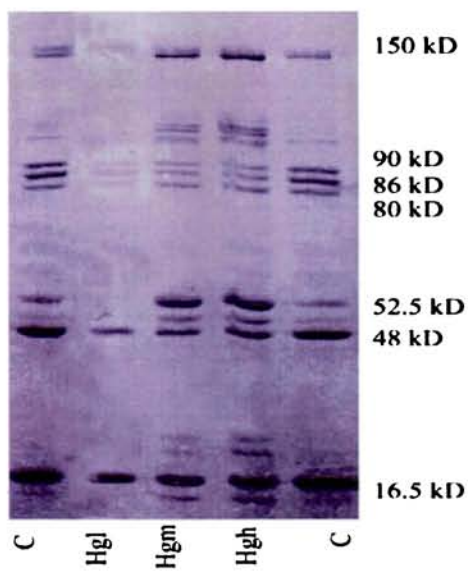


Figure 47.SDS-PAGE of hepatopancreas on the 15th day of the crab *Scylla serrata* exposed to low,medium and high sub lethal concentrations of mercury.

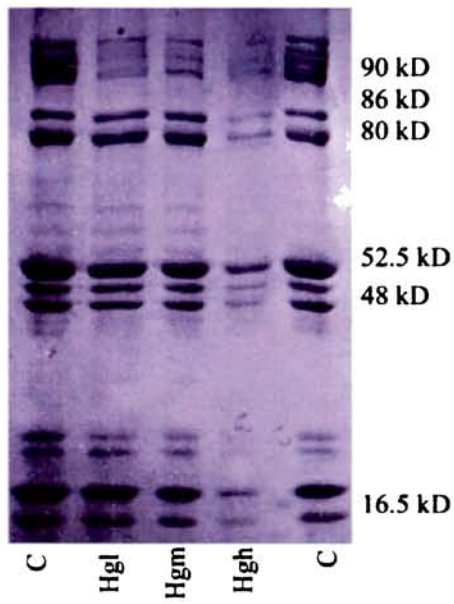


Figure 48.SDS-PAGE of gill tissue on the 1st day of the crab *Scylla serrata* exposed to low,medium and high sub lethal concentrations of mercury.

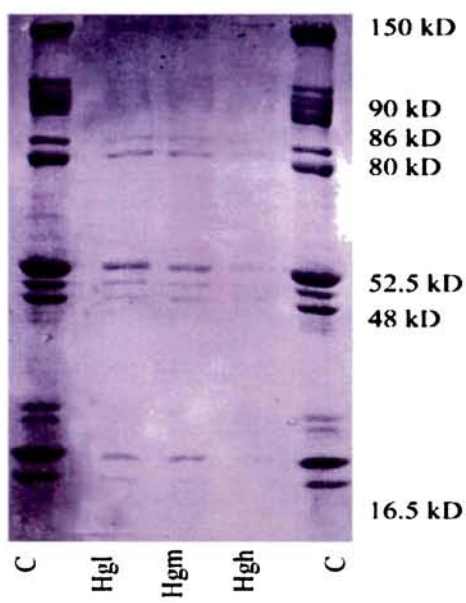


Figure 49.SDS –PAGE of gill tissue on the 7th day of the crab *Scylla serrata* exposed to low,medium and high sub lethal concentrations of mercury.

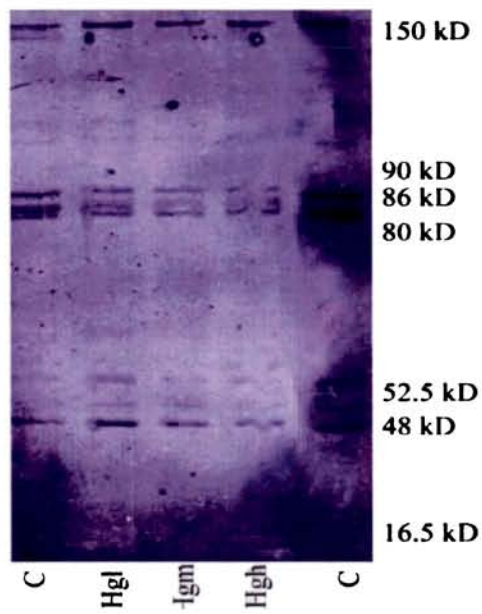


Figure 50.SDS-PAGE of gill tissue on the 15th day of the crab *Scylla serrata* exposed to low,medium and high sub lethal concentrations of mercury.

7.4. Discussion

In the present investigation the protein profiles of abdominal muscle tissue, chelate muscle tissue, hepatopancreas and gill tissues of the crabs exposed to mercury obtained in SDS – PAGE did not give any indication of serious disturbances. Nevertheless, a high molecular weight -150KD- protein did not appear on the profiles of hepatopancreas and gill tissues on the 7th and 15th day of post- exposure. The fractions in the molecular weight of 90KD and 80KD of hepatopancreas and gill tissue appeared faint with less staining intensity, suggesting a possible decrease in their protein synthesis. Similar observations were also reported by Roesijadi and Drum (1982) in the gill tissue of the mussel *Mytilus edulis*. Loganathan(1995) also reported similar observations in mercury and lead treated freshwater prawn *Macrobrachium malcolmsonii*. There are reports on mercury binding proteins in other invertebrates (Roesijadi and Drum, 1982). In the cockroach *Blatella*

germanica the association of soluble mercury with high molecular weight protein components has been reported (Bouqugneau et.al., 1985).

Ramani Bai (1986) and Rajathy (1991) observed a reduction in the number of proteins bands when specimens of *Scylla serrata* were exposed to sub lethal concentrations of copper and mercury. As reported in the present study, the variations in the electrophoretic pattern suggest a qualitative alteration of protein in mercury treated crabs. Similar types of alterations in the electrophoretic pattern of serum proteins due to toxicity were reported by Anee (1974), Ramalingam (1980), and Devaraj (1985). Ahmed et al. (1978) are of opinion that the decrement of protein is due to the increased proteolytic activity that occurs to meet the energy demands under toxic stress. They also suggested that depletion in amino acid pool brought about by enhanced amino transferase activity corroborates with the decreases in the number of protein bands. Holbrook (1980) has stated that the toxicants may directly cease protein synthesis. Any changes in the environment may also alter synthesis and utilization of protein in brain. Ramalingam (1980) reported a significant decrease in the tissue protein during an exposure period of 7 to 15 days. In addition there were also qualitative alteration the serum protein fraction of Tilapia *mossambica,* when subjected to sub lethal concentrations of mercury, DDT and malathion.

Chapter 8

SUMMARY AND CONCLUSION

Since time immemorial, the coastal zone as well as the estuarine zone, throughout the world has been the centre of vigorous human activities. However, during the past few decades the increasing population size has lead to the encroachment of coastal zone also, which has been undergoing increased urbanization and industrialization. Moreover, the coastal zone has become the most convenient 'dump yard' for all sorts of wastes. Industrialization leads to the discharge of treated and/or untreated effluents into the nearby water bodies leading to pollution. The effluents contain loads of heavy metals, pesticides, chemicals etc.

There have been numerous reports on heavy metal pollution and their effects on aquatic organisms. Of late, attention is drawn towards the health status of aquatic organisms, and there have also been increasing concern on the quality of meat of these organisms. Pollutants bring about reversible and irreversible changes in the physiology and biochemistry of organisms, and these changes are definitely affecting the quality of meat.

The Cochin backwaters is one of the most productive and biologically active backwater systems, and is the habitat of varieties of fishes, mollusks, and crustaceans, though this water body also receives tons of effluents from factories located on the banks of the river, Periyar.

Among crustaceans, the mud crabs also have biological and economical importance, as it is becoming the choicest seafood item. The importance of mud crabs as an export commodity has opened up great opportunity for crab farming in several countries, including India. The

growth of export obviously depends on the quality of the items. Since the water bodies where these crabs live are often polluted, it was thought worthwhile to ascertain if heavy metals are likely to cause alarming changes in the quality of the meat, which might even cause as a reason for rejection of the consignment.

With this back ground in mind, a set of experiments was designed to ascertain the effects of mercury at three sub lethal concentrations on the physiology and biochemistry of the estuarine crab, *Scylla serrata,* collected from crab farms in Vypin.

The approach to the present investigation was on the following lines:

i. To evaluate bioaccumulation , and rates of depuration in crabs exposed to three sub lethal concentrations of mercury , at two specific time periods,

ii. To characterize the biochemical changes taking place in selected tissues of the crab exposed to the three sub lethal concentrations of mercury, at specific time periods,

iii. To study the activity levels of acid and alkaline phosphatases in crabs, at three time periods, exposed to three sub lethal concentration of mercury,

iv. To evaluate histopathological changes in selected tissues caused by exposure to mercury at sub lethal levels,

v. To evaluate ionic regulation, and

vi. To evaluate qualitative changes in protein through electrophoresis

Acute toxicity

Acute toxicity tests of static renewal type were carried out. The LC_{50} value for mercury with 95% confidence limits and slope functions for 24, 48, 72 and 96hr were calculated by processing the data for probit analysis. The 96hr LC_{50} value for mercury was 0.09 mg/l. The 3 sub lethal concentrations of mercury chosen for the present investigation were 0.009mg/l, 0.02mg/l, and 0.04mg/l. Time- bound samplings were carried out on 1, 7, 15, and 30 day post- exposures of the crabs to the sub lethal concentrations of mercury for various experimental purposes. Hepatopancreas, chelate and abdominal muscles, and gill were the tissues selected for the present investigation.

Bioaccumulation:

Exposure of the crabs to mercury for a maximum of 30 days, showed bioaccumulation of mercury in the gill, hepatopancreas and muscles. The accumulation of mercury in the four tissues was found directly proportional to the concentration of mercury in the medium. The pattern of accumulation of the metal was in the following order.

Gill > hepatopancreas > muscle

Depuration:

The rate of depuration of mercury showed a linear relationship with the exposure concentration of the mercury. The order of elimination through tissues was similar to that observed for bioaccumulation, and the elimination rate of the metal was very low in the muscle, whereas it was much faster through the gill than hepatopancreas. The temporary adsorption of metal on the gill could be the probable reason for the higher elimination of the same through gill.

Gill > hepatopancreas > muscle

Biochemical composition:

The levels of protein, sugar and lipid were found to be affected in crabs exposed to mercury. The decrease was found to be a function of dose dependence. Hepatopancreas being a detoxifying centre accumulated more metal and this led to severe depletion of nutrients.

Enzyme assay:

Acid and alkaline phosphatase activities were studied in crabs exposed to the three sub lethal concentrations of mercury. Regarding the activity of ALP in hepatopancreas, at low, medium, and high concentrations of mercury, the activity decreased significantly at medium and high concentrations on the 7^{th} day, and at all concentrations on the 15th day. The ALP activity was significantly high in all concentrations on day 1 and in low concentration on the 7^{th} day.

In the case of gill tissue, ALP activity showed increase in low concentrations on the 1^{st}, 7th and 15^{th} days; increase on the 1^{st} day and 7^{th} day but decrease on the 15^{th} day at medium concentration; and slight increase on the 1^{st} day at high concentration but decrease on the 7^{th} and 15^{th} days

Hepatopancreas ACP activity was low in all the three treatments on the 1^{st} day; slight increase in low concentration, but decrease in medium and high concentrations on the 7^{th} day; and slight increase in low concentration, but decrease in medium and high concentrations on the 15^{th} day.

In the case of gill tissue, ACP activity was high in all the three treatments on the 1^{st} day; low at low and high concentrations but high at medium concentration on the 7^{th} day; and low at all concentrations on the 15t day.

Histopathological Studies:

Marked histopathological alterations were observed in gill, hepatopancreas and muscle tissues of the exposed animals. In the gill, in general, these changes included loss of gill structure and necrosis, thickening of gill lamella, bulb-like swelling at the extremities of gill lamellae, perceptible enlargement of intralamellar space, and hemocytic infiltration into gill lamellae. In the hepatopancreas, changes in the constituent cells were observed. The cells were disfigured, clumped and intracellular space reduced, general degeneration of tubular and intertubular tissues, extensive necrosis, and loss of structure of tubular vacuoles. In the abdominal muscles, necrosis was evident, and granular materials were seen in between the muscle fibres, fragmentation, atrophy, and wavy appearance of the muscle fibres were conspicuous. In chelate muscles also atrophy and necrosis were evident.

Ionic Regulation

Alterations in the ionic composition of tissues were added indications of the negative response of the organism to the xenobiotic. Exposures to mercury increased the concentration of sodium and magnesium in all the tissues; on the contrary the levels of potassium and calcium were reduced significantly.

Electrophoresis

Sodium dodecyl sulphate – polyacrylamide gel electrphoresis (SDS-PAGE) was carried out to study the protein profile of muscle tissue, hepatopancreas and gill tissue of the treated and control organisms. The pictures revealed that metal treated crabs exhibited a protein profile different from that of the controls . Intensity of protein fractions in the

mercury treated animals was markedly reduced as compared to the controls.

Concern over heavy metal pollution, especially of aquatic medium, has received greater attention as most of the heavy metals have been proved to be harmful to most organisms at some level of exposure. People and organizations concerned with environmental management and environmental quality have recognized, of late, the need for monitoring the physiological and biochemical effects on aquatic organism, especially of those consumed by humans, as a result of exposure of these organisms to heavy metal contamination in natural systems.

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