Haematological responses of green mussel *Perna viridis* (Linnaeus) to heavy metals copper and mercury

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Certificate

This is to certify that the thesis entitled "**Haematological responses of green mussel *Perna viridis* (Linnaeus) to heavy metals copper and mercury"** is an authentic record of the research work carried out by Ms. Bindya Bhargavan P.V under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Marine Biology of Cochin University of Science and Technology and no part of these has been presented for the award of any other degree, diploma or associate ship in any university.

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Supervising Guide

Declaration

I hereby declare that the thesis entitled "**Haematological responses of green mussel *Perna viridis* (Linnaeus) to heavy metals copper and mercury**" is a genuine record of research work done by me under the supervision of Prof.(Rtd) Dr. K.Y.Mohammed Salih, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in the thesis has not been presented for any other degree or diploma earlier.

Kochi-16
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Preface

Pollution of the ecosystem has always been taking place to a lesser or greater extent. Since the start of the Industrial Revolution, pollution of the ecosystem has obviously increased considerably, and despite the various measures adopted by almost all countries, in one form or the other, to reduce the load of pollutants or minimize it, significant success has always been eluding. Metal contamination of Aquatic ecosystems is a matter of serious concern as it is widespread and many metals are persistent and potentially deleterious to aquatic life.

Bioassay has been a very useful tool for understanding trace metal toxicity. It is simple but the most important reason for the growing application of bioassay test is that it offers rapid, sensitive, cost effective, reliable, predictable, and interpretive values on environmental contamination. Immunotoxicology is now a fertile domain to develop biomarkers as effective tools in biomonitoring studies.

Through this thesis entitled 'Haematological responses of green mussel *Perna viridis* (Linnaeus) to heavy metals copper and mercury 'an attempt is made by me to understand the haematology and hematological responses of this mussel on exposure to the two heavy metals of great concern, and to ascertain the usefulness of haematological parameters as reliable indicators of stress of a general nature, so that preventive measures can be taken before mass mortality takes place.

The thesis begins with an introduction, and the rest is organized into six chapters. Chapter 1 deals with the study of cellular defense mechanism, the 'blood cells'-or haemocytes, of *Perna viridis*. Chapter 2 is on the variations in different parameters of haemolymph organic constituents induced by copper and merury. Chapters 3 and 4 are on the effects of heavy metals on enzyme activity patterns. Chapter 5 examines the histological alterations that have taken place in the gills of metals exposed clams, and the 6^{th} and final chapter explains the bioaccumulation pattern of heavy metals in heamolymph, and other tissues.

This thesis provides base line data on the haematological manifestations of the green mussel *Perna viridis* on exposure to heavy metals, and convincing evidence on the use of bivalve defense mechanisms as immunomarkers in biomonitoring studies.

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Introduction

The aquatic environment is highly fragile, complex and diverse. It includes several distinct ecosystem types such as freshwater streams, lakes, ponds and rivers, estuaries, and marine coastal and deep ocean waters-that encompass many different biotic, and abiotic components of unique characteristics. Coastal seas are characterized by highly productive and economically valuable ecosystems as well as intense human activities capable of interfering with the system function and properties.

Aquatic toxicology has been defined as the study of the effects of chemicals and other toxic agents on aquatic organisms with special emphasis on adverse or harmful effects. A *toxicant* is an agent that can produce an adverse response (effect) in a biological system, seriously damaging its structure or function or resulting in death. A toxicant or foreign substance (i.e., xenobiotic) may be introduced deliberately or accidentally into the aquatic ecosystem, impairing the quality of the water and making it unfavorable for aquatic life. *Aquatic pollution* means the introduction by humans, directly or indirectly, of substances or energy into the aquatic environment resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to aquatic activities including fishing, impairment of quality for use of water, and reduction of amenities (modified from marine pollution definition by (GESAMP,1993).Toxicity is a relative property reflecting a chemicals potential to have a harmful effect on a living organism. Toxicity tests are, therefore, used to evaluate the adverse effects of a chemical on living

organisms under standardized, reproducible conditions that permit comparison with other chemicals or species tested, and comparison of similar data from different laboratories.

Orfila is credited with the modern toxicological study. Though toxicology has a very early history, years prior to and after the World War II saw investigations on the toxicity of various materials and metals to fish, standardizing techniques for acute toxicity testing and suggestions for appropriate test organisms. Since 1960s, biological and aquatic toxicological research on a wide range of pollution problems is being conducted to determine effects on biota and their habitats for each water body receiving wastes (Wells, 1989). The 1970s heralded a new period of awareness and concern about water pollution and saw acute "toxicity" being accepted as a valid parameter for governmental regulation and guidelines for water pollution control. The number, variety and complexity of aquatic bioassays steadily increased during the 1970s.In the 1980s many books were published and journal Aquatic Toxicology came into existence. The 1990's have seen continued growth and activity in the field and there have been significant advances on organismal responses (biomarkers).

For the past many years, biomarkers, defined as "biological responses to a chemical or chemicals that give a measure of exposure or toxic effect" (Peakall, 1992;Peakall and Walker 1994), have been developed to provide signals of detrimental impacts on the marine ecosystem. Biological tests and toxicological end point offer rapid, cost effective, reliable, and unequivocal, predictable and interpretive values. This is the simple but the most important reason for the growing application of biological tests for environmental protection.

Biomonitoring at the lower levels of biological organization allows an "early warning system" whereby stressors can be detected at an early stage, and dealt with before they exert their effects in higher levels of biological organization, *i.e*., at individual or community level. The appropriate use of biomarkers in sentinel organisms provides the first set of tools with which we can measure the actual effects of the chemicals on the biota in the field (Sevendsen, 2004). The class bivalvia is of interest in pollution studies, as it comprises sedentary filter feeding invertebrates, which are likely to accumulate pollutants from the environment. Bivalves have received extensive attention in the literature owing to their reported ability to reflect environmental levels of trace metal contaminants in marine and estuarine ecosystems. Bivalves exhibited several characteristics of an ideal indicator species (Darracott and Watling 1975; Philips, 1977a), including the following: ability to accumulate high concentration of contaminants without dying; a sedentary life history; high numerical abundance; sufficient life span to permit sampling of more than one year and throughout the monitoring period; large size so that ample tissue is available for analysis; and good adaptation to laboratory conditions. Since mussels are a group of major fouling organisms, they have been the subject of many toxicological investigations. The ability of mollusk to concentrate high amount of heavy metals without any apparent bad effects makes these animals very dangerous to their predators (Carpene, 1993).

Many chemicals released into the environment are able to generate toxicity in aquatic organisms. Heavy metals constitute one of the major contaminants, which regularly find their way into the aquatic ecosystem. Metals, being elements, cannot be broken down or destroyed by degradation, instead they accumulate within the environment in different forms. Heavy metals are a group of metallic elements with atomic weights

greater than 40, and are characterized by similar electronic distribution in their external shell. An element, which is required in amounts smaller than 0.01% of the mass of the organism, is called a trace metal. In aquatic systems, the heavy metals of greatest concern are copper, zinc, cadmium, mercury and lead. Many of them are essential for metabolism at lower concentration and are vital for enzymatic activity and as respiratory pigments in organisms. However, all essential trace metals become toxic when their concentration exceeds a threshold value. Heavy metals cause adverse biological effects, (Lawrence, 1981; Zelikoff et al., 1994). Common sublethal effects are behavioral (e.g., swimming, feeding, attraction-avoidance, and prey-predator interactions), physiological (e.g., growth, reproduction, and development), biochemical (e.g., blood enzyme and ion levels), and histological changes (Sheehan et al., 1984). Recent methods for toxicological assessment have implicated the immune system as a target of toxicant insult after exposure to some chemicals and drugs.

With increasing interest in the use of marine aquatic organisms as bioindicators for environmental monitoring (Sanders, 1993), cellular and functional parameters of the immune system can now be monitored in many sentinel species (Wong et al., 1992). Immunotoxicology is now a fertile domain to develop biomarkers as effective tools to detect probably less specific but very sensitive signals for deleterious effects of environmental contamination (Bernier et al., 1995; Zeeman, 1996; Burchiel et al., 1997).

Interest in bivalve “blood cells” has developed for a number of reasons:

a. There has been a recent escalation in research in comparative immunology, including many studies on invertebrates. Bivalves provide relatively simple experimental models, they may supply clues to the ancestry of the lymphoid system, and they may have

some novel defense reactions not yet discovered in more complex immune systems of vertebrates.

b. Many invertebrates, especially the molluscs and crustaceans, are now being extensively farmed to augment the food resources of man. Clearly, a better understanding of the host defense reactions of such species would help avoid and overcome disastrous outbreaks of disease which are likely to occur under the artificial and potentially stressful conditions of commercial culture.

c. Tests of effects of contaminants on simple systems are generally easier, quicker, more replicable, and less expensive. Open circulatory system of bivalves are simple and haemocytes are easily accessible in relatively large numbers in haemolymph fluid drawn from adductor muscle sinuses of bivalves and can be held in vitro for several hours to examine their live properties. Measurements can also be made on biochemical properties of the haemocytes and haemolymph (Chu, 1988). Extraction and analysis of haemolymph for biomonitoring can save these economically valuable species from being sacrificed.

d. The defense function involves transportation of pollutants, detoxification and its elimination, and also protecting the hosts from a variety of infections Invertebrate blood cells not only function in “immune” or host defense mechanisms but also perform functions such as storage, transport and/ or synthesize food, removal of waste products, and distribution of hormones at target sites, and are thus involved in many other vital life processes.

This along with a lack of knowledge on the specific role of invertebrate blood cells in these basic functions provides a major area of research on molluscan haemolymph utilizing modern biochemical and immunological techniques.

Internal defense mechanisms in bivalve molluscs: Haemocytes and Haemolymph

Bivalves possess an open circulatory system with haemolymph (blood) in the sinuses bathing tissues directly. The haemolymph contains cells called haemocytes (blood cells) that float in colorless plasma. Haemocytes are not confined to the haemolymph system but move freely out of the sinuses into surrounding connective tissue, the mantle cavity and gut lumen and hence these cells play important role in physiological processes such as gas exchange, osmoregulation, nutrient digestion and transport, excretion, tissue repair and internal defense. It serves as a fluid skeleton, giving rigidity to such organs as the labial palps, foot, and mantle edges. Haemocytes play a role in wound repair by migrating in great numbers to the damaged area, and plugging the wound while the epithelium regenerates. Haemolymph and haemocytes are responsible for the transport of contaminants from the organ of entry (gill, mantle) to tissues where detoxification or accumulation occur (Ruddel and Rains 1975; George et al., 1978). Because they exhibit transepithelial migration (diapedesis) haemocytes containing contaminants can move into the digestive tract or outward to the external surfaces of the oyster for elimination in the faeces or pseudofaeces. Detoxication and elimination have been attributed primarily to granular haemocytes, and the proportion of this cell type is reported to be fluctuating in polluted environments.

Haemocytes represent the most important internal defense mechanism in marine bivalves. Agranular haemocytes (hyalinocytes)

and granular haemocytes (granulocytes) are classically distinguished and considered by some authors as two distinct cell types. Haemocytes recognise foreign substances and eliminate them by phagocytosis. The process involves several stages: chemotaxis, recognition, attachment, internalization, followed by destruction of foreign particles through release of lysosomal enzymes or by highly reactive oxygen species (ROS).

Various pollutants are known to exert adverse effects on immunity, influencing their survival. Heavy metals, which enter the marine environment from a range of sources, disrupt the various defense parameters and functions such as phagocytosis, and destabilize lysosomal membranes, which play a central role in the degradation of phagocytosed material. Similar effects have been reported for other pollutants such as PAHs (Polycyclic aromatic hydrocarbon), pesticides, fungicides, PCBs (Polychlorinated biphenyls) and tributyl tin. Enhanced production of ROSs in response to accumulation of contaminants in haemocytes has also been reported (Winston et al., 1996). The consequences of this might include release of hydrolytic enzymes into the haemocyte cytoplasm, resulting in enzyme degradation and organelle damage. Heightened defenses, e.g., increase in the density, cell mortality, rate of locomotion, antioxidant enzyme activity and superoxide generation by haemocytes have been reported in animals living in polluted waters. All these cellular activities of haemocytes have the potential as biomarkers.

Understanding the relevance of biomarkers in the current state of affairs of pollution monitoring studies, and the potential of employing defense parameters as immunomarkers in biomonitoring studies, an attempt is made to understand the haematology and haemtological

responses of the bivalve *Perna viridis* (*P. viridis*) on exposure to two heavy metals, copper and mercury.

Perna viridis

The bivalve selected to carry out the experimental study was the Indian green mussel, *Perna viridis* (Linnaeus). This green mussel is essentially an intertidal organism occurring all along the east and west coasts of India. They inhabit primarily estuarine environments, and are often prone to coastal pollution. The mussel forms extensive beds on coastal rocks and other hard substrata beds often extending into estuaries and brackish water areas with an optimal salinity of about 27 to 33ppt, and temperature between 26 to 32°C. They occur from shallow intertidal zone to a depth of 15 m exhibiting wide tolerance for turbidity concentrations and pollution. Wide distribution, easy availability, and easiness to handle make these gregarious invertebrate an ideal candidate in scientific research, in manufacturing industries and in aquaculture activities.

Perna viridis (*P.viridis*) is a reliable and ideal candidate for aquaculture. In 2002 the annual production of mussel was 4,500 tons of which 500 tons come from culture (CMFRI, 2004). Mussel landing was 6909.4 tons in 2003-04 (CMFRI, 2005). The calorific value worked out for green mussel meat has been reported as 6.28 Cal/gm (dry weight) (Kuriakose and Appukuttan 1980). According to them Indian mussels contain 8-10% protein, 1-3% fat and 3-5% glycogen. In addition to this, calcium, phosphorous, manganese, iodine and iron are also present in considerable amount. Across India, mussel is largely a poor man's food. Earlier, Qasim et al. (1977) had stated that if any animal food is going to make substantial difference in the total food production of the world, it is

going to be mussels, particularly in tropical countries, where its production is cheap and their predictions have come true.

By virtue of their filter feeding habit, *P. viridis*, can accumulate high levels of heavy metals, pesticides and hydrocarbons from contaminated waters that can have a severe effect on their physiology and immune mechanism. They are sessile, euryhaline and have a simple life cycle. They are easy to be handled, transported and transplanted for experiments from one site to another. Mussels bioaccumulate almost any pollutant (heavy metals, radionuclides, petroleum hydrocarbons, pesticides etc.), and strains of various viral and bacterial diseases in their tissues, and are capable of responding quickly to pollutants by various behavioural, physiological, biochemical and immunological responses. Like other bivalves, the circulatory system of *P. viridis* is of open-type, which is continuously exposed to changes in environmental factors as well as pollutants. The cellular immune system comprises of haemocytes, which have a variety of functions with close interaction with the humoral factors. These responses not only give a picture of the health of the animal but also a general measure of coastal pollution. So, use of this green mussel can be regarded as a tool to identify 'hot spots' of pollution.

P.viridis has been used in toxicity studies as an environment bio-indicator. Most of the toxicity studies on *P.viridis* have focused on the process of bio-accumulation in this animal, and the organs that help in bio-accumulation of metals. A variety of bio-markers has been used in this mussel to monitor the level of environment pollution (Chan 1988; Krishnakumar et al. 1990; Nicholson 2001, 2003; Siu et al. 2004; Nicholson and Lam 2005; Wang et al., 2005). Previous studies had dealt with the effects of heavy metals, focussing particularly on specific

organs such as the mantle, gills, kidney, digestive gland, and gonad, while the haemolymph was seldom considered as an organ system. Except for a study made by Thiagarajan et al., (2006) there is a dearth of studies on the effects of heavy metal pollutants on the immune competence of this mussel from Indian waters.

Collection site

Specimens of *P. viridis* employed in the study, were collected from Narakkal region of Cochin backwaters. The Cochin estuary/Cochin backwaters, one of the largest tropical estuaries of India (area -256 km^2, 9° 40'-10° 10'N; 76°10' -76°30'E), is facing gross pollution problems following the release of untreated effluents from industries and domestic sectors(Balachandran et al., 2006).The major polluting industries in the region include a fertilizer plant, an oil refinery, rare earth processing plant, minerals and rutile plants, zinc smelter plant ,an insecticide factory, and an organic chemical plant (Balachandran, et al., 2006). Anthropogenic impacts have resulted in the accumulation of heavy metals in the estuarine sediments. Narakkal region is, however, located far from this industrial belt, and is considered as a pristine area of Cochin backwaters. Besides, Narakkal region is a natural bed of the study animal, *P.viridis*, facilitating easy access and abundant supply of species from the same site throughout the experimental phase without depleting the resource. Therefore, specimens of *P. viridis* were collected from Narakkal for the study purpose entirely. Stress factors chosen for the present study were two heavy metals copper and mercury, at sub-lethal levels. Copper is an essential element for bivalve development, and is present in many enzymes But at elevated concentrations it is toxic. Mercury is a trace metal normally presents at very low concentrations in marine environment of no biological importance and is harmful.

In the light of the significance of haemolymph in molluscan immunological studies, and the immense economic importance of *P. viridis*, the following objectives were chosen for the study:

- To understand the immune parameters of *P. viridis*, an important bivalve that has a wide distribution in Indian waters, and is extensively used in aquaculture activities. The data obtained through the present investigation can also be used for future immunotoxicological studies on this organism, and other molluscan species,
- To analyse variations, if any, in the immune mechanism as a result of exposure of the organism to copper and mercury toxicity,
- To ascertain the potential of bivalve defense components for use as possible "immuno markers" in ecotoxicological studies,
- To ascertain if the various assays employed to measure the immunity and antioxidant status can be considered as haemolymph biomarkers,
- To understand the alterations in the gills, which primarily and constantly come into contact with toxins, through histo-pathological studies , and
- To evaluate the possibility of bioaccumulation in the haemolymph in comparison to other tissues through bioaccumulation studies in the haemolyph, and selected tissues.

Chapter 1

Cellular defense mechanisms

A. Haemocytes and their viability

1.1 Introduction

In bivalve molluscs, structural and functional integrity of circulating haemocytes represent the main component of their immune system. Green mussels *P.viridis,* like any other molluscs, possess, an open circulatory system, which is continually exposed to the fluctuations of environmental factors and contaminants. Immunotoxic effects to chemical contaminants can be evaluated by monitoring cellular and functional parameters of the immune system of sentinel species (Wong et al., 1992). Blood cells or haemocytes as they are popularly called move throughout the haemolymph and tissues of bivalves and function in many aspects crucial to the survival of the organism. Physiological functions attributed to haemocytes include internal defense, osmoregulation, digestion of food, nutrient transport, wound and shell recovery, calcium transport, and protein regeneration Cheng, (1981), as well as respiratory burst, encapsulation and phagocytosis (Cheng, 1981; Lopez et al., 1997). Haemocytes also play an active role in heavy metal metabolism, i.e., in the actual uptake (Galtsoff, 1964), distribution to various tissues (Cunningham, 1979), intralysosomal storage (George et al., 1978) and its final elimination. Haemocytes phagocytose pathogens and foreign materials, protecting the animal from adverse environmental conditions. Exposure to environmental pollutants can lead to altered immune function increasing disease susceptibility and can potentially affect survival of the mollusc (Coles, et al., 1994, 1995). Heavy metals get accumulated in the granular haemocytes and overloading results in cell death

due to degradative enzymes and reactive oxygen species. Heavy metals are, thus, known immunotoxic substances and can disrupt several aspects of the immune system.

Given the lack of identification of molluscan haematopoetic tissues Cheng, (1983), the classification and characterization of the haemocytes is still unclear. No uniform nomenclature for different types of observed haemocytes exists. There have been a variety of interpretations as to how many types of haemocytes occur in bivalve molluscs. Further, there has been little agreement as to the designation of these cells. As a result of analyzing these criteria employed by many for distinguishing between the cell types, it becomes very clear that several authors have adopted different criteria for different reasons following different methodology. When the classification systems for bivalve haemocytes employed by several investigators are analyzed, it becomes clear that there are only two principal patterns, one school believing in having two types of cells, granulocytes and hyalinocytes, and the other believing in having three types, the granulocytes divided into two-finely granular haemocytes and coarsely granular haemocytes (Cheng, 1981). At present the haemocytes are classified into two major categories, granulocytes and hyalinocytes, (Cajaraville and Pal, 1995; Cajaraville et al., 1995). Variations in haemolymph cellular composition as well as in total and differential counts, along with significant cell mortality are among the first physiological disturbances described in bivalves exposed to environmental stressors (Anderson 1988; Fisher, 1988).

1.1.1 Review of literature

The field of immunotoxicology of bivalves has grown considerably during the past years due to its significance in fisheries, and aquaculture

sectors, and in ecotoxicological studies (Anderson, 1993). Functions of haemocytes in internal defense Stauber, 1950; Bayne,1983; Feng, 1988), their role in digestion of food Takatsuki, 1934; Cheng, 1977; Feng et al., 1977), and in shell deposition (Wagge, 1951; Watanabe, 1983) were already investigated. In molluscs, principally in bivalves, it has been established that the haemocyte numbers may be affected by environmental factors such as stress (Renwrantz ,1990), pathogens (Anderson et al., 1992;Oubella et al.,1993), and environmental contaminants and physical disturbance (McCormick-Ray, 1987; Renwrantz, 1990; Coles et al.,1994, Santarem et al., 1994; Pipe and Coles,1995; Carballal et al.,1998;Pipe et al.,1999; Fisher et al., 1989, 1996,1999, 2000; Chu 2000; Oliver et al.,2001; Fournier et al.,2002; Lacoste et al., 2002; Soudant et al.,2004). Density of haemocytes was found to be varying with different species, age, physical status and the methods used (Zhang et al., 2006). It has been established that bivalve haemocyte counts may be suppressed by a high dose of selected heavy metals (Fries and Tripp, 1980; Cheng and Sullivan, 1984; Cheng, 1988a, b; Coles et al., 1994, 95). THC was found to decrease following exposure of *Villorita cyprinoides* (*V.cyprinoides)* to very high levels of copper (Suresh and Mohandas, 1990). *In situ* exposures of *Mytilus galloprovincialis* (*M.galloprovincialis*) to chlorinated hydrocarbons and trace metals resulted in impairment in the total and differential haemocyte counts, phagocytosis and the generation and release of reactive oxygen intermediates (Pipe, 1992). Mercury caused significant haemocyte mortality in *Crassostrea gigas* (*C. gigas)* (Thunberg), after 24 h *in vitro* incubation (Gagnaire, 2004). Studies made by Sami et al., (1992) have shown a decrease in the number of haemocytes as a result of exposure to polycylic aromatic hydrocarbons. Mussels exposed to cadmium for 7 days

followed by 7 days exposure to *Vibrio tubiashi* had significantly higher number of circulating haemocytes (Pipe and Coles 1995). Oliver et al., (2001) showed that in oysters with high defense-related activities often had accompanying high tissue concentrations of one or more classes of xenobiotic chemicals. Mechanical disturbance such as shaking reduced the number of circulating haemocytes from $2.62x10^6$ cells/ml to $1.04x10^6$cells/ml (Lacoste et al., 2002).Alterations of total haemocyte counts, was one of the most reliable indicators observed in polluted sites (Auffret, 2006), and he suggested immunosuppressive conditions in contaminated mussels.

The internal defense system and blood cell types present in bivalves have already been reviewed (Cheng 1981; Bayne 1983; Fisher, 1986; Auffret 1988; Feng 1988; Renwrantz 1990). Attempts at developing a uniform classification of bivalve haemocytes have resulted in recognition of two groups of cells; those containing many granules (granular haemocytes or granulocytes), and those with few or no granules (agranular haemocytes or agranulocytes) (Cheng, 1981; Bache`re et al., 1995). From the several categories of haemocytes described by many, the majority of haemocytes belongs to granulocytes. Granulocytes have been reported from many bivalve families except scallops (Auffret, 1988; Henry et al., 1990; Mortensen & Glette, 1996). *Mytilus edulis* (*M.edulis*) and *Mytilus californianus* (*M.californianus*) were found to have eosinophilic granulocytes (Moore and Lowe, 1977, Bayne et al., 1979).Two type of granular haemocytes based on staining and enzyme characteristics, were found in *M.edulis* (Pipe, 1990a, b; Noel et al., 1994 and Friebel and Renwrantz, 1995). Staining of haemocytes showed three types of granulocyte, the basophil, the eosinophil and a mixed type of haemocyte in *M.galloprovincialis* (Carballal et al.,1997). Granulocytes

were identified in *Crassostrea virginica* (*C. virginica*) (Feng et al., 1971; Foley and Cheng, 1972) and in *Mercenaria mercenaria* (*M.mercenaria*) (Cheng and Foley, 1975; Moore and Eble, 1977). Two types of granular haemocytes were also reported from *Tridacna maxima* (*T.maxima*) Reade & Reade, (1976). Ultra structure of *Anadara ovalis* (*A.ovalis*) haemocytes showed a single type of granulocyte (Rodrick and Ulrich, 1984). Wootton et al., (2003) identified 3 type of haemocytes in *Scrobicularia plana* (*S.plana*) as eosinophilic granular haemocytes, basophilic haemocytes with eosinophilic granules, and basophilic agranular haemocytes. Suresh and Mohandas, (1990) found a single type of granulocytes in *Sunetta scripta* (*S.scripta*) and *V. cyprinoides*. Giant clams (*Tridacna crocea*) have eosinophilic phagocytic granulocytes, and non-phagocytic morula-like cells, with large lightly eosinophilic granules, that are discharged on aggregation (Nakayama *et al*., 1997).

Agranular haemocytes are a group characterised by a negative feature, the absence of, or presence of a few, cytoplasmic granules. It has been suggested that these agranulocytes are all hyalinocytes (Cheng, 1981), or developmental stages of hyalinocytes (Foley & Cheng, 1972). However, at the light and electron microscope levels, agranular haemocytes appear to be morphologically heterogeneous (Bachère et al., 1988).They were superficially divided into two groups, blast-like cells with a central ovoid or spherical nucleus surrounded by a rim of scant cytoplasm lacking organelles by (Bachère et al., 1988; Henry et al., 1990) or cells with a larger ovoid, reniform or irregular eccentric nucleus, with or without nucleoli, and copious cytoplasm often containing a variety of organelles. These uncertainties in identification (Feng et al., 1971), and the less frequent occurrence of agranular haemocytes in haemolymph (Renwrantz et al., 1979), have greatly constrained in understanding of

their functions. Agranular haemocytes may be non-phagocytic (Carballal *et al*., 1997; Nakayama *et al*., 1997) or less phagocytic than granulocytes (Renwrantz *et al*., 1979; Tripp, 1992; López *et al*., 1997), produce less superoxide anions than granulocytes (Anderson *et al*., 1992), and have superoxide dismutase associated with the plasma membrane (Pipe *et al*., 1993).

Composition and functions of haemocytes may vary greatly between individual bivalves (McCormick-Ray & Howard, 1991; Mourton *et al*., 1992; Mortensen & Glette, 1996), with collecting site, temperature, salinity changes and time of year (Fisher, *et al*., 1987, Fisher, 1988; Oliver & Fisher, 1995; Carballal *et al*., 1998). As granulocytes are easily recognizable and comprise the majority of haemocytes, in some bivalves they have been well studied (Feng *et al*., 1977; Moore & Eble, 1977; Renwrantz *et al*., 1979; Huffman & Tripp, 1982; Rasmussen *et al*., 1985; Suresh & Mohandas, 1990; Holden *et al*., 1994; Noël *et al*., 1994; Giamberini *et al*., 1996; Carballal *et al*., 1997*a*). The cytoplasmic granules contain acid hydrolases and may, therefore, be considered as a form of lysosome (Moore & Eble, 1977; Bayne et al., 1979; Moore & Gelder, 1985; Gelder & Moore, 1986; Pipe, 1990a; Cajaraville & Pal, 1995; Giamberini et al., 1996; Carballal et al., 1997c; López et al., 1997a). Granulocytes phagocytose bacteria (Cheng, 1975; Feng et al., 1977; Rodrick & Ulrich, 1984; Auffret, 1986; Tripp, 1992; Carballal et al., 1997b; López et al., 1997b; Lambert & Nicolas, 1998), digesting them in secondary lysosomes leaving digestive lamellae (Cheng, 1975; Mohandas, 1985; Auffret, 1986), and resulting in accumulation of cytoplasmic residual bodies and glycogen (Cheng, 1975; Rodrick & Ulrich, 1984; Mohandas, 1985; Carballal et al., 1997b) that may be expelled into the haemolymph (Cheng, 1975; Carballal et al., 1997b).

Granulocytes may be selectively induced by the presence of specific bacteria (Oubella et al., 1996; Paillard et al., 1996). They also phagocytose zymosan and heat - killed yeast (Moore & Eble, 1977; Bachère et al., 1991; Anderson et al., 1992; Tripp, 1992; Anderson, 1994; Cajaraville & Pal, 1995; López et al., 1997b; Lambert & Nicolas 1998), algae (Moore & Gelder, 1983, 1985; Gelder & Moore, 1986), cellular debris (Bubel et al., 1977; Hawkins & Howse, 1982), protozoan parasites (Ford et al., 1989; Mourton et al., 1992), latex beads (Cheng & Sullivan, 1984; Hinsch & Hunte, 1990; Tripp, 1992; Nakayama et al., 1997), erythrocytes (Renwrantz et al., 1979; Tripp, 1992; Russell-Pinto et al., 1994), and carbon particles (Reade & Reade, 1976).

Pipe (1992) has demonstrated that in situ exposures of *M.galloprovincialis* to chlorinated hydrocarbons and trace metals resulted in impairment in the DHC. A range of specific contaminants have been shown to induce alterations in the haemocytes profile of several bivalve species (Anderson, 1993). Studies on the effects of cadmium have demonstrated increased numbers of small cells within the hyalinocyte sub-population of *C.gigas*, following exposure to 0.3ppm cadmium ions with a concomitant decrease in large cells (Auffret and Oubella, 1994). Mussels subjected to cadmium or fluoranthene did not show any alteration in haemocyte profile, however, copper was found to elevate the percentage of basophilic haemocytes as compared with eosinophilic haemocytes (Coles et al., 1994; 1995). Exposure of *C.virginica* to cadmium resulted in increased number of cells with reduced granulocytes, although copper was found to induce a reduction in the percentage of the hyalinocytes (Cheng 1988a). Reports indicate that the relative proportion of both cell types may change with either reproduction or pollution induced stress (McCormick-Ray, 1987; Seiler and Morse,

1988; Cheng 1990; Sami et al., 1992). Hine, (1999) reviewed the morphology, tinctorial properties, ultrastructure and some functions of bivalve haemocytes in relation to simple division of these cells into granular, and agranular as suggested by Cheng (1981).

Thus, bivalve haemocytes have been implicated in diverse functions including internal defence, which encompasses the classical phenomena such as leucocytosis, phagocytosis, encapsulation, and nacrezation Cheng, (1981). Reviews on cell viability in molluscs exposed to contaminants are scarce. Trypan blue exclusion assay carried out (on exposure to 0.5, 1 and 5ppm of Hg^{2+}) resulted in increased cell death in *C.virginica* Cheng and Sullivan, 1984). Fournier et al., 2001 evaluated the effect of *in vivo* exposure to various concentrations of $HgCl_2$ or CH_3HgCl on the viability of *Mya arenaria* (*M.arenaria*) haemocytes and reported decreased viability of haemocytes. Cell viability on *in vitro* exposure of haemocytes to heavy metals Ag, Cd, Hg and Zn was measured by Sauve et al., (2002). Mercury caused a significant mortality of haemocytes after 24h of *in vitro* incubation (Gagnaire et al., 2004).

Despite the large diversity of bivalve molluscs, their immunology has been investigated only in a small number of species. The majority of available information has come from extensive studies on the marine mussels, *M.edulis*, *M .galloprovincialis*, and the American oyster, *C. virginica* (Pipe et al., 1997; Caraballal et al., 1997; Fisher et al., 1999; Hine, 1999;).

The main purpose of this study is to ascertain the alterations encountered in the important haemocytes parameters, such as Total haemocyte Count (THC), Differential Haemocyte Count (DHC), and cell viability in the green mussel, *P. viridis* under stress. The stress factors considered are the two heavy metals, copper and mercury.

1.1.2 Materials and methods

Mussel collection and their maintenance

Animal model adopted for the present study was the Indian green mussel *Perna viridis.*

Specimens of *P. viridis* were collected by detaching them from the rocky intertidal area of Narakkal region of Vembanad Lake. Mussels were quickly transported to laboratory in a plastic bucket. Mussels were moistened with seawater collected from the sampling site instead of submerging them into water to reduce their metabolic rate and reduce excretion of their waste products into water that can contaminate and cause stress to all mussels. Acclimation to laboratory conditions was performed in large FRP tanks that were well aerated, and filled with clean saline water of 30 ± 2 ppt (similar to that of its natural habitat), temperature- 28 ±1° C, and *pH*,- 8.2 ± 0.1. Animals were fed with the algal species *Isochrysis galbana* of density $2x10^6$ cells/ml.

After acclimation for a period of one week, healthy animals were chosen for dosing experiments and subsequent analysis of immune parameters. (Healthy mussels shut the valves rapidly and tightly when solid objects were introduced between their shell valves. They also bind strongly with byssal threads to external objects).

Exposure to Copper and Mercury

Acute toxicity was performed following the standard methodology of EPA/ROC (1998) to determine the lethal concentration (LC_{50}) and the sub- lethal level of copper and mercury.

All experiments were carried out in clean FRP tank of 10 -L capacity. Specimens of *P.viridis* used in the present study were 5±2 cm in

length (weight 25± 3g). Ten mussels each were exposed to 5-L of the required concentrations of copper and mercury. The test solution was prepared using the analytical grade $CuSO_4$ and $HgCl_2$ to get the stock solution. The required concentrations were made by diluting the stock solution. Water was renewed every 24 hours with least disturbance to the animals and they were fed for one hour daily prior to change of water .The LC50 values were calculated using Probit Analysis (Finney 1971). The three sub- lethal concentration selected for the metals were 6 µg/L, 12 µg/L and 30 µg/L of copper, and 10 µg/L, 20 µg/L and 50 µg/L of mercury.

Extraction of Haemolymph for analysis

Metal -exposed mussel were taken out and dried with a tissue paper. Very carefully shells were open apart to a width of 4mm, mantle water drained out, and blotted dry with tissue paper. A hypodermic needle of 23G was inserted into the posterior adductor muscle, and about 1.5ml of haemolymph withdrawn. The collected haemolymph sample was diluted with physiological saline in 1:1 proportion, and stored at 4°C until use. Physiological saline had a composition of 20mM Hepes buffer, 43mM NaCl, 53mM MgSO4, 10mM KCl, and 10mM $CaCl_2$ with a pH of 7.3. Haemolymph extraction and further analysis were carried out every 1^{st}, 7^{th} and 15^{th} day of heavy metal exposure. All the analysis were done using standard procedures.

Total haemocyte count (THC)

Total haemocyte counts (THC) were made using improved Neuber chamber. A 100µl sample of haemolymph was placed on a haemocytometer and haemocytes were counted out and expressed as cells x 10^6 cells/ml haemolymph.

Differential haemocyte count (DHC)

Differential haemocyte counts (DHC) were made by staining with Giemsa stain. A sample of 100µl of haemolymph was taken on a clean, grease- free glass slide and allowed to form a monolayer on the slide by incubating in a moist chamber for 45 minutes. It was fixed with 10% methanol for 15min, air dried, and stained in Giemsa for 20mts. The slides were then viewed under a research microscope (40X) to identify haemocytes.

Cell viability

Viable cell were determined using Trypan blue exclusion assay (Ford and Haskin, 1988). An equal ratio of haemolymph and trypan blue (0.4%) was mixed and left for incubation for about 1minute. Those cells, which appeared blue, are the dead ones while the live cells prevented dye intrusion into cell. A total of 100 cells was counted from each slide, and expressed as percentage of total viable cells.

Statistical analysis

Results are presented as means with standard errors. Data were subjected to two way analysis of variance (ANOVA) with a Tukey post hoc test. For all statistical tests, results were concluded significant with a probability (*P*) value of < 0.05.

1.1.3 Results

THC in unexposed, control mussels was found to be $1.85 \pm 0.031 \times 10^6$ cells / ml. In mussels treated with 6µg/L of copper THC showed a decrease at 24hr, then showed an elevation on 7th and 15th day of observation. Mussels that underwent 12µg/L copper exposure had a biphasic response in their THC, with 1st and 15th day having decreased

THC while 7th day having an elevated THC. At the highest sub- lethal concentration of copper used in this study, i.e., 30µg/L, THC had an increase on all days of exposure with a striking increase of 4.35 ± $0.35x10^6$ on day 7.

In mercury treated bivalves at the lowest sublethal concentration of 10µg/L, mussels had THC lower than the control animals on 1st, 7th and 15th days of observation. In 20µg/L and 50µg/L exposed specimens, the THC was initially increased but more decreased than that of the controls on 7th and 15th day (Table. 1.1 and Figure 1.1 & 1.2).

Giemsa staining for DHC revealed mainly two types of haemocytes. One type with granules in the cytoplasm is the granulocyte, and the other with no granules is the agranulocytes or hyalinocytes. Granulocytes were the most abundant population of haemocytes observed in *P.viridis*.

Hyalinocytes and granulocytes differed in their shape and size. Granulocytes were generally larger than hyalinocytes. Most of the hyalinocytes had a size ranging between 2-4 µm. There were occasionally larger ones too, which had a size of about 10.32 µm. The most abundant among hyalinocytes were the smallest cells having a size of 2µm. Hyalinocytes were concentric in shape. The hyalinocyte had a nucleus which occupied most of the space with a small rim of basophilic cytoplasm.

Granulocytes were of varying sizes, from 6-13 µm. As the size of the haemocyte increased, number of granules was found to be increasing with the largest cell possessing dense granules. Most of the granulocytes had granules which stained dark pink on Giemsa staining, and are therefore eosinophilic. Eosinophilic granular haemocytes dominated more

than the basophilic ones whose granules stained blue on Giemsa staining. Very rarely mixed granules were observed. Though the number of granules varied in granulocytes as few, medium and dense, all the three bearing cells are generally termed granulocytes in the present study. Granulocytes had extending filipodia.

Granulocytes were found to be the major population of haemocytes in *P.viridis*. It consisted of 90% of the total haemocyte counts. As a general observation, the number of granulocytes was found to decrease but small hyalinocyte number was found to increase with increasing exposure in copper and mercury exposed mussels (Table.1.4, Figure 1.3 &1.4 and Plate I).

Trypan blue exclusion assay is to determine the integrity of the plasma membrane and is an important indicator of cell viability. Unexposed control *P.viridis* showed 97% cell viability (almost 100%) but mussels exposed to copper showed a time and dose dependent response of cell mortality. Animals subjected to the highest sublethal concentration of 30µg/L, showed increased cell mortality on 7^{th} and 15^{th} day.

Mercury did not have a dose or time dependent cell mortality in mussel haemocytes. A time dependent decrease in cell viability was observed only in mussels exposed to the highest concentration of 50 µg/L of Hg. Mussels exposed to 10 µg/L and 20 µg/L mercury, had highest haemocytes mortality of 25% and 28%, respectively on 7^{th} day of observation. (Table.1.7, Figure 1.5 &1.6and Plate II).

Staistical analysis showed all variations were significant at $P < 0.05$ probability level (Table. 1.2, 1.3, 1.5, 1.6, 1.8 to 1.11).

1.1.4 Discussion

Changes in haemocyte counts are among the unspecific, but early indications of physiological alterations, which are classified as immunopathological parameters. In the present study copper and mercury were found to alter Total Haemocyte Count (THC) confirming disturbance to the animals. In higher concentrations of copper at 30 µg/L, and mercury at 20 and 50µg/L concentration, there was an increase in THC during the initial days but decreased on prolonged exposure. Elevation of total circulating blood cells appears to be a common response to environmental stressors. The observed increase in the circulating haemocytes indicates a chemical stress induced by the two heavy metals. In bivalves, not all haemocytes are found in the systemic circulation. Bivalves have an open circulatory system and cells may migrate from tissues to the circulatory system or vice versa (Auffret and Oubella, 1994;). This is confirmed through the present investigation, and the increase in THC might be due to migration of haemocytes from tissues to haemolymph as opined by Pipe et al.(1999).Circulating haemocyte density has been found to increase with exposure to copper (Pipe *et al*., 1999).

Elevated THC must be animals' strategy to detoxify the heavy metal stress. When mussels are suddenly exposed to copper and mercury, haemocytes might have moved into the circulation to accumulate and sequester metals. This can be cited as the acute response. Similarly, Auffret and Oubella (1997) report that increase in THC might be due to migration of haemocytes from interstitial tissues into circulation as a consequence of cell disturbance due to loss in adhesion properties. Elevated THC can also be due to haemopoiesis and proliferation of the cells as well (Pipe and Coles 1995). Auffret et al., (2004, 2006) reported

an increase in THC in mussels collected from polluted stations in his two year survey. Pipe et al., (1999)opined elevation of total circulating blood cells to be a common response to environmental stressors. Mussels exposed to heavy metals had elevated circulating haemocyte numbers (Coles et al., 1994, 1995; Pipe et al., 1999). *Mytilus edulis* (*M.edulis*) on exposure to fluoranthene (Coles et al., 1994a, 1994b), cadmium, and temperature stress (Renwrantz, 1990), resulted in increased total cell counts. Exposure of *C. virginica* to cadmium (Cheng, 1988a).

Mussels treated with the lowest concentration of copper and mercury at 6µg/L and 10 µg/L, respectively, had a decrease in THC by 24hour of exposure. But THC increased with days of exposure in 6 µg/L copper treated mussel, with total haemocyte counts more than the control values by 15^{th} day, but not in the case of mercury. Though there was an increase in THC on the first day for higher sublethal concentrations of 20 and 50µg/L mercury, it decreased with respect to controls on day 7 and 15, and for all days in 10 µg/L mercury. This shows a general immuno suppression in *Perna viridis* on mercury exposure. It is possible that heavy metals in higher concentrations might have caused decreased heart rate resulting in lower number of haemocytes in circulation, or due to cell death or withdrawal of haemocytes to reservoirs to protect them from direct exposure. Studies of Cheng, (1984) on a series of heavy metal toxicity on the American oyster *C. virginica* revealed Hg^{2+} as highly toxic for oyster haemocytes. This was attributed to cell death and/ or low inflow of haemocytes from other sites into the haemolymph. The other reason cited for reduced THC could be enhanced infiltration of haemocytes from circulation to tissues. Migration of cells involved in immune defense (haemocytes and brown cells) to connective tissues has been described in molluscs on exposure to pollutants (Seiler and Morse,

1988; Marigo´mez *et al.,* 1990; Zaroogian and Yevich, 1993).During stress immunoactive haemocytes are mobilized to certain tissues where injury or pathogen attacks are most probable. This has been described in several marine molluscs under stress condition (Seiler and Morse 1988; Cajaraville et al., 1990 a, b ; Marigomez et al., 1990).A very convincing evidence for this is given in another chapter of this thesis. Histology of gills confirms the infiltration of haemocytes into gill tissue as a result of copper and mercury stress. Another hypothesis as given by (Lacoste et al., 2002) is that since mollusc haemocytes are involved in nutrient transport these cells play a role in the redirection of bioenergetic resources and leave main haemolymph vessels to convoy nutrients to certain tissues involved in adaptation and survival. The reduction in haemocyte number as a result of exposure to organic pollutants was reported by Fisher et al., (1990); Pipe et al.,(1995) and Fournier et al., (2002), whereas copper was reported to be lethal to the haemocytes (Cheng 1998). A reduction in the haemocyte count can affect immunity adversely.

Time dependent decrease of haemocyte count, especially in mercury exposed *P. viridis*, may result in immune depression. A decrease in phagocytic granulocytes was observed in the current study along with decrease in cell viability, both pointed out as reason for a decrease in THC in metal exposed mussels. Decrease in THC can also be a result of reduced proliferation of haemocytes or movement of cells from circulation into damaged tissues (Pipe and Coles 1995). Generally in mollusc number of circulating haemocytes decreases with starvation and exposure to heavy metals are found to decrease the filtration efficiency leading to starvation which can also alter circulating haemocyte count.

A notable elevated THC of $4.35x10^6$ was obtained for copper exposed mussel at 30µg/L on day 7. Similar increase in THC was obtained by Pipe et al., (1999) when *M.edulis* was exposed to copper at 0.02ppm (20µg/L) for a period of 7days. For the lowest concentration of 6 µg/L copper, THC kept on increasing with time of exposure which reflects increasing toxicity to animals on prolonged copper exposure.

Several morphologically distinct categories of haemocytes have been described, but to date there is no universally accepted system of classification (Gosling, 2003). Attempts at developing a uniform classification of bivalve haemocyte have resulted in recognition of two groups of cells; those containing many granules, the granulocytes, and those with no granules, the hyalinocytes (Cheng 1981, 1984; Hinne, 1999). In accordance with these works for the present study haemocytes were recognized as two groups: granulocytes and agranulocytes.

As in many other observations, the majority of haemocytes in *P.virdis* was granulocytes which contributed 90% of total cell count. DHC in *M. edulis* revealed a dominance of granular haemocyte, varying between 70% and 93% in total, within which the eosinophilic and basophilic granular cells comprised similar proportions (Wootton et al., 2003). Hyalinocytes in *M. edulis* and *Ensis siliqua* (*E. siliqua*) and *Crassostrea edule* (*C.edule*) comprised between 6.13 and 15.75% (Wootton et al., 2003).Itoop et al., (2006) identified granulocytes, and agranulocytes in Indian edible oyster *Crassostrea madrasensis* (*C.madrasensis*). In the scallop *Chlamys ferreri*(*C. ferreri*), hyalinocytes of 5.93 µm in size comprised of about 34%, and granulocyte of 14.43µm in size constituted about 66% of cells in the haemolymph (Xing, et. al., 2002).

Granulocytes of *P.viridis* observed in the present study were larger in size with cell diameter varying from 6-13µm. Hyalinocytes were much smaller with their size ranging from 2-4 µm in diameter. This observation on size was common to many bivalve species, e.g., *M.marcenaria* (Foley and Cheng , 1974),*C.virginica* (Feng,1965), *C.edule* (Russel-Pinto et al.,1994), *M.edulis* (Rasmussen et al., 1985,Friebel et al.,1995), *M. lusoria* (Wen et al.,1994) and *C.madrasensis* (Itoop et al., 2006) . In *M.edulis* the eosinophils were the largest haemocyte with10-12µm diameter, and were characterized by large numbers of granules and a round nucleus with a low nucleus: cytoplasmic ratio (Wootton et al., 2003). In the clam *Tridacna crocea* (*T.crocea*) eosinophilic granules were 9.6 ± 0.1 µm in diameter, while agranular cells were 8.2 ± 0.2 µm in diameter (Nakayama et al., 1997).

Alternations in the relative proportion of blood cell types in bivalves have been observed previously in response to various stressors, including hydrocarbons and metals (Anderson, 1993). Copper exposure was found to reduce the percentage of hyalinocytes (Cheng, 1988a) and stimulate the percentage of granulocytes in oyster (Ruddell and Rains, 1975) and mussels (Pickwell and Steinert, 1984). It has been reported that the granulocyte/ hyalinocyte ratio increases on exposure to some metallic pollutants but decreases on exposure to others (Cheng, 1990). However, the present study has shown a drastic decrease of granulocytes and a predominant increase in small hyalinocyte of 2 µm in copper and mercury exposed mussels, by the end of the experiment.

The granular haemocytes of bivalves carry out functions such as phagocytosis, and are known to possess a number of lysosomal and antioxidant enzymes, as well as antimicrobial factors localized in the

cytoplasmic granules. (Anderson, 1993; Pipe , 1990; Pipe et al ., 1997; Cajaraville, 1995; Hubert 1996). The dominance of granular haemocytes in *P.viridis*, therefore, suggests that they are likely to be more capable of active defense reactions. Predominance of granulocytes may contribute greatly to the resilience of *P.viridis* in terms of its wide habitat range, close associations with diverse macro and microorganism, and tolerance of adverse conditions in intertidal zones or in polluted environments. In this study, granulocytes which are endowed with phagocytic activity were seen to decrease on exposure to copper and mercury. Such variations may be either due to differential blood cell mortality or migration of granulocytes into or out of tissues.

Trypan blue exclusion assay for *P.viridis* exposed to Cu and Hg revealed decreased cell viability. It has been reported that haemocyte viability *in vitro* is depressed in polluted or stressed bivalve molluscs (Cheng, 1990; Alvarez and Friedl, 1992). In the present study when mussels exposed to copper showed increased cell mortality (20%) as its chronic effect, 14% mortality of haemocytes were observed as its acute effect in mercury treated mussels. This discrepancy in behaviour of animals towards the two metals can be explained by the toxic mechanism of the metals. Hg^{2+} has a strong affinity for proteins bearing sulfhydryl groups, and its binding to the specific –SH site leads to denaturation of structural proteins leading to cell death. This is supported by Cheng and Sullivan (1984) for the decreased haemocyte viability in *C.virginica* on exposure to Hg^{2+}. Lack of mortality on the first day of copper exposure might be due to a lower affinity for such binding sites. Cell mortality observed in copper exposed *P.viridis* is brought about by its accumulation in the lysosome leading to cell death at higher concentration, and longer exposure times.

A dose dependent increase of mortality was observed when mussel *M.galloprovincialis* was exposed to copper (Olabarrieta et al., 2001). Similar pattern was observed in the present study with increased cell mortality on prolonged exposure to copper. The viability of haemocytes decreased in clams exposed to 10^{-6} M CH_3HgCl for 7 days, and $HgCl_2$ for 28 days (Fournier et al., 2001). Metal related cytotoxicity, expressed as decreased haemocytes viability, was noted for $HgCl_2$ at 10^{-3}M. Gagnaire et al., 2004, reported mercury to be an active pollutant that causes cell death and modulation of haemocyte activity, and also hypothesize that mercury has a direct action by inducing cell death. Mercury causes effects in a short period of time. Cheng, (1990) and Alvarez and Friedl, (1992) also reported haemocyte viability to be depressed in polluted or stressed bivalve mollusc.

1.1.5 Conclusion

The highly regulated nature of bivalve immune system renders it quite vulnerable to toxicants. Internal defense functions are critical to bivalves because suppression of their activities could lead to greater vulnerability to natural pathogens and environmental contaminants .Sub-lethal toxicity of copper and mercury showed, in general, a decrease in circulating haemocytes especially in mercury exposed mussels. This reduction in THC adversely affects the competitiveness of the affected species, which will affect their populations through higher rates of disease, parasitism and predation in the population. DHC revealed two types of haemocytes: granulocytes and agranulocytes. Granules present were either eosinophilic or basophilic or rarely of a mixed type. DHC showed a marked decrease in granulocytes and an increase of hyalinocyte in *P. viridis* exposed to copper and mercury, and therefore appears as useful indicator of heavy metal stress. Use of haemocytes and the

monitoring of haemocytes viability may, thus, represent an ideal biological endpoint to determine the level of toxicity linked to aquatic contaminants. Cell viability is an easy, cheap and reliable assay to understand cell mortality in *P.viridis*.

This study presents a baseline data for future biomonitoring studies employing *P.viridis*. The heavy metals, copper and mercury, interfering with the internal defense functions of haemocytes may have a profound effect on long-term survival of highly valued green mussel, the *P. viridis*. The affected species will no longer be able to fully enact its role in its ecological niche, further affecting the other inter-dependent species in the ecosystem, ultimately affecting the fragile balance of aquatic ecosystem.

Table.1.1 Total Haemocyte counts (THC) in Copper and Mercury exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	1.862± 0.042	1.215± 0.228	1.705± 0.266	2.600± 0.274
	7th day	1.877± 0.045	1.816± 0.176	2.485± 0.322	4.350± 0.345
	15th day	1.853± 0.061	3.372± 0.371	1.584± 0.192	2.473± 0.391
TOXICANT	**Exposed days**	**Control**	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	1.801± 0.046	1.542± 0.274	2.266± 0.479	2.455± 0.177
	7th day	1.883± 0.051	0.738± 0.068	1.717± 0.159	1.343± 0.100
	15th day	1.827± 0.046	1.135± 0.334	1.705± 0.112	1.535± 0.141

Table.1.2 ANOVA Table for THC in Copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	7.516	2	3.758	57.395	.000
Concentration	19.131	3	6.377	97.402	.000
Day * Concentration	23.412	6	3.902	59.599	.000
Error	3.928	60	.065		
Total	423.749	72			

Table.1. 3 ANOVA Table for THC in Mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	4.705	2	2.352	53.625	.000
Concentration	6.713	3	2.238	51.007	.000
Day * Concentration	2.723	6	.454	10.345	.000
Error	2.632	60	.044		
Total	215.711	72			

Table.1.4 Differential Haemocyte counts (DHC) in Copper and Mercury exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	**1th day**	90.193± 0.160	71.169± 3.633	67.111± 7.073	70.486± 7.338
	7th day	90.653± 0.592	42.155± 3.161	67.162± 1.558	41.493± 0.917
	15th day	90.603± 0.938	41.493± 0.917	41.845± 1.192	60.553± 2.403
TOXICANT	**Exposed days**	**Control**	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	**1st day**	90.945± 1.094	84.900± 2.020	75.198± 10.057	73.423± 5.577
	7th day	90.565± 1.145	42.948± 2.116	63.483± 2.473	53.508± 1.891
	15th day	90.238± 0.437	53.623± 1.683	56.052± 2.361	51.933± 1.829

Table.1.5 ANOVA Table for DHC in Copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	3755.080	2	1877.540	161.350	.000
Concentration	16628.231	3	5542.744	476.326	.000
Day * Concentration	4855.135	6	809.189	69.539	.000
Error	698.186	60	11.636		
Total	326185.844	72			

Table.1.6 ANOVA Table for DHC in Mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	5372.896	2	2686.448	195.442	.000
Concentration	11571.485	3	3857.162	280.613	.000
Day * Concentration	3172.808	6	528.801	38.471	.000
Error	824.729	60	13.745		
Total	362756.196	72			

Table.1.7 Cell Viability in Copper and Mercury exposed *P.viridis*

TOXICANT	Exposed days	Control	6µg/L	12µg/L	30µg/L
COPPER	**1day**	97.40± 1.3842	95.80± 3.7459	98.00± 1.6371	96.50± 2.0030
	7day	97.50± 1.2900	97.00± 2.8355	84.40± 5.2437	78.00± 4.2849
	15Day	97.50± 1.1815	84.35± 6.2737	83.68± 4.4229	79.77± 5.8205
TOXICANT		**Control**	**10µg/L**	**20µg/L**	**50µg/L**
MERCURY	**1day**	97.82± 1.0167	86.25± 6.4476	91.83± 4.8558	83.30± 4.6721
	7day	97.50± 1.3668	15.85± 6.0787	72.35± 5.4376	83.38± 5.9570
	15Day	97.50± 1.2506	81.67± 7.6259	85.80± 8.3523	76.20± 5.5982

Table.1.8 ANOVA Table for Cell Viability in Copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	1440.480	2	720.240	50.134	.000
Concentration	1582.516	3	527.505	36.718	.000
Day * Concentration	1176.322	6	196.054	13.647	.000
Error	861.977	60	14.366		
Total	599002.300	72			

Table.1.9 ANOVA Table for Cell Viability in Mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	6821.111	2	3410.555	116.038	.000
Concentration	12074.886	3	4024.962	136.942	.000
Day * Concentration	13195.134	6	2199.189	74.823	.000
Error	1763.508	60	29.392		
Total	503771.290	72			

Table. 1.10 Multiple Comparison Test for Copper

		THC	DHC	Cell Viability
Tukey	**Control Vs 6μg/L**	0.612	0.000	0.001
	Control Vs 12μg/L	0.515	0.000	0.000
	Control Vs 30 μg/L	0.000	0.000	0.000
	6 ppm Vs. 12 μg/L	0.054	0.000	0.025
	6 ppm Vs. 30 μg/L	0.000	0.000	0.000
	12 ppm Vs. 30 μg/L	0.000	0.720	0.014
		THC	**DHC**	**Cell Viability**
Tukey	**1 day Vs 7 day**	0.000	0.000	0.000
	1 day Vs 15 day	0.028	0.000	0.000
	7 day Vs 15 day	0.001	0.189	0.027

Table. 1.11 Multiple Comparison Test for Mercury

		THC	DHC	Cell Viability
Tukey	**Control Vs 10μg/L**	0.000	0.000	0.000
	Control Vs 20μg/L	0.000	0.023	0.000
	Control Vs 50 μg/L	0.000	0.000	0.000
	10 ppm Vs. 20 μg/L	0.004	0.000	0.000
	10 ppm Vs. 50 μg/L	0.895	0.000	0.000
	20 ppm Vs. 50 μg/L	0.000	0.441	0.561
		THC	**DHC**	**Cell Viability**
Tukey	**1 day Vs 7 day**	0.000	0.000	0.000
	1 day Vs 15 day	0.000	0.000	0.015
	7 day Vs 15 day	0.947	0.066	0.000

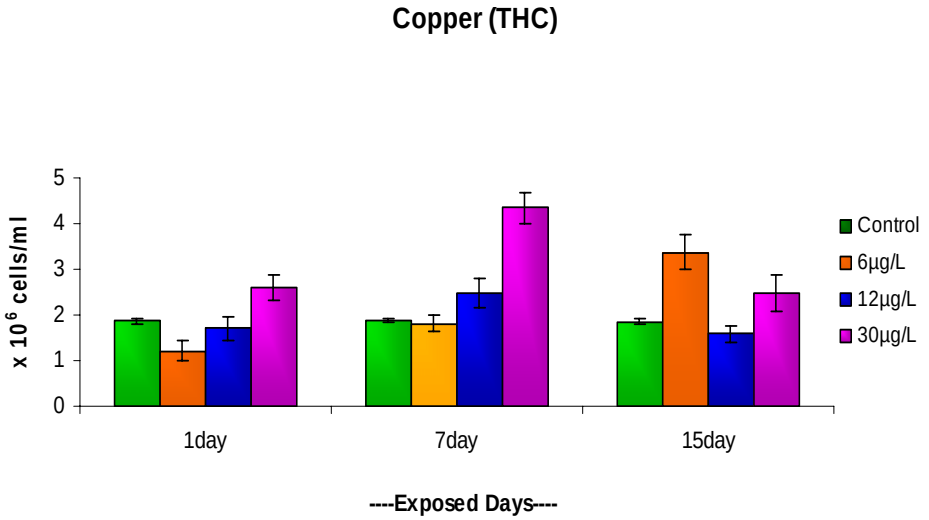


Fig. 1.1 THC in Copper exposed *P.viridis*

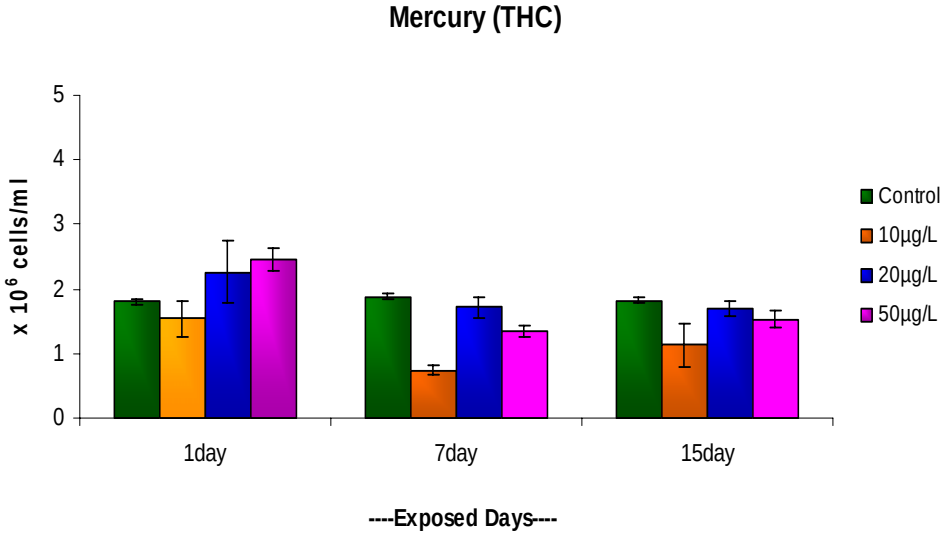


Fig. 1.2 THC in Mercury exposed *P.viridis*

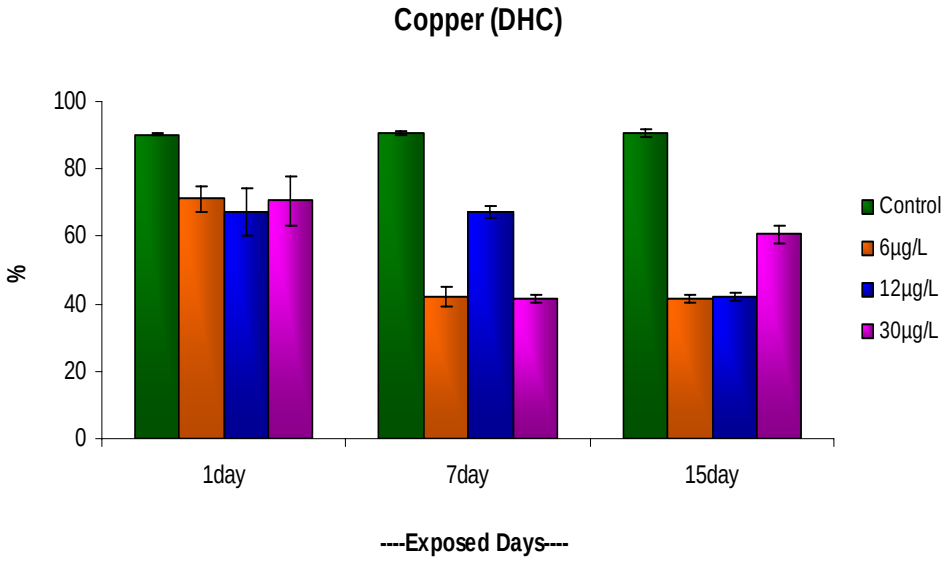


Fig. 1. 3 DHC in Copper exposed *P.viridis*

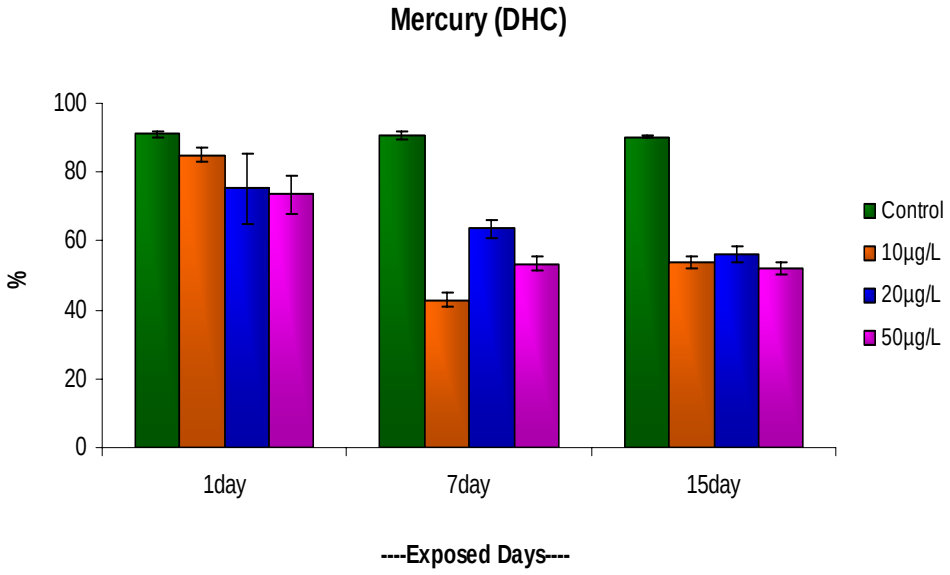


Fig. 1.4 DHC in Mercury exposed *P.viridis*

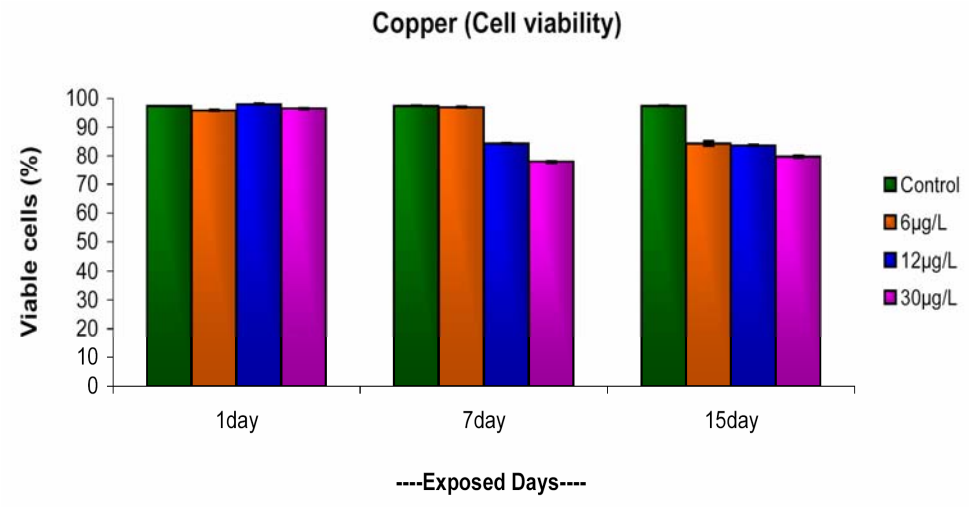


Fig. 1.5 Cell viability in copper exposed *P.viridis*

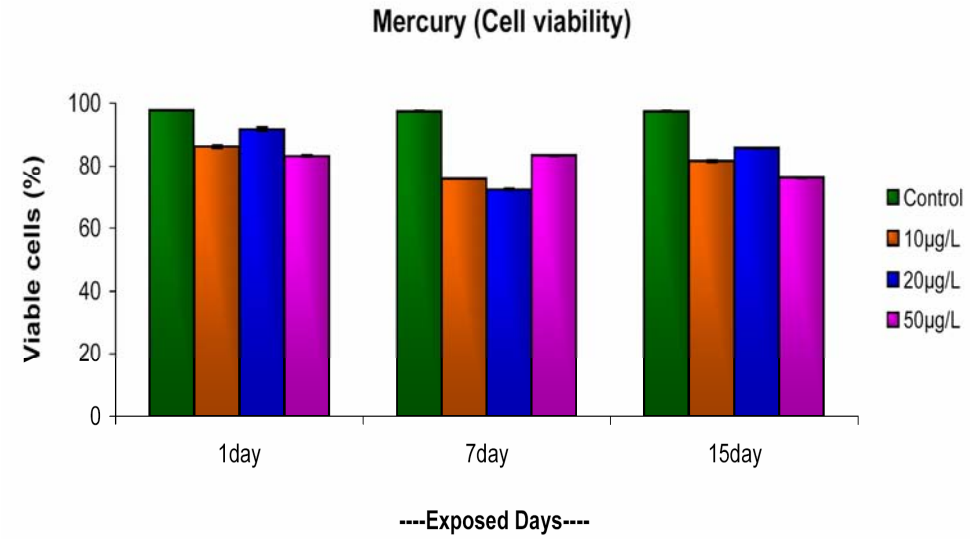


Fig. 1.6. Cell viability in Mercury exposed *P.viridis*

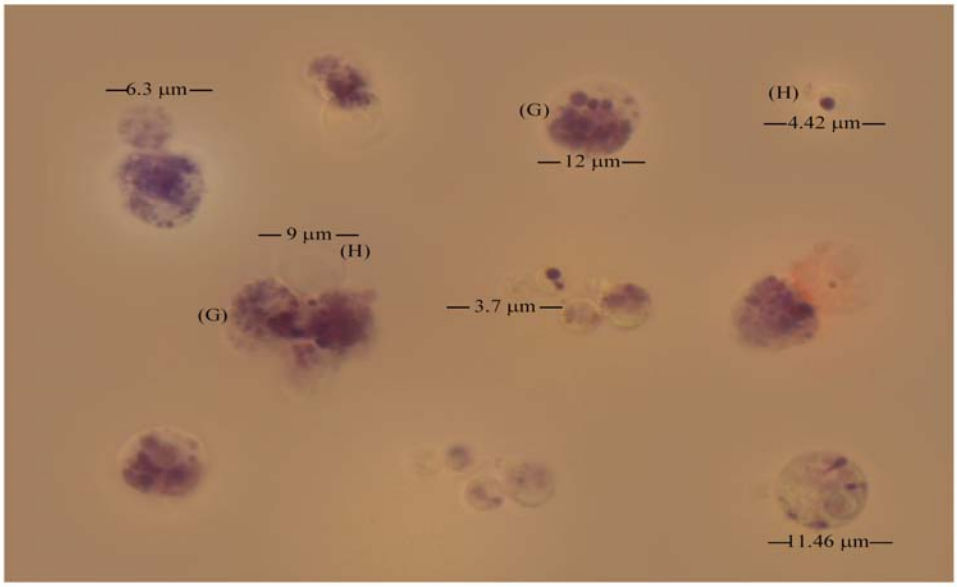


Plate I: Different types of Haemocytes in *P.viridis* as observed under light microscope (40x)
H: Hyalinocyte **G:** Granulocyte

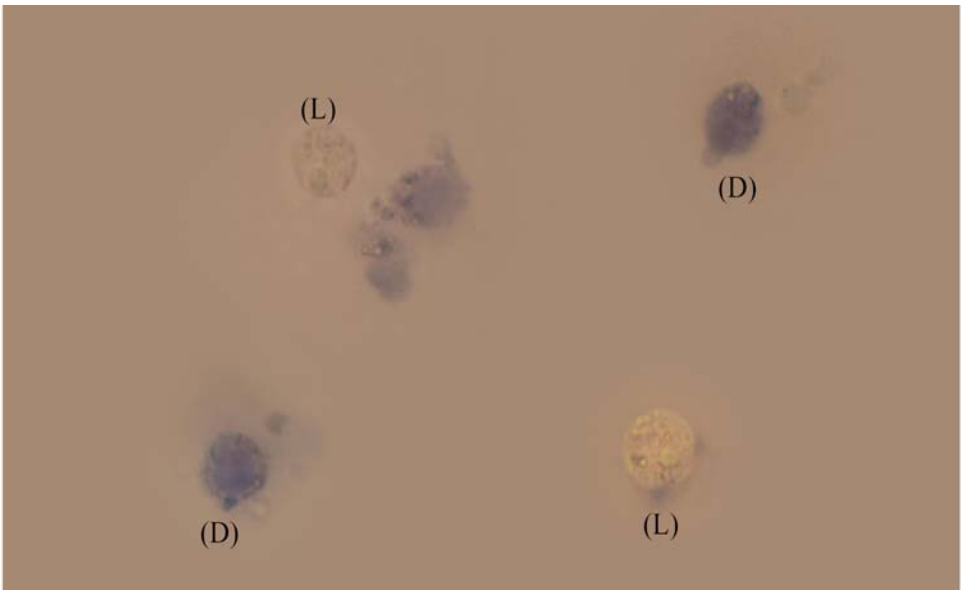


Plate II: Dead and live Haemocytes on trypan blue exclusion assasy (40x)
L: Live **D:** Dead

B. Neutral Red Retention Assay of Haemocytes

1.2 Introduction

Haemocytes, of bivalves play an active role in heavy metal metabolism, i.e., in the actual uptake of certain metals (Galtsoff, 1964), distribution to various tissues (Cunningham, 1979), and in the intra lysosomal storage of metals (George et al., 1978). Furthermore, phagocytic molluscan haemocytes are also known to participate in a number of important physiological functions, e.g., defense against invasion by pathogens, wound and shell repair, and nutrient transport (Cheng, 1981), and consequently toxic effects of heavy metals on these cells could potentially affect survival of the animal itself . Therefore, it appeared to be of interest to ascertain the effect of selected heavy metal ions on the normal function of these cells.

During the last decades, considerable research efforts have been made towards developing sensitive early warning biomarkers of pollutant exposure and effects.Cytological biomarkers allow the determination of pollutant impact on living organism in aquatic system and provide early warning signals of detrimental impacts on coastal water. Metals and other contaminants are accumulated and sequestered within the mussel lysosomes, and excessive accumulation alters the permeability of the membrane initiating increased autophagy and also the release of several hydrolytic enzymes. Therefore, the degree of membrane labilisation can be considered as proportional to the magnitude of stress.

Lysosomes are a heterogeneous group of cytoplasmic organelles, which contain a large number of hydrolytic enzymes and are enclosed by a unit membrane. Lysosomes are involved in a broad spectrum of physiological and pathological functions (Moore, 1982; Cheng 1983). In

molluscs lysosomes play a central role in innate defense (Cheng, 1981, Adema et al., 1991).Various important functions of lysosomes include cell detoxification (Owen, 1972), food degradation (Owen, 1972), protein and organelle turnover, (Owen, 1972), gamete resorption (Bayne et al., 1978), and shell formation. Lysosomes can release lysosomal enzymes and reactive oxygen species (ROS) (Cheng, 1983; Austin and Paynter, 1995), to destroy foreign organisms such as bacteria and viruses. Lysosomes have the remarkable ability to accumulate toxic metals, organic chemicals and hydrocarbons in them and act like a 'sink'.Excessive accumulation ultimately results in the impairment of the single membrane bound around them, increased permeability to substrates takes place (Moore, 1980) This response can be enhanced and lead to lysosomal membrane fusion resulting in the formation of larger lysosomes, and can cause deleterious cellular effects due to autophagy. (Lowe et al., 1981; Tremblay and Pellerin- Massicotte, 1997).

1.2.1 Review of literature

Elucidation of lysosomal membrane integrity utilizing neutral red assay (NRR-assay) has been applied successfully in pollution monitoring studies. Many molluscan cell types are very rich in lysosomes (Sumner, 1969; Moore, 1976).Excessive accumulation of many metals can induce alterations in the structure of lysosomes, which may result in the labilisation of the membranes (Moore and Stebbing, 1976). Determining lysosomal membrane responses using marine mussels has proven to be effective tools in the elucidation of cytotoxicity, (Lowe et al., 1995b; Cheung et al., 1998; Nicholson, 1999b), and is being applied increasingly as biomarkers in pollution monitoring. Published NRR assay data on oysters indicate that the stability of the lysosomal membrane is associated with stress factors the animals are experiencing, such as temperature and

salinity changes, spawning, bacterial inoculation, environmental contaminants, grading, starving etc. (Hauton et al., 1998, 2001; Butler and Roesijadi, 2001; Ringwood *et al*., 2002; Cho and Jeong, 2005; Zhang *et al*., 2006, Zhang and Li 2006). Many investigators have shown that lysosomal destabilization is intimately associated with pollutant exposure (Moore 1982;Regoli, et al., 1998;Ringwood et. al., 1998). Micromolar concentrations of Hg^{2+} and Cu^{2+} are able to rapidly destabilize lysosomal membranes of mussel haemolymph cells (Marchi et.al., 2004). Copper elicited significant destabilization of haemocyte lysosomal membrane (Nicholson 2003). Lowe and Pipe (1994) found probe retention time significantly reduced in lysosomes of cells isolated from mussels exposed to hydrocarbons. A significant decrease of lysosome stability was observed in spawned oyster (Cho, 2005). Bhargavan et al., (2006) found lysosomal membrane destabilisation in *V.cyprinoides* haemocytes, on exposure to mercury.

NRR assay is simpler, more convenient and more cost efficient, and has been widely applied to evaluate the effects of environmental and physiological changes and mechanical stresses on molluscan species (Lowe and Pipe, 1994; Lowe et al., 1995 b; Fernley et al., 2000; Grøsvik et al., 2000; Da Ros et al., 2002; Castro et al., 2004; Harding et al., 2004a, b; Zhang and Li, 2006). The neutral red retention (NRR) assay measures the speed of the efflux of lysosomal contents into the cytosol (Lowe et al., 1995).

The main objective of this study is to understand the response of haemocyte lysosomes on exposure to the heavy metals copper and mercury, using NRR time as the indicator of stress.

1.2.2 Materials and Method

Neutral Red Retention Assay

Lysosome membrane stability was measured using methods described previously by Lowe *et al*. (1995). Dissolving 20mg of dye in 1ml of Dimethy Sulfoxide (DMSO) a stock solution of Neutral Red was prepared. A working solution was then prepared by diluting 15µl of the stock solution with 5ml of physiological saline. It was filtered and stored in a clean cool place. A 50µl sample of haemocyte cell suspension was dispensed onto a microscopic slide and kept in a humid, dark incubation chamber. The cells were allowed to attach for 15minutes. The excess solution was then tipped off, and fresh 50µl of neutral red working solution was added to the attached cells, and incubated for another 15minutes. A cover slip was placed on the layer and viewed under a light microscope (x 400 magnification). It was transferred to the incubation chamber after observation and thereafter examined at every 30minutes interval. Lysosomes take up neutral red dye and turn red in colour, helping its easy identification. In unstressed cells, lysosomes will accumulate and retain dye for an extended period of time. Experiment was terminated by 180 minutes or when 50% cells lost membrane stability. Number of cells destabilized at the end was counted out from a total of 100 cells. Controls were run simultaneously.

Statistical analysis

All data are presented as means with standard errors. Data were subjected to two-way analysis of variance (ANOVA) with a Tukey post hoc test. For all statistical tests, results were considered significant with a probability (*P*) value of < 0.05.

1.2.3 Results

On the 1st and 15th day of exposure to sub-lethal concentrations of copper, the number of cells with destabilized lysosomal membrane increased with increasing copper concentration. Animals exposed to the lowest sub-lethal concentration of copper alone had a time dependent increase in cells with damaged lysosomal membrane. On day 7, mussels exposed to 12µg/L and 30 µg/L copper did not have much of their cell membranes damaged with respect to first day observation.

Mercury exposed animals showed a different pattern of NR retention when compared to copper exposed ones. On the first day, haemocytes with destabilized cells were more or less the same for all concentration tested. Mussels exposed to the lowest concentration had increased number of destabilized cells on day 7 and 15. At medium and highest sublethal concentrations of 20 µg/L and 50 µg/L of mercury, mussels had increased number of injured cells on 7th day when compared to day 1 and 15. (Table.1.12 Figure 1.7 and 1.8 & Plate-III)

Statistical analysis showed (Table 1.13-1.16) all variations were significant at probability level ($P < 0.05$).

1.2.4 Discussion

Neutral red is lipophilic and as such will freely permeate the cell membrane (Lowe et al., 1992). Within lysosomes, dyes become trapped by protonisation imparting lysosomes a red colour that can be visualized microscopically. Injury to their single membrane results in free passage of lysosomal contents (neutral red dye) into cytosol, which is the measure of stress. In unstressed cells lysosomes will accumulate and retain the neutral red dye for an extended period of time. As a weak cytotoxic compound neutral red is an additional stressor to the cells (Lowe et al., 1995a).

Lysosome prevents cytotoxicity through metal sequestration, and excess metal concentration makes lysosomal membranes destabilized. The pH of lysosomes is maintained by membranes Mg^{2+}ATPase dependent H^{+} ion pump (Peek and Gabbott, 1989). Any impairment in these pumps destabilizes the membrane and will result in a reduction of retention time. The present investigation shows lysosomal membrane stability is significantly affected on exposure to heavy metals copper and mercury. Generally, the reduction in NR uptake after *in vivo* exposure to pollutants might result from cell damage or death (Borenfreund and Babich, 1993). Reduced dye retention may have also occurred through copper mediated intra lysosmoal formation of highly reactive radicals, which induce membrane lipid peroxidation (Chavpil et al., 1976).

In this study though more than 50% of the metals-exposed animals could maintain Neutral red dye for more than 3hours, there was always a significant ($P< 0.05$) hike in the number of cells with their membrane damaged when compared to the controls.. The response, however, was not entirely dose and time dependent. A similar observation by Nicholson, (2001) on *P.viridis* exposed to copper tended to have lower lysosomal retention of neutral red, although responses were not always entirely exposure-concentration dependent. Copper induced haemocyte lysosomal membrane injury was evident using NR probe in *P.viridis*. Exposure to elevated copper concentrations may overload their sequestering capacity thereby rendering the membrane particularly susceptible to excess ambient concentrations (Viarengo et al., 1981). Lowe et al., (1995) examined contaminant impact on mussel, and the presence of organochlorines, cobalt and mercury appeared to be a major contributing factor to toxicity resulting in blood cell lysosomal pathology.

In mercury exposed mussels there was not always a direct relationship between metal concentration and NR retention as the lowest concentration of Hg showed greatest number of cells destabilized. It is probable that the lower mercury exposures impaired the lysosomal membrane at concentrations which preceded metallothionein induction thereby eliciting cytotoxicity (Ringwood *et al*., 1998).Verity and Brown (1968) demonstrated destabilization of lysosomal membranes in mice following mercury exposure. Working with the colonial hydroid *Campanularia flexuosa*, Moore and Stebbing (1976) demonstrated elevation of free lysosomal hydrolases following exposure to mercury, which they attributed to decreased membrane stability.

When comparing the effects on exposure to sublethal concentration of copper and mercury, haemocytes of mussels exposed to mercury had more cells with their lysosomal membrane destabilized by day 7, while in copper, more cells got destabilized on prolonged exposure. This reveals that mussels have a better copper detoxifying mechanism as it is an essential heavy metal, whereas mercury proves to be toxic. (Lowe et al., (1995) found mercury and cobalt to be toxic to lysosomal membrane. They reported a significant decrease in retention time by lysosomes of blood cells from the industrial and domestic waste prone sites (Lowe et al., 1995).

In the current study lysosomes could retain neutral red for more than 3hours when mussels were exposed to copper and mercury .This might be because of the low sublethal concentrations used for the study, which the mussels might have sequestered and detoxified before severe disruption to lysosomal membrane integrity occurred. In an earlier work by Bhargavan et al., (2006), in the black clam *V. cyprinoides* using 100 µg/L, 300µg/L and 600µg/L, found reduced retention time to 30 minute

for the highest concentration within a 96 hr exposure. This implies that lysosoml membranes can tolerate acute exposure to sublethal levels of copper and mercury at their sublethal concentrations, and severe disruption occurs at higher or at lethal concentrations. As stated by Nicholson, (2001), the present study also confirms haemocyte lysosomal membrane biomarkers in *P.viridis* to be robust to stressors within normal range. Hence, NRR assay can be considered as a reliable biomarker to assess the health of mussels.

1.2.5 Conclusion

Haemocytes represent the main component of the mussel immune system, and have highly developed lysosomal system. Furthermore, mussel haemocytes can be obtained without sacrificing the animal, thus affording the opportunity of conducting non-destructive monitoring. The present study confirms that elucidation of lysosomal membrane stability from living cells will afford a better indication of animal condition. In the present study more than 50% of the cells could retain neutral red dye for more than 180 min., which might be due to low sublethal concentration employed for the study. A singular use of membrane biomarkers in natural condition is not preferred and suites of biomarkers are therefore, preferable to assess sublethal toxicity. However, NRR assay proves to be robust for contaminants and environmental parameters within normal range, but deleterious at extreme concentrations. To conclude, *P.viridis* lysosomal membrane markers are nevertheless easily ascertained and may be useful as a rapid, inexpensive, screening tool prior to measuring higher order physiological dysfunction.

Table.1.12 NRR-Assay of metal - stressed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	3± 1.329	9± 1.033	11± 4.278	17± 4.834
	7th day	3± 1.722	12± 2.066	10± 0.516	9± 1.941
	15th day	5± 2.168	18± 3.189	20± 3.077	21± 2.066
TOXICANT	**Exposed days**	**Control**	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	3± 0.516	18± 1.265	16± 4.457	17± 5.785
	7th day	4± 1.414	18± 4.179	26± 4.690	29± 1.941
	15th day	5± 1.871	29± 2.098	15± 4.309	19± 2.757

Table. 1.13 ANOVA Table for NRR-Assay in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	679.361	2	339.681	48.315	.000
Concentration	1711.819	3	570.606	81.161	.000
Day * Concentration	444.306	6	74.051	10.533	.000
Error	421.833	60	7.031		
Total	12941.000	72			

Table. 1.14 ANOVA Table for NRR-Assay in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	370.083	2	185.042	16.509	.000
Concentration	3946.264	3	1315.421	117.361	.000
Day * Concentration	1008.028	6	168.005	14.989	.000
Error	672.500	60	11.208		
Total	25897.000	72			

Table. 1.15 Multiple Comparison Test for Copper

		NRR-Assay
Tukey	**Control Vs 6 μg/L**	0.000
	Control Vs 12 μg/L	0.000
	Control Vs 30 μg/L	0.000
	6 ppm Vs. 12 μg/L	0.994
	6 ppm Vs. 30 μg/L	0.001
	12 ppm Vs. 30 μg/L	0.003
		NRR-Assay
Tukey	**1 day Vs 7 day**	0.015
	1 day Vs 15 day	0.000
	7 day Vs 15 day	0.000

Table. 1.16 Multiple Comparison Test for Mercury

		NRR-Assay
Tukey	**Control Vs 10 μg/L**	0.000
	Control Vs 20 μg/L	0.000
	Control Vs 50 μg/L	0.000
	10 ppm Vs. 20 μg/L	0.081
	10 ppm Vs. 50 μg/L	1.000
	20 ppm Vs. 50 μg/L	0.090
		NRR-Assay
Tukey	**1 day Vs 7 day**	0.000
	1 day Vs 15 day	0.006
	7 day Vs 15 day	0.036

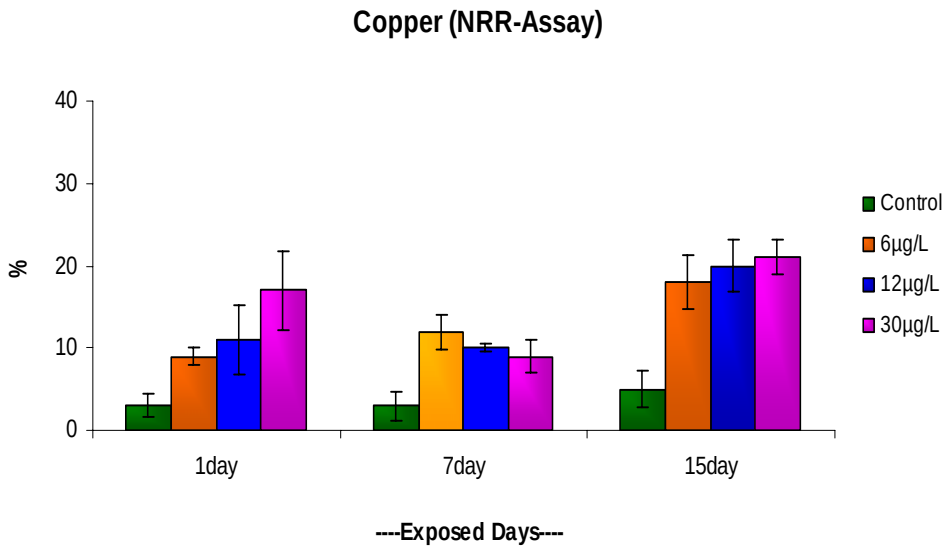


Fig. 1.7 NRR assay of copper exposed *P. viridis*

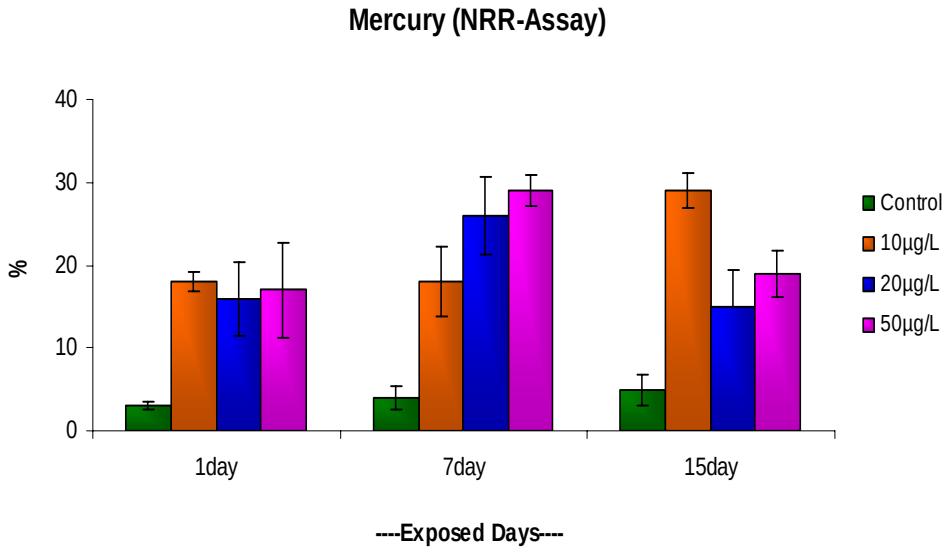


Fig. 1.8 NRR-assay of mercury exposed *P. viridis*

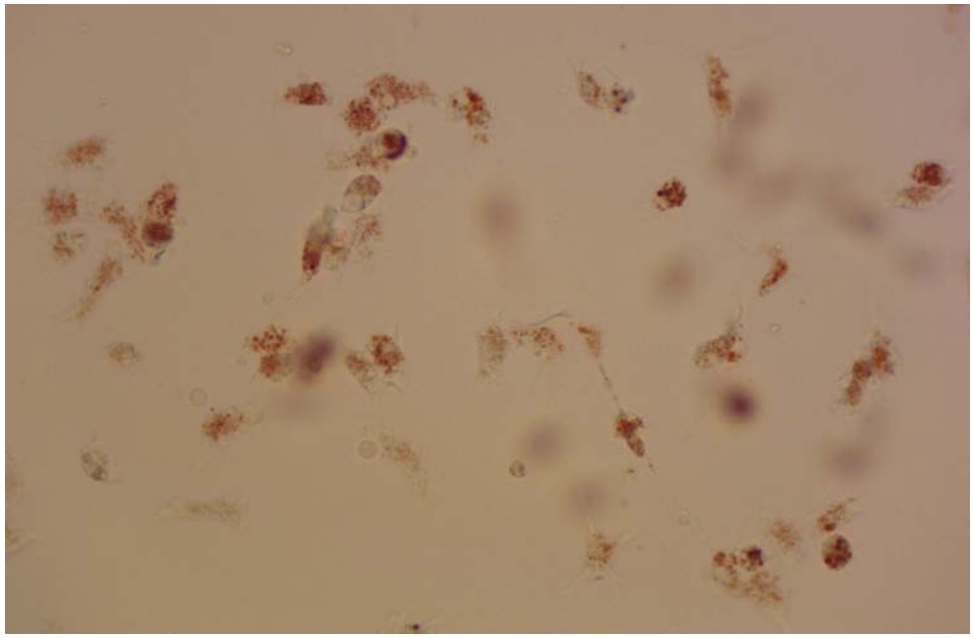
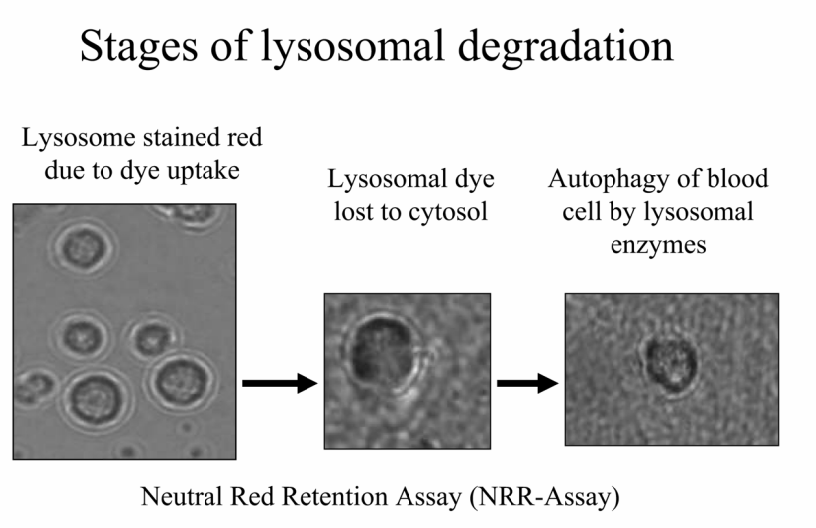


Plate III: Plate showing neutral red dye uptake by lysosomes of haemocytes



Chapter 2 Metals induced biochemical changes in the haemolymph organic constituents

2.1 Introduction

Exposure to heavy metals evokes several behavioral, physiological, and biochemical changes that appear to be closely related. To counteract any stress, energy reserves, which might otherwise be utilized for growth, and reproduction will have to be diverted towards enhanced synthesis of detoxifying ligands (metal binding proteins, granules), or expended in order to maintain an elevated efflux of metal. Consequently, various enzymes related to energy metabolism alter their activity pattern depending on the nature of stress. Excess energy is required to carry out defensive behavioral responses that help animal to adapt and survive. This confers some confidence in quantifying metabolic changes in the energy parameters, and related enzyme activities as integrated markers of healthy physiological status.

Mollusc use carbohydrates as the main source of energy for their metabolic processes. Carbohydrates are the central point in energy production because of its great mobility in the living systems, together with its capacity to get compartmentalized within the cells and tissues. The mobility is provided by glucose and compartmentalization by glycogen. In most marine bivalves, glycogen is the major carbohydrate storage reserve. Electron microscopy of *S.plana* (Wootton and Pipe, 2003), haemocytes described granular haemocytes with small granules characterized by large deposits of glycogen with an associated ring of mitochondria, termed as 'glycogen lake'. The presence of glycogen and

SER in the cells suggests a role for storage and maintenance of blood glucose levels. Proteins are the most abundant organic molecules of living system and form the basis of structure and function of life. Proteins have many different physiological functions in bivalves. They are associated with enzymes, transport, and regulation of metabolism, defense, structural elements, and storage, and hence represent an important biochemical constituent in mussel haemolymph.

Organisms, in response to carbohydrate depletion, use other substrates to obtain the energy needed for its maintenance through gluconeogenesis. Alanine and aspartate are precursor for gluconeogenesis, among amino acids, and the initial reaction is catalysed by alanine aminotransferase (ALT), and aspartate amino transferase (AST). Amino transferases, also called transaminases, constitute a group of enzymes that catalyzes the inter conversion of amino acids in α-ketoacids by transferring amino groups. ALT is also known as glutamic pyruvate transaminase (GPT), and AST as Glutamic oxaloacetate transaminase (GOT). ALT and AST which serve as a strategic link between carbohydrate and protein metabolism, play as an essential group of enzymes in the gluconeogenesis pathway. Beyond this, the aminotransferases are good indicators of tissue lesions. They are known to be altered during various physiological and pathological conditions making it a possible biomarker.

2.2 Review of Literature

In the case of bivalves the haemolymph carbohydrate content is very low (Winners et al., 1978). Bayne (1973) has reported low blood sugar levels in bivalves. The blood sugar levels of all classes of molluscs thus far investigated appear to be glucose (Goudsmit, 1972). Wootton and Pipe (2003), described granular haemocytes with small

granules in *S. plana* characterized by large deposit of glycogen. Toxicity of endosulfan impairs the metabolic function in *Macrobrachium malcolmsonii* (*M.malcolmsonii*) resulting in an increase in haemolymph proteins and total sugars (Bhavan and Geraldine, 1997). Reduction of nutritional reserves during starvation in either blood or tissues has been observed in several crustacean species, and has been used as an indicator of nutrient metabolism (Pascual, et al., 2006).Glycogen depletion and hyperglycemia have been observed in cadmium exposed fish and explained as an effect of cadmium on the hormonal regulation of the glucose level (Larsson and Haux, 1982; Sastry and Subhadra, 1982). Glycogen reserves were found to be reduced in the oyster, *C.virginica,* on exposure to PCB (Encomio and Chu, 2000). Investigations on haemolymph proteins were made by Bayne (1973a), Santarem et al. (1992), and Robledo et al. (1995), among others.ALT and AST are widespread in animal tissues and they are of considerable importance in metabolic economy. Transaminations have been shown to occur in bivalves and in gastropods (Read, 1962; Awapara and Campbell, 1964). Transaminations might be of particular importance under conditions that impose a heavy drain on the animal's store of metabolites. Hammen and Wilbur, (1959) have shown that in oyster they are involved in manufacture of shell material. They probably contribute to the supply of acid intermediates from which the molluscs synthesize their considerable stores of glycogen. All tissues of all molluscs assayed so far had ALT and AST activities Bishop, (1983). Aminotransferases activity has been shown by some authors in the haemolymph and tissues of molluscs infected with larval trematodes (Manohar et al., 1972; Christie and Michelson, 1975). Transaminases

are widely reported in gastropod tissues also (Sollock et al., 1979; Mohan and Babu , 1976). Effects of heavy metals on ALT and AST activities have been studied by Blasco and Puppo (1999). Aminotransferases activity in the haemolymph of snails was reported by Nabih et al. (1990), El-Emam and Ebeid (1989), and Pinheiro et al. (2001). Information on aspartate transaminase in flat oyster *Ostrea edulis* (*O.edulis*) was given by Culloty et al. (2002). Activity pattern of haemolymph transaminases in the fresh water gastropod *Pila virens* (*P.virens*) exposed to pesticide was made by (Reju et. al., 1993).

Though changes in activity pattern of glucose and other carbohydrates are known to occur depending upon the nature of environmental stress, only a few studies have been conducted on toxicant induced effects on glucose in invertebrates. Transaminases in molluscan tissues and haemolymph have been assessed by some workers but ALT and AST activity pattern in the haemolymph of the green mussel *P. viridis* on exposure to heavy metals is yet to be investigated.

Tissue glycogen serves as an immediate source of reserve energy via its component glucose, and can, therefore be used as biomarkers of environmental stress. Similarly, the enzymes involved in carbohydrate metabolism are potent biomarkers of pollution in invertebrates (Lagadic et.al., 1994).

Heavy metal mediated biochemical alterations in the haemolymph organic constituents, and aspartate and alanine amino transferases activity patterns in the haemolymph of the commercially important bivalve *P. viridis* on exposure to copper and mercury were investigated, and the results are presented in this section.

2.3 Materials and Methods

Maintenance of animals, methods of exposure to heavy metals, and haemolymph extraction procedure were the same as described in Chapter 1.

Glucose was estimated using Di-nitro salicylic acid test. A sample of approximately 0.1ml of haemolymph was added to 1ml of 5% TCA and centrifuged at 2500rpm for 5mts.To 0.2ml of supernatant 2ml of DNS reagent was added and kept for boiling for 10mts.It was then cooled to room temperature and absorbance was measured at 575nm using a HITACHI-U-2001- UV-Vis Spectrophotometer. A reagent blank and a standard of glucose solution were also maintained. Glucose content was expressed as mg/ml haemolymph.

Glycogen was estimated following the method described by Caroll *et al*. (1956).

A sample of approximately 0.1ml of haemolymph was added to 1ml of 5% TCA and centrifuged at 2500rpm for 5mts.To 0.2ml of supernatant; 1ml of absolute ethanol was added and left overnight in cold. It was then centrifuged for about 15mts at 3000 rpm. Precipitate obtained was dissolved in 1ml of dissolved water. A reagent blank (1 ml of water) and a standard (1ml of glucose solution containing 30mg of glucose) were also prepared. Two ml of anthrone reagent were added to all samples and kept for boiling for 10mts, and then cooled at room temperature in dark. Optical density was read at 620nm in a HITACHI-U-2001- UV-Vis Spectrophotometer. Glycogen content was expressed as mg/ml haemolymph.

Estimation of Protein

Proteins in the haemolymph were estimated following the method of Lowry et al. (1951).

Haemolymph was de- proteinised in equal volume of 5% TCA. Contents were allowed to stand for 30 minutes at room temperature for precipitating proteins, and were centrifuged at 3000rpm for 10 mts. The precipitate was dissolved in 1ml of 0.1 N NaOH. Four ml of alkaline copper reagent were then added and shaken to mix well. After 10 mts, 0.5 ml of Folin–Ciocalteau reagent added and the tubes were kept undisturbed for 30 mts. A reagent blank devoid of sample was maintained along with the experimental tubes. The absorbance was measured at 500nm in a HITACHI-U-2001- UV-Vis Spectrophotometer. Bovine Serum Albumin was used as the standard. Protein was expressed as mg/ml of haemolymph.

Estimation of Aspartate amino transferase (AST)

Haemolymph ALT and AST activities were carried out following the by the method described by Mohun and Cook (1957)

To estimate haemolymph AST activity, a 0.2 ml sample of haemolymph was added to a test tube containing 1ml of phosphate buffer-substrate containing 0.1Mphosphate, 0.1M L-aspartate, and 2mM 2-oxoglutarate at pH 7.4. It was mixed well and incubated at 37ºC for 1hour.To this were added 0.5ml of 2, 4 DNPH and 2.5ml of 0.4M NaOH, and the red colour developed was measured in a HITACHI-U-2001- UV-Vis Spectrophotometer at 510nm, and the enzyme activity was expressed in terms of U/ml.

Estimation of Alanine amino transferase (ALT)

To estimate haemolymph AST activity, a 0.2 ml sample of haemolymph was added to a test tube containing 1ml of phosphate buffer-substrate containing 0.1M phosphate, 0.2 M L-alanine,and 2mM 2-oxoglutarate at pH 7.4.It was mixed well and incubated at 37ºC for 1hour.To this were added 0.5ml of 2, 4 DNPH and 2.5ml of 0.4M NaOH, and the red colour developed

was measured in a HITACHI-U-2001- UV-Vis spectrophotometer at 510nm, and the enzyme activity expressed in terms of U/ml.

Statistical analysis

Statistics Data were analyzed by two factor ANOVA for effects of heavy metals on varying days and concentration. The Tukey test for multiple comparisons compared means when ANOVA was significant. Data is represented as its mean and standard errors.

2.4 Results

Glucose

Glucose level in the controls was 1.06mg/ml of haemolymph. Copper and mercury exposed animals showed difference in glucose values at various days of observation.

Copper exposed animals showed hyperglycemic condition on the initial day of exposure, but the values decreased well below that of the controls by day 15. On the 1st day at the lowest concentration used, i.e., 6 µg/L copper, haemolymph had the highest glucose content of 1.98 ± 0.375 mg/ml. By 15^{th} day glucose values in all sub lethal concentrations were significantly lower than that of the control values.

Mercury exposed *P. viridis* individuals differed from copper exposed mussels in that they showed a bi-phasic response in their glucose concentration. The 1^{st} and the 15^{th} day observation showed hyperglycemic condition. A marked increase from the control values was shown on the first day. This was followed by a steep drop in glucose levels in all the three concentrations on the 7^{th} day, which again tended to increase slightly than the control values by the15^{th} day (Table 2.1, Figure. 2.1, 2.2).

Glycogen

Glycogen value in the controls was 2.32±0.02 mg/ml.

Glycogen content decreased in copper-stressed mussels. The high reduction of glycogen values seen in the initial stage altered and increased on the 7th and 15th day. The increase, however, was significantly far below the control values. .

Mercury-exposed *P.viridis* specimens had reduced glycogen levels throughout the experimental period, though a definite pattern was not observed. Mercury toxicity decreased haemolymph glycogen content into half (Table 2.4, Figure. 2.3, 2.4).

Protein

Total protein content in the controls of *P. viridis* was 1.76mg/ml of haemolymph.

Copper exposed mussel showed day dependent increase in its haemolymph protein content in all three concentrations. But a concentration dependent elevation was not observed. A slight decrease in protein content, with respect to control, was observed on day 1 in only those mussels which were exposed to 6µg/L copper.

Mercury exposed mussels also had concentration and day dependent increase in its total haemolymph protein content. On the 1st day at 10 and 20 µg/L mercury concentrations, haemolymph protein contents decreased beyond control level. Haemolymph protein was found to be highest in animals exposed to the highest concentration of mercury (Table. 2.7, Figure. 2.5, 2.6).

Aspartate amino transferase (AST)

AST activity level in the unexposed *P. viridis* specimens was found to be 4.55I ± 0.2175 U/ml.

At 6 and 12 µg/L copper there was an initial decrease in enzyme activity. Highest AST activity was observed in mussels exposed to the highest concentration of copper, 30µg/L. For this concentration, the 1st and 7th day haemolymph analysis gave increased AST activity, which decreased by the end of the 15th day.

In the case of mercury treated mussels, the initially decreased AST activity diminished further by the end of the experimental period, except for an observed increase in animals at 10 and 50µg/L on the 7th day. Inhibitory effect as a result of chronic exposure to mercury was evident (Table 2.12, Figure 2.7, 2.8).

Alanine amino transferase (ALT)

ALT activity level in the unexposed *P.viridis* specimen was 1.609 ± 0.055. On exposure to copper, *P. viridis* exhibited significantly increased haemolymph ALT activity on the 1st and 7th day. At 6µg/L copper, on the 7th day mussels gave the highest value of 2.09 U/ml. ALT activity decreased on prolonged exposure of 15days.

Exposure to mercury resulted in a decrease in haemolymph ALT activity. On the 1st day, the activity was almost halved in mussels exposed to 20 and 50 µg/L copper when in compare with the control values. ALT activity decreased at almost all concentrations on days 7 and 15 (Table 2.15. Figure.2.9, 2.10)

Two way ANOVA test carried out on haemolymph glucose, glycogen and protein values showed that the values obtained for all

energy parameters were significant when compared with the control values at $P<0.05$ level (Table. 2.2, 2.3, 2.5, 2.6, 2.8, 2.9, 2.10, 2.11,2.13, 2.14, 2.16 to 2.19) .

2.5 Discussion

Energy levels vary when there is difference in energy demand during growth, reproduction or when they are under stress. Results clearly show heavy metals affect blood glucose levels. Hyperglycemia on day 1 at all concentrations of copper might be due to glycogenolysis and gluconeogenesis. This is proved in the light of the results of the experiments conducted on the activity levels of glycogen and transaminases subsequent to heavy metals exposure. Glycogen levels decreased which might be due to its breakdown, and AST levels have increased resulting in gluconeogenesis both resulting in increased glucose content in the haemolymph. Mussels exposed to 6 µg/L of copper gave the highest glucose level. This may be because the generated glucose might not have been be used up readily in the case of 6 µg/L copper due to its low toxicity.

Mercury showed a biphasic response with significantly reduced haemolymph glucose level only on 7^{th} day with respect to control mussels. The elevation in glucose level must be due to glycogenolysis alone, for ALT and AST activity levels were seen to be inhibited on exposure to mercury. Glucose transported through haemolymph from metabolically less active tissues to metabolically more active tissues through haemolymph can lead to a temporary increase in glucose level (Suresh, 1993).Another reason might be channeling of protein reserves. Cu, Hg, and Cd induce hyperglycemia in the freshwater prawn *Macrobrachium kistenensis*, and the crab *Barytelphusa canicularis*

(Nagabhushanam and Kulkarni, 1987; Machele et al., 1989).Glucose -6-phosphate formed from the breakdown of glycogen or from gluconeogenic pathways can be dephosphorylated to glucose and transported around the body in the haemolymph (Gabott, 1976).

A decrease in glycogen content by day 1 shows larger mobilisation of energy to overcome stress reflecting the toxicity of heavy metals. In shrimps under starvation haemolymph glycogen depleted fast, evidencing haemolymph reserves were not enough to compensate starvation thereby inducing use of muscle reserves. This substantiates the biphasic response of glucose in mercury treated mussels though haemolymph glycogen was depleted, and ALT and AST activities were partially inhibited. Glycogen depletion and hyperglycemia have been observed in cadmium exposed fish and explained by an effect of cadmium on the hormonal regulation of the glucose level (Larson and Haux 1982; Sastry and Subhadra, 1982).Glycogen represents the readily mobilizable storage form of glucose for most organisms. The increased energy demand associated with stress usually results in depletion of glycogen reserves (Lagadic *et al.*, 1994).

A total absence of glycogenesis has to be ruled out as glycogen can be synthesized from different metabolic pathways. Glycogen could be obtained from protein when muscle proteins get transferred to blood to be processed in digestive gland via gluconeogenesis pathway (Kucharski and Da Silva, 1991; Oliveira and Da Silva, 1997). Cheng and Cali, (1974) also observed glycogen deposits in the haemocytes of *C.virgiica*, and it was suggested that the intracellular digestion of phagocytosed material results in the isolation of carbohydrate constituents and conversion of these to glycogen deposits within granulocytes. This interpretation can explain the increased haemolymph glycogen content even after s15 days

of exposure to copper. At the same time glucose concentration was found to be very low, suggesting the inhibition of glycogenolysis, which is brought about by enzymes phosphorylase, phosphoglucomutase, and glucose-6-phosphatase (Wootton and Pipe, 2003).Ultra structural studies on haemocytes of *S. plana* made by Wootton and Pipe (2003) revealed considerable amounts of SER throughout the cytoplasm. One of the functions of SER is in glycogen catabolism for releasing stored glycogen as glucose through the action of various enzymes. The presence of glycogen and SER in the cells suggests a role for storage and maintenance of blood glucose levels.

Animals undertake adaptive responses to save nutritional reserves. In the present study with *P. viridis*, a reduction in filtration rate, byssal thread production, and minimal shell opening in heavy metal exposed mussels might be the animals' adaptation to save energy. On the other hand, mucous secretion was obvious in copper and mercury treated mussels. Again for the production of mucous, glucose is essential, the requirement being met by chanellising from metabolically less active tissues.

Transitory nature of changes in glucose concentrations in haemolymph limits its usefulness as an indicator of stress but glycogen is not as transient or sensitive to non toxicant stress and, therefore, is more promising as biomarkers of environmental pollution (Lagadic et al., 1994). However, in the present study chronic effect was a significant decrease in glucose content in *P. viridis* exposed to copper and mercury evidencing haemolymph glucose level as a biomarker. Whereas glycogen had no particular pattern in its alteration, though significant decrease in glycogen content was observed in mercury exposed animals.

In *M. edulis* protein concentration was found to be ranging between 115-282 mg $100ml^{-1}$ (Bayne, 1973a- cited from Gosling, 2002). Protein value for healthy mussels obtained in the present study was 1.76 ± 0.004 mg/ml, and this falls in the range given above. In bivalves, haemolymph proteins have been implicated to perform a variety of functions.

Many molluscs have a remarkable permeable epidermis across which metals are transported across the epithelial cells, and exocytosed into the blood on the basal side of the cells, from which they are circulated around the body in amoebocytes and retained or excreted.

When heavy metals or any xenobiotic is in higher concentration, circulating haemocytes may not be sufficient to decrease the cytotoxic effect. In such circumstances the permeability of cell membrane is altered, and seepage of protein occurs from within the tissue cells into the haemolymph. An increase in haemolymph protein will thus result as obtained in the present study, where protein concentration kept on increasing with increasing exposure time in days. The neo-synthesized metallothionein-like protein in response to metal ions may be contributing to this trend in protein increase (Suresh, 1988).Total protein concentration was found to be higher in the haemolymph of prawns, *M.malcolmsonii* exposed to endosulfan (Bhavan and Geraldine, 1997).

Bivalves require energy to surpass any physiological stress. During copper and mercury toxicity in the present study, the animal uses glucose, the immediate energy increases rapidly, and its deficiency is overcome by gluconeogenesis. Alanine and aspartate are the major glucogenic amino acids, which give rise to energy precursors by the activity of transaminases. The synthesis of glucose from non-carbohydrate precursors, from the substrates pyruvate, lactate, ketoglutarate, L-alanine,

L-aspartic acid and L-glutamate, has been demonstrated in the whole tissue of *Biomphalaria alexandrina* and *Bulinus truncatus* (Ishak et al., 1975; Sharaf et al., 1975). Hence, changes in ALT and AST activities are often associated with changes in metabolic functions and may thus represent widespread alterations in the organism's physiological state. ALT and AST activities in copper treated mussel did not show drastic inhibition as seen in mercury treated ones. AST tended to reach control values by 15th day of exposure. This could be explained as the conditioning of mussels to copper toxicity or acclimatization after prolonged exposure. Similar result was obtained by Blasco and Puppo (1999) when the clam, *Ruditapes philippinarum* (*R.philippinarum*) was exposed to copper, when no much change in AST activity was observed. A significant increase in AST activity in 50µg/L mercury exposed mussels can be considered as a stress induced immediate reaction of the organism to compensate for the drain of metabolites (Reju et al., 1993). Elevated AST and ALT activities seen in copper exposed animals on day 7 can be attributed to the same reason. An increased trnasaminases could also be due to anaerobic state persisted due to closure of valves to prevent the entry of toxic substances into the body.

Both AST and ALT had a general inhibitory or decreased activity in the green mussels on contact with mercury. Inhibitory effect persisted till the 15th day for AST while ALT had stimulation on 15th day with values in animals exposed to highest concentration of mercury nearing to those for the control specimens. This inhibitory activity is either due to inactivation of enzyme as a direct effect of mercury or due to lesser availability of aspartate and alanine precursors because of the heavy uilisation of aminoacids to counter the stress (Chow and Pond, 1972; Reju et al., 1993).

2.6 Conclusion

Alterations in major biochemical constituents in the haemolymph, such as glucose, glycogen and protein indicate disturbance of the oxidative carbohydrate metabolism. Apart from evaluating energy content as biomarkers, this experiment shows how the animal undergoes metabolic alteration to overcome stress. Glucose and glycogen decreased on prolonged exposure to heavy metals. Haemolymph protein values, however, significantly increased, which might be due to channelising tissue proteins to blood thereby decreasing tissue proteins. It is a matter of concern when biochemical constituents get decreased in *P.viridis* since it reflects a loss of nutritive value of this highly cherished seafood. Nevertheless, these parameters should be carefully assessed for field studies, and may not produce the same result as energy parameters change according to seasons, growth pattern, and reproduction and many biotic and biotic factors act together giving an entirely different result and picture.

Table.2.1 Haemolymph Glucose values in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	1.064± 0.253	1.978± 0.375	1.642± 0.092	1.806± 0.440
	7th day	1.019± 0.225	1.183± 0.387	1.117± 0.228	1.386± 0.321
	15th day	1.029± 0.259	0.917± 0.102	0.392± 0.059	0.557± 0.113
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	1.050± 0.263	1.785± 0.205	2.216± 0.292	2.615± 0.104
	7th day	1.096± 0.248	0.924± 0.174	0.910± 0.066	1.068± 0.184
	15th day	1.036± 0.212	1.173± 0.135	1.407± 0.179	1.180± 0.105

Table.2.2 ANOVA Table for Glucose level in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	12.668	2	6.334	88.780	.000
Concentration	.594	3	.198	2.777	.049
Day * Concentration	4.257	6	.710	9.945	.000
Error	4.281	60	.071		
Total	114.056	72			

Table.2.3 ANOVA Table for Glucose level in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	11.164	2	5.582	151.195	.000
Concentration	3.315	3	1.105	29.931	.000
Day * Concentration	5.344	6	.891	24.125	.000
Error	2.215	60	.037		
Total	157.455	72			

Table.2.4 Haemolymph Glycogen values in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	**1st day**	2.285± 0.317	1.003± 0.084	1.055± 0.251	1.530± 0.283
	7th day	2.331± 0.125	1.738± 0.064	1.608± 0.161	1.745± 0.262
	15th day	2.344± 0.221	1.888± 0.223	2.246± 0.216	1.608± 0.256
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	**1st day**	2.331± 0.178	1.269± 0.176	1.165± 0.209	1.732± 0.221
	7th day	2.337± 0.285	0.937± 0.099	1.178± 0.287	1.152± 0.230
	15th day	2.311± 0.289	1.380± 0.274	1.478± 0.476	1.237± 0.209

Table.2.5 ANOVA Table for Glycogen level in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	3.869	2	1.935	40.268	.000
Concentration	7.047	3	2.349	48.891	.000
Day * Concentration	3.242	6	.540	11.246	.000
Error	2.883	60	.048		
Total	245.586	72			

Table.2.6 ANOVA Table for Glycogen level in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.724	2	.362	5.359	.007
Concentration	15.036	3	5.012	74.230	.000
Day * Concentration	1.467	6	.244	3.620	.004
Error	4.051	60	.068		
Total	192.550	72			

Table. 2.7 Table for Protein values in metal exposed *P.viridis*

TOXICANT	Exposed days	Control	6µg/L	12µg/L	30µg/L
COPPER	**1st day**	1.755± 0.0298	1.708± 0.0148	1.870± 0.0164	1.826± 0.0157
	7th day	1.746± 0.0167	2.116± 0.0475	2.710± 0.1097	3.723± 0.1212
	15th day	1.753± 0.0230	3.832± 0.1022	3.514± 0.1768	3.679± 0.0785
		Control	**10µg/L**	**20µg/L**	**50µg/L**
MERCURY	**1st day**	1.753± 0.0256	1.062± 0.0483	1.090± 0.0353	1.431± 0.0799
	7th day	1.752± 0.0229	1.235± 0.1559	1.820± 0.0113	2.070± 0.0379
	15th day	1.753± 0.0256	3.723± 0.1013	4.122± 0.0453	4.356± 0.0716

Table. 2.8 ANOVA Table for Protein level in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	516.477	2	258.238	163.231	.000
Concentration	428.226	3	142.742	90.226	.000
Day * Concentration	555.504	6	92.584	58.522	.000
Error	94.923	60	1.582		
Total	8286.157	72			

Table.2.9 ANOVA Table for Protein level in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	63.382	2	31.691	6899.610	.000
Concentration	7.786	3	2.595	565.074	.000
Day * Concentration	21.677	6	3.613	786.567	.000
Error	.276	60	.005		
Total	435.450	72			

Table. 2.10 Multiple Comparison Test for Copper

		Glucose	Glycogen	Protein
Tukey	**Control Vs 6 μg/L**	0.318	0.000	0.000
	Control Vs 12 μg/L	0.999	0.000	0.000
	Control Vs 30 μg/L	0.091	0.000	0.000
	10 ppm Vs. 12 μg/L	0.395	0.582	0.000
	10 ppm Vs. 30 μg/L	0.913	0.656	0.000
	20 ppm Vs. 30 μg/L	0.124	0.999	0.000
		Glucose	**Glycogen**	**Protein**
Tukey	**1 day Vs 7 day**	0.000	0.000	0.000
	1 day Vs 15 day	0.000	0.000	0.000
	7 day Vs 15 day	0.000	0.029	0.000

Table. 2.11 Multiple Comparison Test for Mercury

		Glucose	**Glycogen**	**Protein**
Tukey	**Control Vs 10 µg/L**	0.003	0.000	0.000
	Control Vs 20 µg/L	0.000	0.000	0.000
	Control Vs 50 µg/L	0.000	0.000	0.000
	10 ppm Vs. 20 µg/L	0.007	0.804	0.000
	10 ppm Vs. 50 µg/L	0.000	0.180	0.000
	20 ppm Vs. 50 µg/L	0.326	0.659	0.000
		Glucose	**Glycogen**	**Protein**
Tukey	**1 day Vs 7 day**	0.000	0.012	0.000
	1 day Vs 15 day	0.000	0.951	0.000
	7 day Vs 15 day	0.002	0.026	0.000

Table.2.12 Haemolymph AST values in metals exposed *P.viridis*

TOXICANT	**Exposed days**	**Control**	**6 µg/L**	**12 µg/L**	**30 µg/L**
COPPER	**1st day**	4.509± 0.465	3.791± 0.412	3.950± 0.331	5.367± 0.244
	7th day	4.537± 0.060	5.265± 0.109	4.406± 0.253	5.335± 0.296
	15th day	4.484± 0.150	4.593± 0.170	4.583± 0.091	4.523± 0.438
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	**1st day**	4.671± 0.312	2.777± 0.281	2.922± 0.155	1.777± 0.517
	7th day	4.452± 0.181	4.212± 0.530	2.654± 0.127	3.212± 0.452
	15th day	4.523± 0.137	1.749± 0.398	1.495± 0.270	1.254± 0.223

Table.2.13 ANOVA Table for AST activity in copper exposed *P. viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	2.939	2	1.469	18.121	.000
Concentration	5.727	3	1.909	23.542	.000
Day * Concentration	7.626	6	1.271	15.676	.000
Error	4.865	60	.081		
Total	1552.544	72			

Table.2.14 ANOVA Table for AST activity in mercury exposed *P. viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	22.895	2	11.447	105.508	.000
Concentration	65.895	3	21.965	202.446	.000
Day * Concentration	14.846	6	2.474	22.805	.000
Error	6.510	60	.108		
Total	747.295	72			

Table.2.15 Haemolymph ALT values in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	**1day**	1.618± 0.101	1.777± 0.309	1.636± 0.256	1.382± 0.159
	7day	1.597± 0.053	2.095± 0.763	1.696± 0.075	1.897± 0.079
	15Day	1.589± 0.018	1.141± 0.065	0.844± 0.148	0.746± 0.053
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	**1day**	1.602± 0.038	1.230± 0.118	0.735± 0.104	0.742± 0.103
	7day	1.623± 0.044	0.724± 0.148	0.530± 0.107	1.286± 0.219
	15Day	1.625± 0.078	1.237± 0.109	0.933± 0.169	1.503± 0.081

Table.2.16 ANOVA Table for ALT activity in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	5.821	2	2.911	42.451	.000
Concentration	.870	3	.290	4.228	.009
Day * Concentration	2.483	6	.414	6.035	.000
Error	4.114	60	.069		
Total	169.797	72			

Table.2.17 ANOVA Table for ALT activity in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	1.176	2	.588	40.725	.000
Concentration	7.211	3	2.404	166.494	.000
Day * Concentration	2.258	6	.376	26.071	.000
Error	.866	60	.014		
Total	106.536	72			

Table. 2.18 Multiple Comparison Test for Copper

		AST	ALT
Tukey	**Control Vs 6 μg/L**	0.975	0.969
	Control Vs 12μg/L	0.174	0.088
	Control Vs 30 μg/L	0.000	0.021
	6 ppm Vs. 12 μg/L	0.071	0.220
	6 ppm Vs. 30 μg/L	0.000	0.066
	12 ppm Vs. 30 μg/L	0.000	0.938
		AST	**ALT**
Tukey	**1 day Vs 7 day**	0.000	0.176
	1 day Vs 15 day	0.206	0.000
	7 day Vs 15 day	0.000	0.000

Table. 2. 19 Multiple Comparison Test for Mercury

		AST	ALT
Tukey	**Control Vs 10μg/L**	0.000	0.000
	Control Vs 20μg/L	0.000	0.000
	Control Vs 50 μg/L	0.000	0.000
	10 ppm Vs. 20 μg/L	0.000	0.000
	10 ppm Vs. 50 μg/L	0.000	0.022
	20 ppm Vs. 50 μg/L	0.069	0.000
		AST	**ALT**
Tukey	**1 day Vs 7 day**	0.000	0.551
	1 day Vs 15 day	0.000	0.000
	7 day Vs 15 day	0.000	0.000

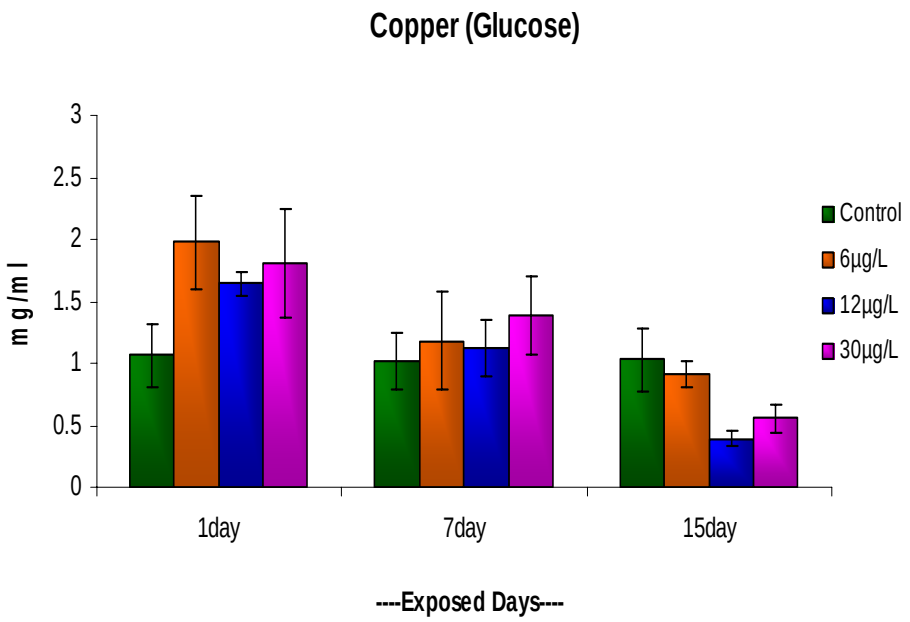


Fig. 2.1. Haemolymph glucose levels in copper exposed *P.viridis*

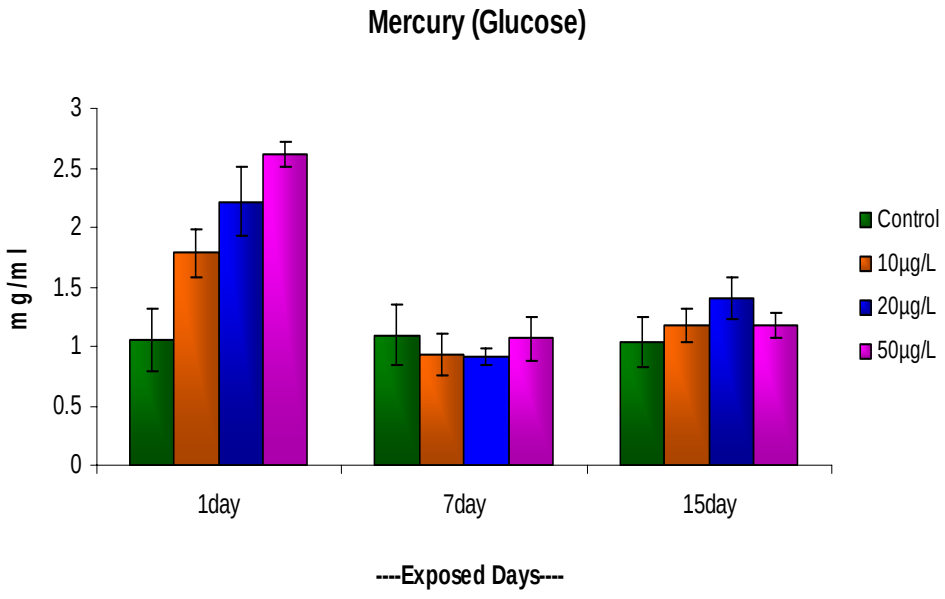


Fig. 2.2. Haemolymph glucose levels in mercury exposed P.viridis

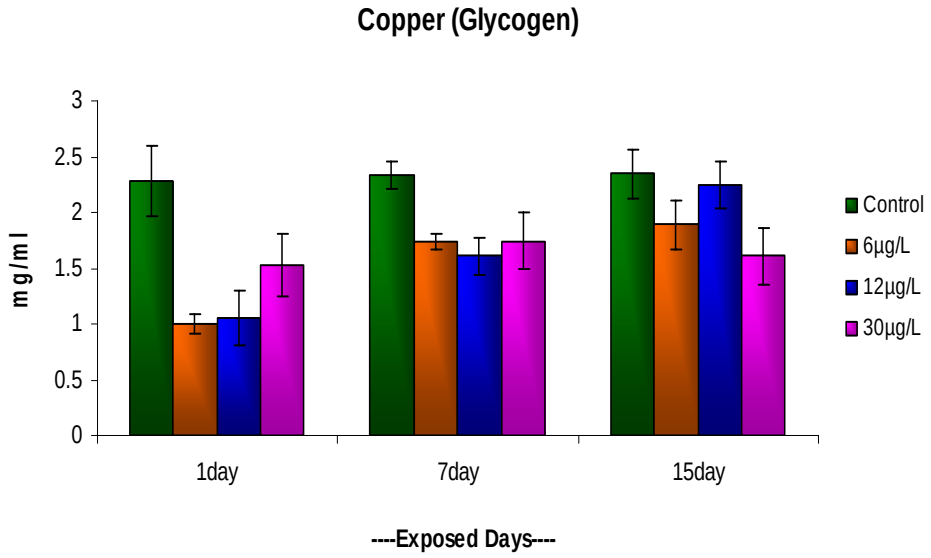


Fig. 2.3. Haemolymph glycogen levels in copper exposed *P.viridis*

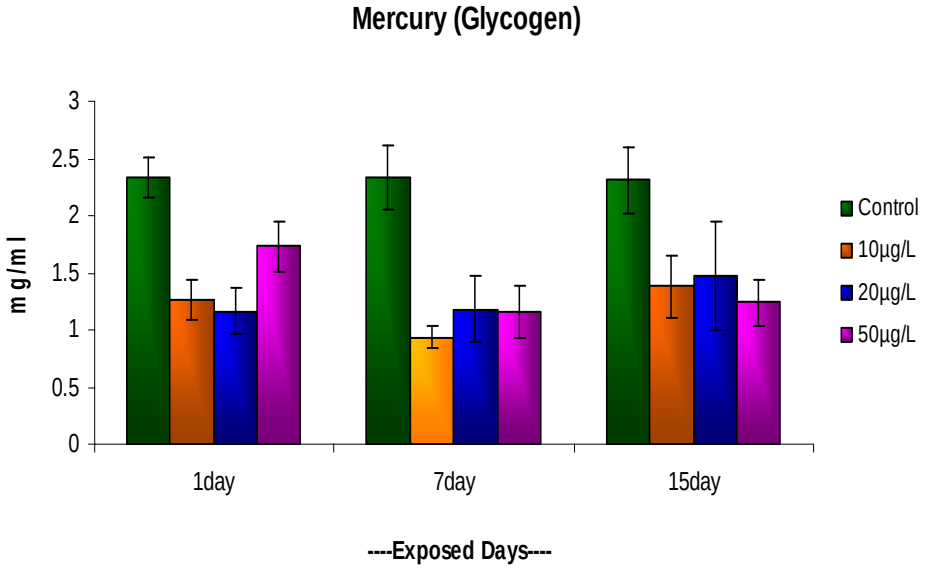


Fig. 2.4. Haemolymph glycogen values in mercury exposed *P.viridis*

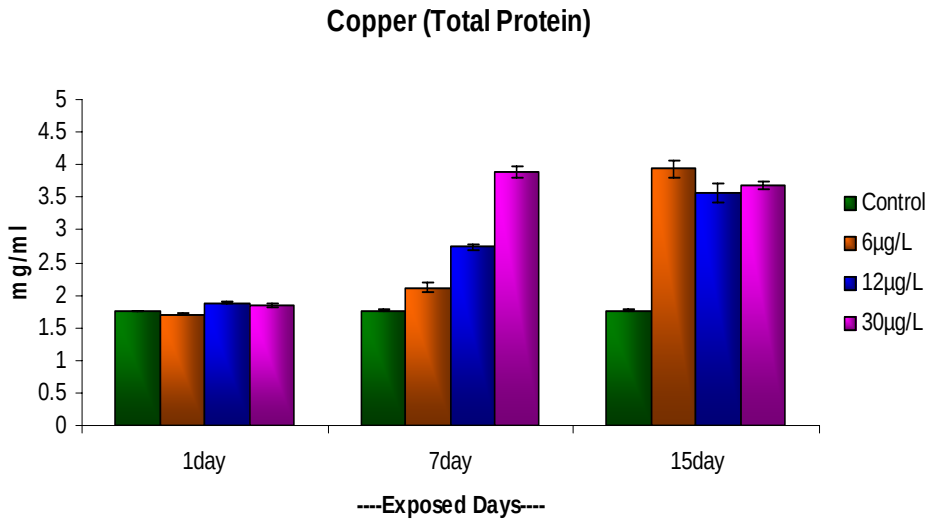


Fig. 2.5. Haemolymph protein values in copper exposed *P.viridis*

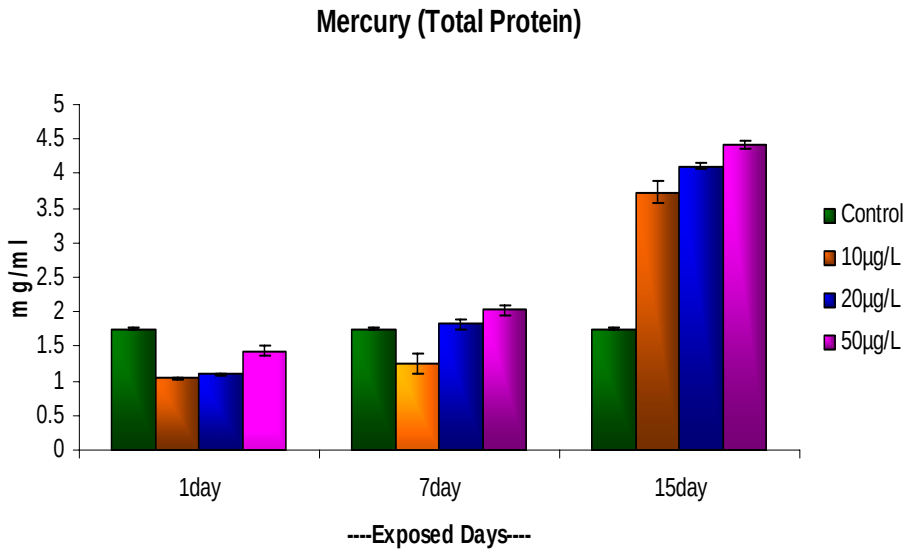


Fig. 2.6. Haemolymph protein values in mercury exposed *P.viridis*

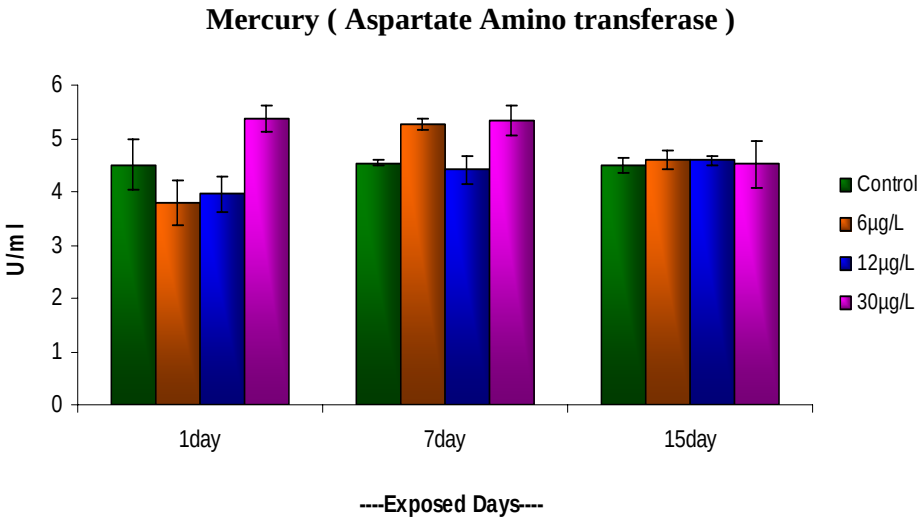


Fig. 2.7. Haemolymph AST values in copper exposed *P.viridis*

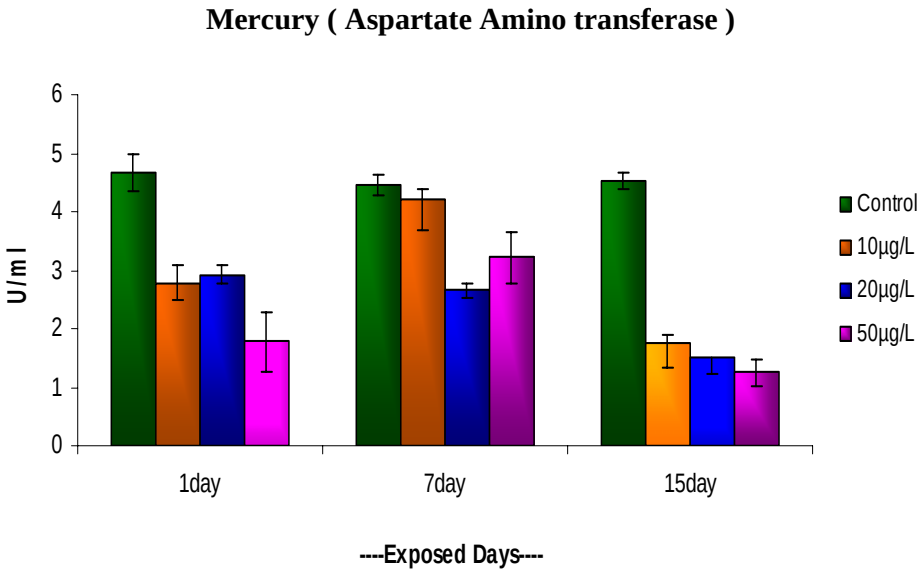


Fig. 2.8. Haemolymph AST values in mercury exposed *P.viridis*

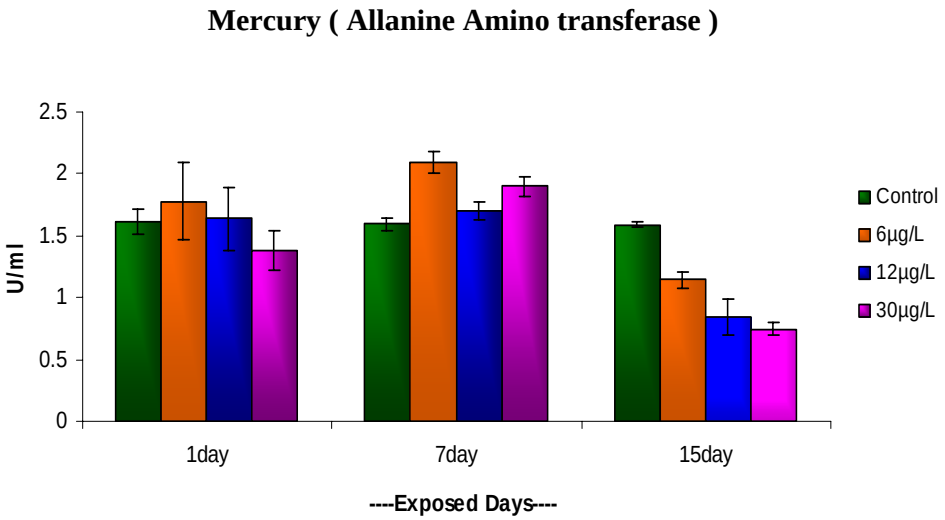


Fig.2.9 Haemolymph ALT values in copper exposed *P.*viridis

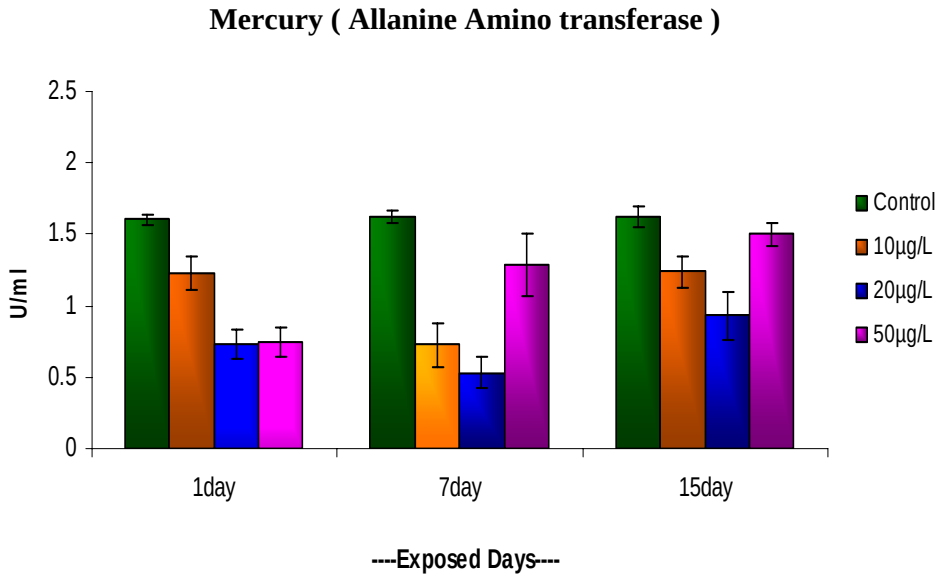


Fig.2.10 Haemolymph ALT values in mercury exposed *P.viridis*

Chapter 3

Metals induced changes in the haemolymph enzyme activity

A. Acid and Alkaline phosphatases

3. 1 Introduction

Quantitative assessment of enzymes is a reliable indicator of stress imposed on the organism by environmental pollutants such as heavy metals (Cheng, 1983a). Many physiological processes including activity of many lysosomal hydrolytic enzymes are inhibited by heavy metals even though these metals may also activate certain enzymes. Two important phosphatases are Acid Phosphatase (ACP) and Alkaline phosphatase (ALP), both differing in their sub cellular distribution. ALP activity was found to be highly concentrated in plasma membrane enriched fraction, where as ACP is associated with lysosomes. They have a very significant role in bivalve immunity. These enzymes are involved in a variety of metabolic activities such as permeability, growth and cell differentiation, protein synthesis, absorption and transport of nutrients, gonadal maturation, and steroidogenesis (Ram and Sathyanesan, 1985).

A useful biochemical indicator of lysosomal stability is the specific activity of suitable lysosomal enzyme, and acid phosphatase (ACP) (EC 3.1.3.2) is a major marker enzyme. Material to be hydrolyzed is taken into lysosomes by endocytosis and the enzymes catalyze the hydrolysis of most of the major polymeric compounds as well as foreign bodies entered into animal body. Lysosomal enzymes are mainly acid hydrolases and ACP is known to hydrolyse the phosphomonoesters, which are produced by hydrolysis of other major phosphates of the cell. Heavy metals

accumulate to a relatively high concentration in lysosomes destabilizing its single membrane. But Mohandas and Cheng (1985) have demonstrated through an electron microscope study that the lysosomes released from the granulocytes of *M.mercenaria* have two membranes.

ALP (EC 3.1.3.) is a polyfunctional enzyme, present in the plasma membrane of all cells. (32) It hydrolyses a broad class of phosphomonoester substrates, and acts as a transphosphorylase at alkaline *p*H, 9. It also acts as an early marker of cell differentiation in the osteogenic lineage in bivalve mollusc (Mourie`s et al., 2002). ALP activity has been reported to be sensitive to heavy metal pollutants (Regoli and Principato, 1995). In *Venus gallina* alkaline phosphatase activity is implicated in shell formation Carpene et al. (1979). ALP in serum and haemocytes of *C. farreri* were more important than any other enzymes in immune defense Mu et al. (1999), Wootton and Pipe (2003), Zhang et al., (2005), Pan et al., (2005).

3.1.1 Review of literature

The involvement of lysosomal system in the metabolism of many metals, either through sequestration and binding of metals within the lysosome or as a target of their toxicity has been reviewed by Moore and Stebbing (1976). ACP activity pattern in the haemolymph of copper exposed clam was investigated by Suresh and Mohandas (1989). Rajalakshmi and Mohandas (2005) suggested ACP as a reliable marker tool for the biological assessment of metal pollution. ACP as a lysosomal marker enzyme that exists in the haemocytes and serum of bivalve is suggested by Sun and Li. (1999), Mazorra, et. al.,(2002), and Wootoon and Pipe (2003). Acid phosphatases are involved in the immune defense of the oysters (Das, et al., 2004; Munoz et al., 2006). Various

concentrations of copper and mercury at varying length of exposure were found to influence ACP enzyme activity in *Lamellidens corrianus* (*L.corrianus*). (Rajalakshmi and Mohandas 2005, 2007).

Michelson and Dubois (1973) demonstrated the presence of alkaline phosphatase in some haemolymph samples and in all digestive gland extracts from the snail *Biomphalaria glabrata* (*B.glabrata*). Calorimetric techniques demonstrated increased alkaline phosphatase levels in both the haemolymph and digestive glands from *Schistosoma mansoni* (*S.mansoni*) infected snails. Characterization and effect of heavy metal on ALP was made by Mazorra et al. (2002) in the clam *S. plana,* and mercury showed highest inhibitory effects on ALP activity in various tissues analysed. ALP, which is sensitive to metals, gives a better picture of the general metabolic condition of the organisms (Regoli and Principato, 1995; Xiao et al., 2002). Intestinal and serum ALP activities were stimulated at 10 µM Cu exposure (Atli and Canli, 2007) in the fresh water fish, *Oreochromis niloticus* (*O.niloticus*).

The sensitivity of ALP and ACP as immune defense biomarkers is verified in this study, and observations are reported in this chapter.

3.1.2 Materials and Methods

The maintenance of the target animal and extraction procedure of haemolymph was the same as explained in details in Chapter 1.

Alkaline and Acid phosphatase activities were determined by following the methods of Anon (1963).

For ALP analysis, reaction mixture containing 0.1 ml of haemolymph and 1ml of PNP (1%) substrate in 0.1 M Glycine/ NaOH buffer at pH-9 was incubated at 37ºC for 30 min.

Reaction mixture for ACP analysis contained 0.1ml of haemolymph and 1ml of PNP (1%) substrate in citrate buffer at pH-4, which was incubated at 37ºC for 30 min. To both the mixtures for ALP and ACP measurements, 1.5 ml of 0.1N sodium hydroxide was added to stop the reaction. The hydrolytic product, yellow *p*-nitrophenol, was measured at 405 nm in a UV- Vis HITACHI-U-2001 spectrophotometer. Protein estimation was performed as per the method of Lowrys et al. (1951).Enzyme activity is expressed as mg PNP released/ml.

Statistical analysis

Two way ANOVA, followed by Tukey's post hoc mean comparison test was used to assess for significant differences between variables. Data are reported as mean and its standard error. The level of significance was considered at probability level ($P < 0.05$).

3.1.3 Result

Generally, ALP and ACP values revealed an increase in activity with time and concentration on exposure to the heavy metals Copper and Mercury.

Alkaline phosphatase activity in copper- exposed animals showed a decrease in all concentrations applied, on the 1st and 7th day of observation. On the 15th day, a drastic increase was observed compared to healthy control mussels. Highest activity was observed in mussels exposed to the highest concentration of copper, i.e., 30µg/L.

In mercury- exposed animals, 1st day showed a decrease in ALP activity in all the three sublethal concentration used. A concentration dependent increase in ALP activity was observed at 7th and 15th days of

exposure, when compared with the control values. Highest concentration of 50 µg/L showed the highest ALP activity (Table 3.1, Figure. 3.1 & 3.2).

A decreased ACP activity was detected in copper- exposed mussels on the first day of observation. As the concentration of copper and days of exposure increased, ACP activity also increased. ACP activity gave the highest value in mussels exposed to 30 µg/L, the highest concentration of copper used in study.

Low activity of ACP was observed in *P. viridis* on the 1st day of exposure to mercury. ACP activity was increasing on the 7th day of exposure when compared to the value of the 1st day, though the value remained still lower to that of control mussels. Long-drawn-out exposure of 15 days, however, increased the ACP activity above that of the control mussels (Table 3.4, Figure 3.3 & 3.4.)

Statistical analysis of ACP activity in copper and mercury exposed *P. viridis* showed all variables vary significant with concentration. It can be seen that for ALP activity, concentration has no significant impact on the dependent variable at probability level ($P<0.05$) (Table 3.2,3.3, 3.5, 3.6,3.16 & 3.17).

3.1.4 Discussion

The increase in activity of ACP and ALP reveals that the animals are under metal stress. Bivalves subjected to metals avoid the toxicant intake by valve closure and hence reducing the filtering activity. The decrease in ACP and ALP activities at the initial stages of exposure to copper and mercury can be attributed to this avoidance behavior of bivalves. Similar conduct was exhibited by *Anodonta cygnea* (*A.cygnea*) and *M.edulis*, where filtering activity was reduced and the period of rest increased correspondingly on

exposure to mercury and copper (Davenport (1977), Langston and Spence, (1995). Higher concentrations of metals lead to quicker valve closure (Tran et al., 2003). Prolonged valve closure would prove lethal due to respiratory stress and animals are forced to open valves, which will again permit contact with metals resulting in stress.

Lysosomal response was considered as the most reliable effect observed in mussels during stress (Grundy et al., 1996) and ACP is a lysosomal marker enzyme that exists in the haemocytes and serum of bivalve (Sun and Li. 1999; Mazorra, et.al., 2002; Wootoon and Pipe, 2003). The decrease in ACP activity at the initial stages of exposure may be due to the reason that, the available enzyme must be used up in sequestering the metals that have already made entry into the cell, and also due to shell closure, the first strategy of organisms to prevent metal toxicity (Langston and Spence,1995;Davenport, 1977). Lower ACP activity in copper and mercury treated mussels indicates detoxification of metals with the available enzyme without hyper production. Metallothionein might have been present sufficiently to sequester the metal accumulated (Rajalakshmi and Mohandas, 2007). A low ACP activity shows that tissue damage is less compared to those exposed to higher concentrations. Severe damage to tissues results in seepage of enzymes from cells and tissues to haemolymph resulting i.n high levels of enzymes in the haemolymph compartment. Decreased lysosomal membrane stability was observed by Regoli et al (1998) on exposure to copper and mercury.

Haemocyte lysosomal membranes were not found to be damaged in the early days of exposure (as observed in NRR assay and cell viability test in Chapter I). It implies that metal ions were getting loaded in haemocyte lysosomes at a tolerable concentration preventing the release of hydrolytic

enzymes, the ACP. Acid phosphatase activity and metallothionein synthesis are so closely related that binding of metals to the lysosomal membranes causes increased loading of metal binding proteins within the lysosomal compartment (Regoli et al., 1998), detoxifying the heavy metal, which justifies the decrease in ACP activity as immediate response to heavy metals in *P.viridis* as observed in the present work.

The excess engulfing into and storing of metals in the lysosome of hemocytes and other cells lead to membrane labilisation and hydrolases are released, which are capable of cellular component lysis. Hydrolases are, therefore, predominantly sequestered in an inactive form within a thick membrane in order to prevent free access to cellular constituents. This property is termed structure-linked latency. Toxicity of metals disrupts lysosomal membrane integrity and causes its destabilization followed by release of stored lysosomal hydrolases into the haemolymph thereby increasing the activity of the enzyme in haemolymph. The above explanation justifies the hyper activity of ACP as observed at high concentration of metals, and the extended period of exposure. The higher activity observed in the present study can be due to the hyper- synthesis of acid phosphatase, and its subsequent release into the haemolymph to meet metal insult. Hyper synthesized enzymes play a protective role in the removal of inflammation-provoking agents (Cheng 1983a). Hyper synthesis of enzyme underlines the involvement of enzyme in defense reaction, so as to destroy the foreign bodies, biotic and abiotic (Cheng 1983b). Previous studies show that the ACP activity in serum can reflect the immune state of the two species of scallop *A.irradians* and *C.farreri* (Liu et al., 2004; Mu et al., 1999; Zhang et al., 2005). Inhibition of ACP activity in digestive cells by copper has been reported in mussels (Rajalakshmi and Mohandas, 2005).

ALP activity has been reported to be sensitive to heavy metal pollutants (Regoli and Principato, 1995) the higher activities of ALP as observed on the 15th day of exposure to copper and mercury suggest that an extended period of contact can lead to stress in mussel. Decrease in ALP activity may result from disturbance of membrane transport system, while enhancement of enzyme can be observed as a signal of tissue damage (Atli and Canli, 2007; Karan et al., 1998)

The concentration of enzyme in haemolymph of *B.glabrata* infected with larval trematodes of *S.mansoni* appeared greater, and was 0.65±0.37units/ml per mg, as shown by (Michelson and Dubious, 1973). In the present observation, the ALP activity in highly stressed mussel at 50µg/L was 0.655 mg *p*-nitrophenol released /ml haemolymph. This increase in ALP activity on exposure to copper and mercury may represent an "overflow" into the open circulatory system from various tissues and haemocytes that might have got damaged due to metal toxicity. Muller (1965) reported that tissue destruction by the parasite released a rich content of enzyme bound in the digestive gland of *B. glabrata*

Vallee and Ulmer (1972) reported that mercury can inactivate ALP by binding to the sulfhydryl group of catalytic cysteine groups in the protein backbone. Such enzyme inactivity was observed in the form of decreased activity on the first day of exposure, which later increases on extended exposure. In fish, the serum ALP activity generally increased following metal exposures. (Atli and Canli , 2007) and this can be correlated with tissue alterations and cell damage (Karan et al., 1998).

3.1.5 Conclusion

The apparent sensitivity of Alkaline and Acid phosphatase activities suggests that analysis of these enzymes in haemolymph can be used as

biomarker in metal pollution monitoring. This base line data together with a comparison of haemolymph enzyme levels from field samples in pollution monitoring studies endow on them the status of a promising biomarker in aquatic toxicology.

Table.3.1 Haemolymph Alkaline phosphatase activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	0.442± 0.0289	0.281± 0.007	0.221± 0.005	0.294± 0.004
	7th day	0.448± 0.028	0.387± 0.004	0.330± 0.007	0.410± 0.002
	15th day	0.442± 0.028	0.692± 0.004	0.947± 0.002	1.054± 0.004
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	0.431± 0.013	0.266± 0.073	0.300± 0.052	0.213± 0.050
	7th day	0.415± 0.019	0.443± 0.036	0.462± 0.096	0.512± 0.070
	15th day	0.448± 0.014	0.555± 0.090	0.598± 0.029	0.655± 0.030

Table.3.2 ANOVA Table for Haemolymph Alkaline phosphatase activity in copper exposed *P. viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	3.073	2	1.537	99.056	.000
Concentration	.228	3	.076	4.904	.004
Day * Concentration	1.322	6	.220	14.204	.000
Error	.931	60	.016		
Total	23.250	72			

Table.3.3 ANOVA Table for Haemolymph Alkaline phosphatase activity in mercury exposed *P. viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.829	2	.415	59.260	.000
Concentration	.018	3	.006	.852	.471
Day * Concentration	.306	6	.051	7.286	.000
Error	.420	60	.007		
Total	15.609	72			

Table.3.4 Haemolymph Acid phosphatase activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	**1st day**	0.554± 0.002	0.215± 0.005	0.186± 0.008	0.197± 0.046
	7th day	0.545± 0.002	0.601± 0.007	0.639± 0.004	0.521± 0.004
	15th day	0.559± 0.002	0.828± 0.016	0.687± 0.003	1.028± 0.006
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	**1st day**	0.510± 0.002	0.289± 0.078	0.325± 0.057	0.232± 0.055
	7th day	0.526± 0.002	0.448± 0.039	0.463± 0.123	0.475± 0.076
	15th day	0.528± 0.001	0.625± 0.110	0.650± 0.031	0.764± 0.032

Table.3.5 ANOVA Table for Haemolymph Acid phosphatase activity in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	2.900	2	1.450	340.894	.000
Concentration	.049	3	.016	3.849	.014
Day * Concentration	1.314	6	.219	51.493	.000
Error	.255	60	.004		
Total	25.919	72			

Table.3.6 ANOVA Table for Haemolymph Acid phosphatase activity in mercury *exposed P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	1.101	2	.551	129.831	.000
Concentration	.041	3	.014	3.257	.028
Day * Concentration	.409	6	.068	16.090	.000
Error	.254	60	.004		
Total	18.828	72			

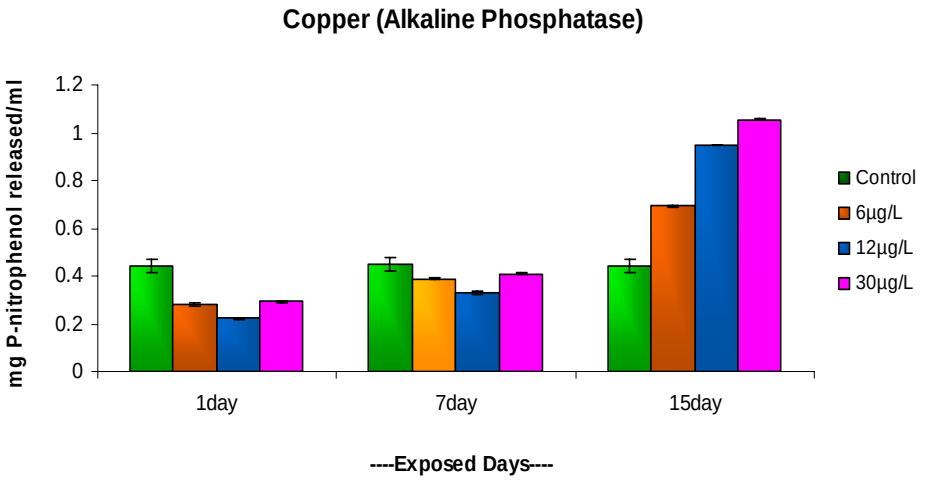


Fig. 3.1. Haemolymph Alkaline phosphatase activity in copper exposed *P.viridis*

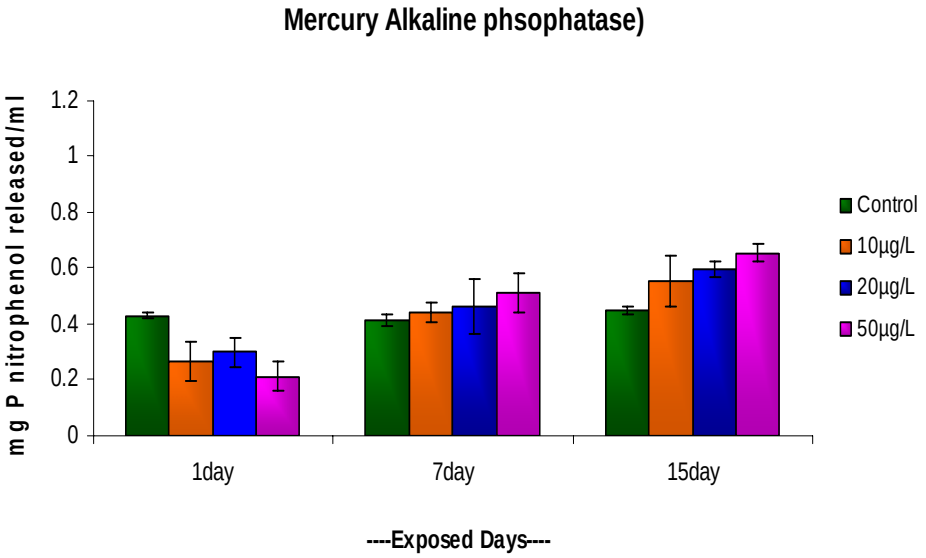


Fig. 3.2. Haemolymph Alkaline phosphatase activity in mercury exposed *P.viridis*

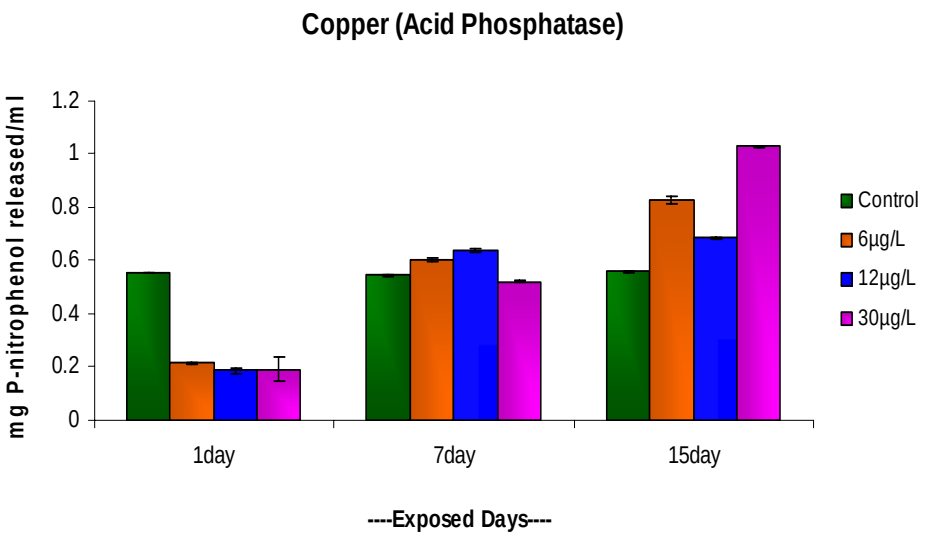


Fig.3.3. Haemolymph Acid Phosphatase activity in copper exposed *P.viridis*

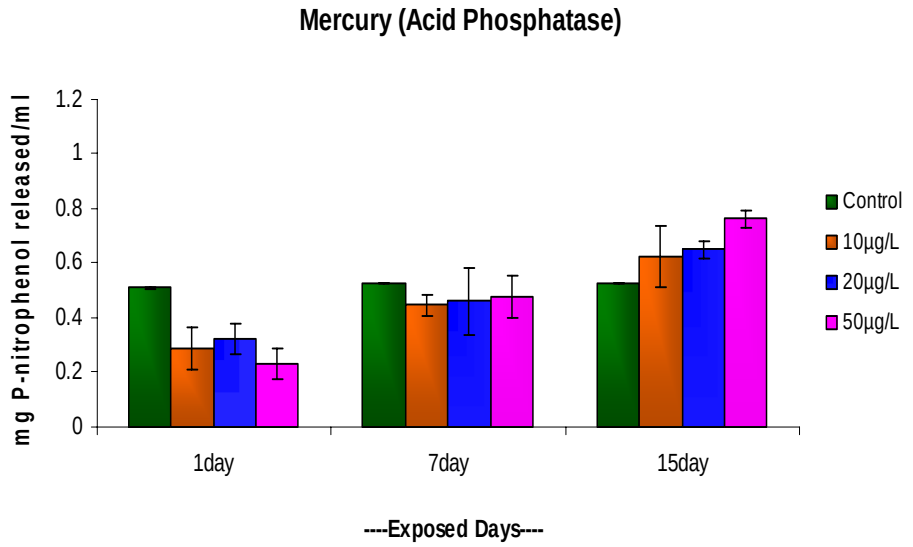


Fig. 3.4. Haemolymph Acid Phosphatase activity in mercury exposed *P.viridis*

B. Acetylcholinesterase-AChE

3.2 Introduction

The enzyme acetylcholinesterase (AChE) which catalyses the hydrolysis of acetylcholine is ubiquitous in the animal kingdom (Massoulie *et al*., 1993, Walker and Thomson, 1991). It is a well characterised enzyme in the vertebrates because of its critical catalytic function at the cholinergic synapses. The enzyme acetylcholinesterase (AChE) (EC. 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine to acetate and choline at the cholinergic synapses, terminating nerve impulse transmission. AChE as a potential cell membrane marker enzyme is already approved (Mitchell 1965; Severson *et al*., 1972, Steck *et al*., 1974; Watts *et al*., 1978).

3.2.1 Review of Literature

Since the initial work of Ellman *et al*.,1961, the use of acetylcholinesterase activity as biomarkers of pollution is well documented by Winners *et al*. (1978), Verma *et al*.(1979), Olson and Christensen (1980), Singh and Agarwal (1983), Habig *et al*.(1988), Day and Scott (1990), Payne *et al*.(1996), Lundbeye *et al*.,(1997), Owen *et al*.,(2002), Dailianis,(2003), and Brown et al.,(2004).

The effect of heavy metal exposure on AChE activity in mussel was evaluated by Najimi *et al*., (1997) and Bainy *et al*., (2006). Most studies on acetyl cholinesterase inhibition in marine molluscs have focused on whole organism or muscle extracts of sacrificed bivalves (Bocquene, 1997, Galgani and Bocquene,1990, Bocquene *et al*.,1990, 1993, Radenac *et al*.,1998 and Bainy *et al*.,2006). Use of whole tissue is, however, destructive in nature and non-destructive techniques involving haemolymph and plasma are scarce. A few studies made by Winners *et al*.,(1978), and Owen *et al*.,(2002) have confirmed the presence of cholinesterase enzymes in molluscan adductor

muscle haemolymph. AChE is present in the haemolymph of *Aplysia*, a marine gastropod, and in the cholinergic as well as non- cholinergic neurons (Srivatsan *et al*., 1992). In the haemolymph of *Mytilus* a true acetyl cholinesterase exists with the highest specificity as observed by Winners *et al*. (1978). The use of AChE as biomarker is documented in crustacea as well (Fossi *et al*., 1996; Lundebye *et al*., 1997).

This part of the thesis depicts AChE activity pattern in the haemolymph of *Perna viridis* exposed to copper and mercury, a less investigated aspect.

3.2.2 Materials and methods

Collection of animals, methods of their maintenance and acclimatization together with the haemolymph extraction procedure was the same as described in detail in Chapter 1.

AChE was analyzed following the procedure which is an optimized version of Ellman *et al*., (1961). Principle underlying the reaction is that the substrate acetylthiocholine when hydrolysed by the enzyme acetylcholinesterase yields thiocholine. This, on subsequent combination with DTNB, forms the yellow anion 5-thio-2nitrobenzoic (TNB) acid, which absorbs strongly at 412nm. Extracted haemolymph samples were centrifuged at 10,000 rpm to remove hemocytes. A 100 µl sample of the resulting supernatant was added to 1.45 ml of 0.27mM DTNB-5’5’dithiobis (2-nitrobenzoic acid) made up in phosphate buffer at *p*H 7.4, and mixed well. This was followed by addition of 50 µl of the substrate analogue, 0.075mM acetylthiocholine iodide, and the rate of production of the yellow anion 5-thio-2-nitrobenzoic acid was measured at 412nm over 1min in a HITACHI-U-2001- UV-Vis Spectrophotometer,

and expressed as U/mg protein. Protein estimation was done following the method of Lowry *et al*.(1951)

Statistical analysis

All assays were performed in triplicate and the mean and standard error were calculated. Data were analysed statistically by two-way analysis of variance (ANOVA) and Tukeys test to find significant changes. Differences were considered significant when $P < 0.05$.

3.2.3 Result

Results clearly indicate stimulation of AChE activity on exposure to copper and mercury, which went on decreasing as duration of exposure increased. AChE assay seemed to be very sensitive and rapid as the prominent yellow end product forms instantly on addition of enzyme source (Table 3.7, and Figure. 3.5 & 3.6.).

At all the three sublethal concentration of copper, elevated activity was observed in the haemolymph, with a two fold increase in the values when compared with that of the controls on the 1st day of analysis. Highest activity measured on the 1st day was 0.227±0.05 U/mg protein in the haemolymph of animals exposed to 30µg/L copper. By the 7th day AChE activity decreased, still remained higher than the control values. On the 15th day, activity reduced further, and values nearing to that of the control, which was shown to be 0.093± 0.002 U/mg proteins. For all the concentrations haemolymph AChE activity decreased from the initial stimulation as days of toxicity increased.

Specimens of *P. viridis* exposed to mercury showed an initial increase in AChE activity. While the 10 µg/L and 20 µg/L mercury-exposed mussels showed a 3 -fold increase in enzyme activity, those at

the highest concentration of 50 µg/L had only 0.166± 0.019 U/mg protein, which is almost 2 times higher than that of the controls. This hike was followed by a decrease in AChE on the 7th and 15th day. At 10 µg/L, there was a gradual decrease in enzyme activity, whereas at 20µg/L and 50 µg/L mercury there was a steep decrease by the 15th day. Inhibitory effect was observed in 20µg/L and 50 µg/L exposed mussels with activity values of 0.054± 0.002 and 0.059 ± 0.015 U/mg protein, respectively, which is below that of the control value of 0.093 (± 0.002) U/mg proteins.

All variations were statistically found to be significant at probability level $P < 0.05$ (Table 3.8, 3.9, 3.16, 3.17).

3.2.4 Discussion

Results strongly prove a stimulatory effect on haemolymph AChE activity as an immediate response to stress, which then decreases on extended exposure to Cu and Hg. Though there are classic studies to confirm inhibitory effect of AChE activity, a handful of studies support an increased AChE activity as a result of heavy metal toxicity. Gill *et al*., (1991) reported increased AChE activity in skeletal muscles and brain of fish *Barbus conchonius* when exposed to cadmium. A stimulatory effect of lead in rats and oligochaetes was described by Flora and Seth, (2000). Thaker and Haritos (1989) demonstrated that mercury (0.4mg/L) causes a significant increase in esterase activity in shrimps while Dailianis *et al.*, (2003) reported increase in AChE activity in *Mytilus edulis* collected from polluted areas.

Elevated AChE content observed initially in the present study might be a response of heavy metal toxicity. Heavy metals alter enzyme activity by binding to the functional group of proteins like imidazole, sulphydryl,

carboxyl, and peptide groups. Heavy metals can alter the AChE activity not only by inhibiting but also by stimulating the catalytic function of the enzyme (Jackin, 1974). Romani et al., (2003) based on their observation of increased AChE in Copper exposed fish for a period of 20 days proposed that Cu could enhance the formation of the enzyme-substrate complex, increasing the activity of AChE. Results of the present study are similar to that of Najimi *et al*., 1997, who found a significant increase in AChE in *Perna perna* after 3 and 7day- exposure to cadmium, and 3and 4days to zinc. They also found that by the end of experimental period (11 days), AChE activity decreased significantly and tended to return to control value.

Another possibility for AChE activation by metals could be related to *de novo* synthesis of the enzyme (Romani et al., 2003).They further suggested that metals under laboratory condition, could interact with AChE receptor, and thereby affect its binding efficiency, leading to an increase in AChE synthesis, to decompose the higher levels of neurotransmitter as an acute response.

In the present study, inhibitory effect when compared to control group was observed in 20 and 50 µg/L mercury- exposed group of mussels. This along with decreasing activity on the 7^{th} and 15^{th} day exposure to copper and mercury points to the fact that prolonged exposure inhibits AChE activity. Hence, the inhibitory effect indicated by researchers cannot be ruled out. Olson and Christensen (1980) determined the order of effect of several heavy metals on AChE activity in fish, and concluded that copper had inhibitory effect.

From the suite of biomarkers analysed in this study, haemolymph AChE is highly supported as a marker enzyme as it is simple, sensitive,

and rapid in its action. The rapid hydrolysis of acetylthiocholine iodide to form yellow coloured 5 Thio- 2 nitrobenzoic acid gives a visual measurement of enzyme reaction indicating the sensitive activity of AChE unlike certain other enzymes tested, which are either gradual or time consuming, or the end product is faint. Dailianis *et al.*, (2003) strongly recommend AChE as a biomarker especially in haemolymph and digestive gland. Chad and Srivatsan (2003) has shown that the cholinesterase present in the haemolymph of *Aplysia* is acetylcholinesterase due to its high rate of hydrolysis towards the substrate acetylthiocholine iodide. AChE activity in the haemolymph of *Aplysia* is of a greater magnitude than in other tissues (Srivatsan, 1992). The high AChE activity in the haemolymph in comparison with the activities in other tissues probably indicates that, in molluscs, the role of this enzyme may not be related to nerve impulse transmission. Furthermore, the existence of cholinergic transmission in the peripheral nervous system in molluscs has not so far been demonstrated (Heyer,et.al.,1973; Mercer and McGregor, 1982).

3.2.5 Conclusion

In agreement to the other workers, the present data once again confirm AChE to be a sensitive enzyme marker using mussel haemolymph, as it has been demonstrated to be a non destructive method of pollution monitoring. Acetylcholinesterase, due to its high rate of hydrolysis towards the substrate acetylthiocholine chloride, cofirms its presence in the green mussel *P.viridis,* a highly valued aquaculture candidate in Indian waters. It is highly sensitive and is recommended as a useful biomarker in biomonitoring studies.

Table. 3.7 Haemolymph AChE activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	0.094± 0.052	0.207± 0.009	0.188± 0.024	0.227± 0.050
	7th day	0.091± 0.034	0.127± 0.019	0.128± 0.011	0.149± 0.025
	15th day	0.094± 0.046	0.091± 0.004	0.090± 0.004	0.101± 0.047
		Control	10 µg/L	20 µg/L	50 µg/L
MERCURY	1st day	0.093± 0.029	0.307± 0.011	0.318± 0.035	0.166± 0.019
	7th day	0.093± 0.025	0.150± 0.036	0.111± 0.012	0.070± 0.022
	15th day	0.095± 0.024	0.145± 0.049	0.054± 0.002	0.059± 0.015

Table.3.8 ANOVA Table for Haemolymph AChE activity in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.090	2	.045	43.588	.000
Concentration	.043	3	.014	13.746	.000
Day * Concentration	.030	6	.005	4.871	.000
Error	.062	60	.001		
Total	1.480	72			

Table.3.9 ANOVA Table for Haemolymph AChE activity in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.254	2	.127	176.428	.000
Concentration	.149	3	.050	68.960	.000
Day * Concentration	.125	6	.021	28.859	.000
Error	.043	60	.001		
Total	1.939	72			

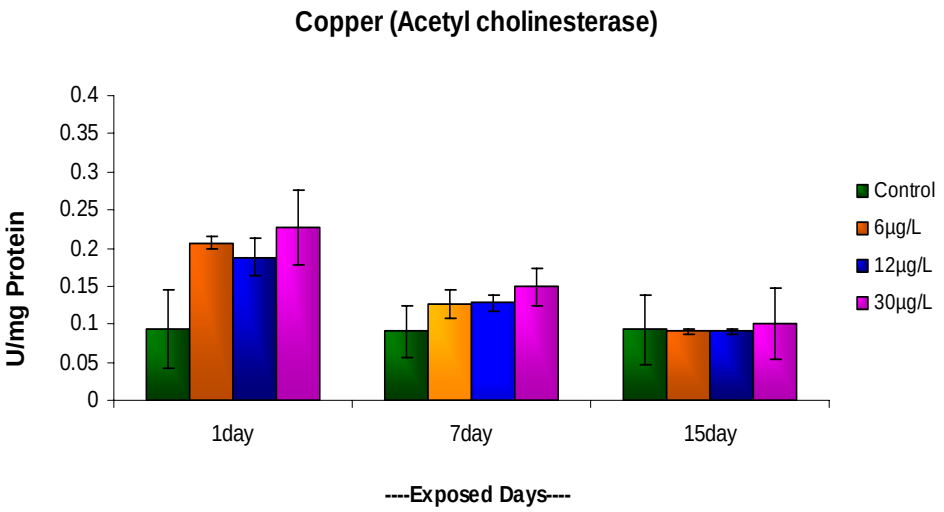


Fig . 3.5. Haemolymph AChE activity in copper exposed *P.viridis*

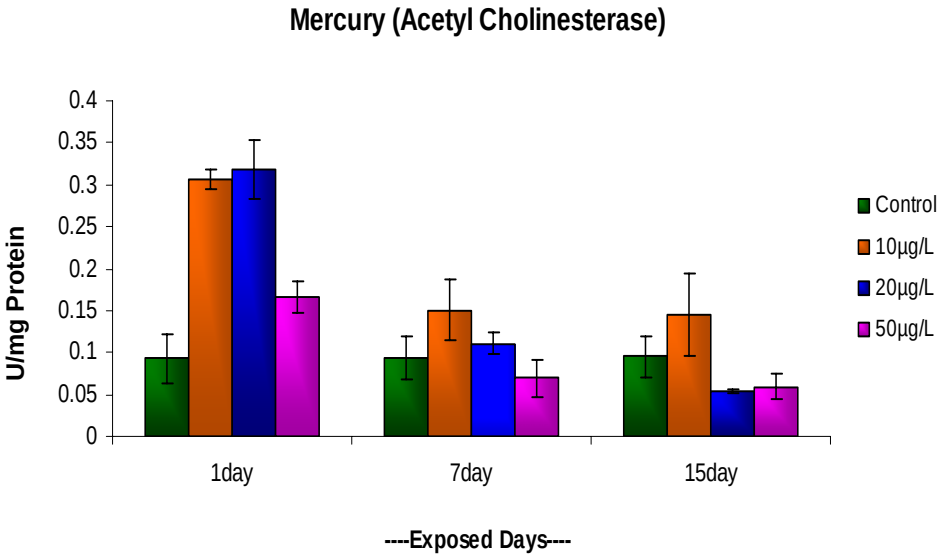


Fig. 3.6. Haemolymph AChE activity in mercury exposed *P.viridis*

C. Na^+/ K^+ - ATPase

3.3 Introduction

Metals exert their toxicological activity through nonspecific binding of the metal to physiologically important target molecules. The disturbed functioning of a number of critical cellular macromolecules and organelles has been shown to occur in response to copper and mercury exposure. Metal toxicity is observed when lysosomal or metallothionein detoxification systems are overwhelmed, which causes destabilization of lysosomal membranes, reduced ATPase function, and interactions of free metal ions with essential enzyme systems (Moore, 1985; Roesijadi, 1996b; Ringwood et al., 2004).For example, Na^+ K^+/Ca^+ Mg-ATPase inhibition, and it appears that a variety of metals can alter the permeability characteristics of membranes and disrupt the ionic balance of cells by acting upon this and other metal sensitive ion translocating enzymes.

Na^+/ K^+ ATPase (E.C 3.6.1.3) is a membrane bound enzyme, which has important functions such as ion transport, maintenance of the electrochemical gradient and regulation of cell volume (Heath, 1987; Canli and Stagg, 1996). The concept that Na^+/K^+ATPase, being intimately involved in active transport of ions across biological membranes, has gained wide acceptance in recent years. Gills with their high levels of Na^+/K^+- ATPase activity constitute the primary site for ion uptake from the medium to the haemolymph. Na-pump is mainly involved in the regulation of the composition and volume of the cell compartment. ATPase is located in cell membrane and has been implicated in the active transport of Na^+/ K^+ across cell membrane.

3.3.1 Review

Na^+ /K^+ ATPase is located in the cell membrane and has been implicated in the active transport of Na^+ and K^+ across cell membrane. Na^+ /K^+ ATPase has been known to play an important role in maintaining haemolymph ion concentrations different from ion concentrations of their environment (Quinn and Lans, 1966). Their use, as carrier enzymes in ionic exchange mechanisms, has been described by Towle et al., (1976). Alteration in the activity of this enzyme causes elevation of electrolytes in the blood, which indicates acute stress response (Larson et al., 1981). Na^+ /K^+ ATPase was detected in tissue homogenates of outer mantle epithelium of *Anodonta cygnea* (Costa et al., 1999).

A wealth of data indicates that metal ions pass across biological membranes, subsequently enhancing or inhibiting cellular activities like enzyme inhibitions. Na^+/ K^+ ATPase, which is involved in osmoregulation and intracellular functions i.e. sodium pump, is a very sensitive indicator of trace metal toxicity. Atli and Canli (2007) have reported that *Oreochromis niloticus* exposed to copper exhibited decreased Na^+/ K^+ ATPase activity in the gill and intestine. The inhibition of Na^+/ K^+ ATPase activity by xenobiotics might generate perilous toxic effect in the cell. This study explores the effect of different sublethal concentration of two heavy metals on haemolymph Na^+ /K^+ ATPase activity of *Perna viridis* over an extended period of exposure.

3.3.2 Materials and methods

Na^+ /K^+ ATPase activity was estimated by the method of Bonting (1970). A reaction mixture containing 1.0 ml of Tris-HCl buffer (30mM, pH 7.5), 0.2 ml each of magnesium sulphate (50mM), potassium chloride (50mM), sodium chloride (600mM), EDTA (1mM), and ATP (40mM)

was incubated at 37 °C for 15 min followed by the addition of enzyme source (haemolymph), which was left to incubate for another 30min. Reaction was arrested by adding 1.0 ml of 10% cold TCA. The reaction mixture treated similarly was taken as the blank, but TCA was added in the beginning itself. The reaction mixture was centrifuged and the supernatant was estimated for the phosphorous content. The amount of phosphorous liberated was estimated according to the method of Fiske and Subbarow (1925). The enzyme activity was expressed as µmoles of phosphorous liberated/min/mg protein under incubation conditions.

Two way Analysis of Variance (ANOVA) was carried out to find out significance at $P < 0.05$, and summary of data represented as mean and standard error.

3.3.3 Results

Copper and mercury showed a similar pattern in Na^+ /K^+ ATPase activity reflecting the sensitivity of the enzyme towards heavy metals.

On the 1st day of exposure there was a hike in haemolymph ATPase activity in copper exposed *P. viridis* compared to the control values. Reduced enzyme activity observed on the 7th day further decreased on the 15th day.

Specimens of *Perna viridis* exposed to mercury, on 1st day showed increased haemolymph ATPase activity when compared to the control values. On the 7th and 15th day observation, ATPase activity was further inhibited. Na^+/ K^+ ATPase activity was almost completely inhibited by the end of the whole experiment (Table. 3.10 and Figure.3.7 & 3.8).

On statistical analysis all the values were found to be significant at $P < 0.05$ probability level (Table.3.11, 3.12, 3.16 & 3.17).

3.3.4 Discussion

The Na^+ /K^+ ATPase channel is probably the most well studied ion pump, which is electrogenic, that it transports $3Na^+$ out and $2K^+$ in across a membrane for each ATP molecule that is hydrolyzed. Results from the present study showed that Na^+/ K^+ ATPase activity was generally inhibited by copper and mercury on prolonged exposures, though there was an initial enhancement in activity on the 1st day of observation. This observation supports the previous study conducted by Atli and Canli (2007) in copper exposed *Oreochromis niloticus*. The inhibition of ATPase activity can be related to the levels of metal ions to which the organisms were exposed. The decrease in enzyme activity could be related to the high affinity of metals to -SH groups on the enzyme molecule, membrane rupture or disturbance of the ion homeostasis. Metals may alter the enzyme activity or function in several ways. They can bind to a number of sites on proteins including imidazole, histidyl, carboxyl, and especially sulphydryl side chains (Verma et al.1983). Metals may also bring about changes in concentration of cofactors or reactants by altering membrane permeability, including that of mitochondria, again indirectly affecting enzyme activity. The initial enhancement of Na^+ / K^+- ATPase activity could possibly be due to an adaptation period dependent upon the continuing metal effect or maintenance of the ion flux. Ay et al. (1999) reported a decrease in the branchial Na^+/ K^+-ATPase activity in Tilapia *zillii* after 14 days of exposure to copper and lead suggesting that metals can alter the enzyme activity by binding to a number of sites on proteins, especially on sulphydryl groups that cause conformational

changes. A decrease in activity was observed in the present investigation also, when *Perna viridis* was subjected to copper stress for a period of 15 days.

The Na^+/K^+-ATPase activity measured in the present study was 0.023 µmolPi/mgprotein/min, while Rebelo, et.al. (1999) observed an activity of 0.053µmolPi/mgprotein/min in the homogenate of outer mantle epithelium of *Anodonta cygnea.* The difference observed can be due to the change in the tissue studied. Kamunde and Wood, (2003) explained tissue-specific differences in Na^+/K^+-ATPase enzyme kinetics. Rameshthangam et al., (2006) found highest Na^+ K^+ ATPase activities in healthy prawn tissues. In tune with this, the present observation reveals that metal exposure for 7 and 14days showed a drastic decrease compared to control values.

Higher Na^+/ K^+-ATPase has more potent Na^+K^+ pump activity; consistent with high tissue concentrations of inorganic phosphorus and high-energy charge values. High-energy charge favours Na^+/ K^+- ATPase activity (Wang et al., 2003). The present study has shown a substantial decrease in the activity of membrane bound ATPases on exposure to copper and mercury indicating that energy production is apparently very low during heavy metal stress, disrupting active transport of ions and osmo effector exchanges. The fatty acid composition of cellular membranes can modify permeability (Bell et al., 1986; Haines, 1994; Porter et al., 1996), and can modulate the activity of Na^+/K^+-ATPase and other enzymes embedded in the membranes (Gibbs, 1998; Turner et al., 2003).

3.3.5 Conclusion

ATPase is an integral part of the membrane and when its activity gets altered, movement of substance by active transport would be blocked, disrupting the functions of the organs where ATPase has been inhibited. The results of the present study indicate that inhibition of haemolymph Na^+/K^+-ATPase activity occurs following copper and mercury exposure. Inhibiton of this ion transport enzyme in haemolymph/haemocytes definitely disrupts osmoregulation of haemocytes, which play a pivotal role in immune defense, failing or weakening them to survive in nature, which is a 'cocktail' of pollutants. Results of the present investigation highlight the importance of addressing the sensitivity of these enzymes when they are used as a bioindicators of metal contamination.

Table.3.10 Haemolymph Na^+/K^+ ATPase activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	0.029± 0.005	0.062± 0.003	0.067± 0.018	0.050± 0.001
	7th day	0.030± 0.004	0.019± 0.006	0.017± 0.002	0.009± 0.001
	15th day	0.025± 0.005	0.011± 0.002	0.008± 0.003	0.011± 0.002
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	0.028± 0.003	0.091± 0.017	1.138± 0.322	0.064± 0.002
	7th day	0.028± 0.005	0.037± 0.007	0.011± 0.003	0.021± 0.001
	15th day	0.027± 0.003	0.015± 0.003	0.009± 0.002	0.008± 0.003

Table.3.11 ANOVA Table for Haemolymph Na^+/K^+ATPase activity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.021	2	.010	254.643	.000
Concentration	.001	3	.000	5.806	.001
Day * Concentration	.007	6	.001	28.707	.000
Error	.002	60	4.08E-005		
Total	.088	72			

Table.3.12 ANOVA Table for Haemolymph Na^+/K^+ATPase

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	1.550	2	.775	89.260	.000
Concentration	1.664	3	.555	63.901	.000
Day * Concentration	3.571	6	.595	68.560	.000
Error	.521	60	.009		
Total	8.395	72			

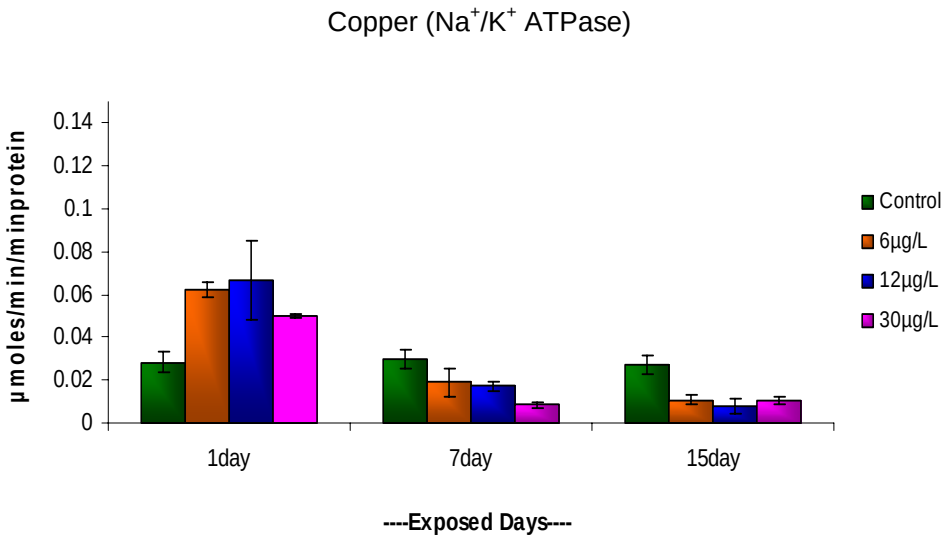


Fig. 3.7. Haemolymph Na^+/K^+ ATPase activity in copper exposed *P.viridis*

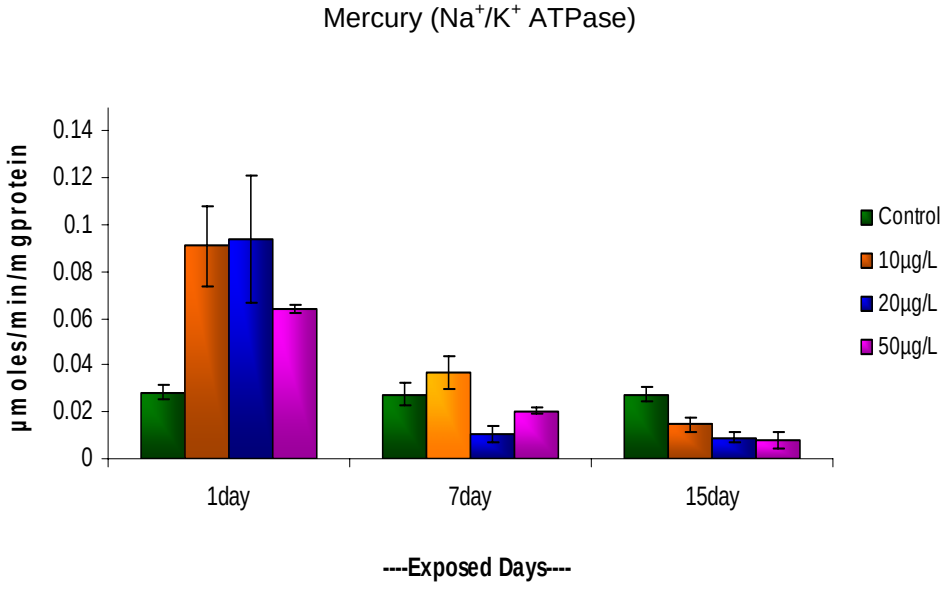


Fig. 3.8. Haemolymph Na^+/K^+ ATPase activity in Mercury exposed *P.viridis*

D. Ca^{2+} ATPase

3.4 Introduction

Ca^{2+} ATPase, a membrane bound enzyme, which is ubiquitous in animal cell, plays a significant role in the homeostasis mechanism of calcium. Transient elevations of cytosolic Ca^{2+} levels are an important component of cell signaling, whereas sustained Ca^{2+} increases are not tolerated by cells and generally lead to apoptosis or cytotoxicity and necrosis. The cytosolic concentration of free Ca^{2+} is maintained at low levels (10^{-7} M) by extrusion and compartmentalization systems.An important component of the extrusion process is the plasma membrane Ca^{2+}-ATPase pump showing high Ca^{2+} affinity.

It has been shown that heavy metal ions can alter Ca homeostasis in a number of cell types (Abramson et al., 1983; Nathanson et al., 1995; Viarengo et al., 1996; Marchi et al., 2000), thereby leading to the disruption of cell Ca^{2+} regulation (Viarengo and Nicotera, 1991; Viarengo, 1994). These findings are relevant for environmental studies on the effects of heavy metals in living organisms, and the use of Ca^{2+}-ATPase as biomarkers. *P. viridis* is a preferential model organism for investigations on biomarkers of stress and their application in ecological risk assessment (Bayne et al., 1979). In addition, mussels have been also used in studies on cellular calcium. It has been demonstrated that heavy metals are able to inhibit in vitro the Ca^{2+}-ATPase activity present in purified plasma membranes obtained from mussel gills cells.

3.4.1 Review of literature

Ca^{2+}-ATPase pump, the main component of Ca^{2+} extrusion process is localized at sarcoplasmic reticulum tubules and it removes Ca ions from the cytoplasm for preserving the low Ca levels in the cell (Watson

and Beamish, 1981; Saxena et al., 2000) thus playing a significant role in the maintenance of the physiological Ca^{2+} level in the cytoplasm.

Occurrence and relative activities of Ca stimulated ATPase in different species of molluscs have been reported by Viarengo et al. (1993), and Da Silva et al., (2002). Burlando et al. (2004) reported inhibition and stimulation of enzyme activities by Cu^{2+} and Hg^{2+} , respectively, in the gills of *Mytilus galloprovincialis.* Heavy metal toxicity in Ca homeostasis has been shown by a number of cells types (Abramson et al., 1983; Nathanson et al., 1995; Viarengo et al., 1996; Marchi et al., 2000), thereby leading to the disruption of cell Ca^{2+} regulation (Viarengo and Nicotera, 1991). Na^{+}/K^{+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities have been reported in many crustaceans. They are used as carrier enzymes in ionic exchange mechanisms (Towle et al., 1976). *Oreochromis niloticus* exposed to copper showed an increased muscle Ca^{2+}-ATPase activity (Atli and Canli 2007).

3.4.2 Materials and methods

Ca^{2+}-ATPase activity was estimated following the method of Bonting (1970). To the reaction mixture containing 1.0 ml of Tris-HCl buffer (0.125M, pH 8) 0.1 ml of $CaCl_2$ (50mM), 0.1ml of ATP(10mM) and 0.1ml of distilled water, 0.1ml of enzyme source (haemolymph) was added, and the mixture was left to incubate at 37^{o}C for 15 minutes. The reaction was arrested by the addition of 1.0 ml of 10% cold TCA. The blank used was similar but the TCA was added in the beginning itself. Reaction mixture was centrifuged and the supernatant was taken for determining the phosphorous content. The amount of phosphorous liberated was estimated according to the method of Fiske and Subbarow (1925). The enzyme activity was expressed as µmoles of phosphorous liberated/min/mg protein under incubation conditions.

Statistical analysis

The two way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons was checked at probability level ($P< 0.05$). Summary of data as mean and standard errors is represented.

3.4.3 Result

Results showed a minute increase in Ca^{2+}-ATPase activity on the first day of exposure in the lowest concentration of copper (6µg/L), which increased drastically by 7th day followed by a sharp decline in activity. On exposure to 12 µg/L concentration of copper, Ca^{2+}-ATPase activity decreased compared to the control level on 1st and 15th day of treatment, while it showed an increased activity on the7th day of observation. In mussels exposed to the highest concentration of copper, a slight initial decrease (1st day) in Ca^{2+}-ATPase activity was noted, which declined further (7th day), and then slightly increased (15th day), which was still well below the control levels.

Mussels exposed to mercury showed more inhibitory effect on enzyme activity. Those animals exposed to 10 and 20 µg/L of mercury had elevated enzyme activities on the 7th day of observation. A slight inhibitory effect shown on the 1st day for 10 and 20 µg/L concentrations declined profusely on the 15th day. After an initial increase on the first day, Ca^{2+}-ATPase activity showed a sharp decline by the 15th day in mussels exposed to the highest concentration of 50 µg/L (Table. 3.13, Figure 3.9 &3.10).

Statistical analysis showed that al data obtained were significant at $P<0.05$ probablity level (Table 3.14 to 3.17).

3.4.4 Discussion

Inhibitory effects of mercury and copper on continued exposure are clear from the data presented. This must have resulted from the breakdown of the active transport mechanism depending upon the altered membrane permeability and also due to the disturbed Ca homeostasis. Slight disturbances of Ca^{2+} homeostasis, like those deriving from low-dose heavy metal contamination, can affect the cellular ability to maintain and modulate Ca^{2+} signaling over a period of time. Though there was rise and fall in Ca^{2+}-ATPase activity on short period of exposure (1^{st} and 7^{th} days), inhibition was well marked on extended exposure to 15 days. Increase in inhibition was proportional to days of exposure in mussels exposed at 50 µg/L of mercury. The toxic effect of copper and mercury can be explained as follows. Hg^{2+} and Cu^{2+} act by preventing the formation of the phosphorylated intermediate, an essential step in the process of Ca^{2+} transport (Viarengo et al., 1993, 1996). Hg^{2+} shows a greater effect than Cu^{2+}, but the inhibition induced by copper is enhanced in the presence of ascorbic acid, suggesting different mechanisms of action for the two metal ions. The toxicity of mercury is generally ascribed to its high affinity for nucleophilic groups such as sulfhydryls. Copper also shows an affinity for SH groups (Viarengo and Nott, 1993), but its toxicity is thought to be primarily related to redox cycling properties (Stohs and Bagchi, 1995; Viarengo et al., 2002).

At the highest concentration of both metals, mussels exposed to mercury after an initial increase, had decreasing enzyme activity. Copper exposed mussels showed inhibitory effect from day 1 to day 15. Mercury had the least observed activity of 0.005 (± 0.001) µmP/min/mg protein, whereas in copper exposed mussels activity was not as low as that of

mercury exposed ones. This gives a picture of the magnitude of stress of both the metals tested. As these concentrations, 30 µg/L, 50µg/L, are likely to occur in natural conditions, whichever animal species comes across, the observations made can be imperative. Results show that at this concentration of copper, mussel is likely to adapt in natural condition, while the activity range of 50 µg/L mercury, can harm animal beyond recovery.

Burlando et al. (2004) reported that plasma membrane Ca^{2+}-ATPase activity in mussel digestive gland on 4 days of consistent exposure to copper proved that Cu^{2+} is inhibitory, whereas Hg^{2+} stimulated the plasma membrane Ca^{2+}-ATPase activity (PMCA). Inhibition of PMCA on Hg^{2+} treatment was reported by Viarengo et al., (1993). When living cells were exposed for minutes to Hg^{2+}, an activatory effect on Ca^{2+}-ATPase was observed (Burlando et al., 2003a,). In the present study, concentration of mercury that was inhibitory on 1st day showed a stimulatory effect by 7th day.

Healthy prawn tissues showed higher Ca^{2+}-ATPase activity than that of WSSV infected prawn (Rameshthangam et al., 2006). Biochemical data concerning the in vitro effect of Cu^{2+} on mussel PMCA showed a maximum inhibitory effect on the fourth day (Viarengo et al., 1993). In this study inhibition due to copper for short duration (1st Day) was observed in 12 and 30 µg/L of copper, which increased in 12 µg/L by 7th day. Ca^{2+}-ATPase activity in the muscle tissue of *Oreochromis niloticus* was reported to increase on Cu exposure (Atli and Canli 2007). The redox cycling of copper metal might have produced toxic hydroxyl radical causing enzyme damage resulting in weak enzyme activity on the 15th day of exposure.

3.4.5 Conclusion

Ca^{2+}-ATPase is indispensable for calcium homeostasis in cell. Calcium ions are important for maintaining homeostasis of haemocytes, and its increase lead to apoptosis or cytotoxicity and necrosis. Overall study points out on the degrading health of green mussel *P.viridis* exposed to copper and mercury for more than 15 days due to disruption in Ca^{2+}-ATPase pump.

Table. 3.13 Haemolymph Ca^{2+} ATPase activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	0.016± 0.001	0.016± 0.002	0.008± 0.002	0.014± 0.004
	7th day	0.016± 0.001	0.042± 0.001	0.018± 0.001	0.007± 0.002
	15th day	0.016± 0.001	0.011± 0.003	0.010± 0.002	0.011± 0.001
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	0.016± 0.001	0.014± 0.004	0.013± 0.002	0.016± 0.006
	7th day	0.016± 0.001	0.025± 0.005	0.017± 0.007	0.013± 0.001
	15th day	0.016± 0.001	0.010± 0.002	0.010± 0.004	0.006± 0.001

Table.3.14 ANOVA Table for Ca^{2+} ATPase activity in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.001	2	.001	161.908	.000
Concentration	.002	3	.001	157.483	.000
Day * Concentration	.003	6	.000	137.531	.000
Error	.000	60	3.34E-006		
Total	.023	72			

Table.3.15 ANOVA Table for Ca^{2+} ATPase activity in mercury exposed *P. viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.001	2	.001	161.908	.000
Concentration	.002	3	.001	157.483	.000
Day * Concentration	.003	6	.000	137.531	.000
Error	.000	60	3.34E-006		
Total	.023	72			

Table.3.16 Multiple Comparison Test for Copper

		ALP	ACP	AChE	Na^+/K^+ ATPase	Ca^{2+} ATPase
Tukey	**Control Vs 6 μg/L**	0.996	0.997	0.000	0.436	0.000
	Control Vs 12μg/L	0.548	0.128	0.001	0.498	0.000
	Control Vs 30 μg/L	0.006	0.690	0.000	0.152	0.000
	10 ppm Vs. 12 μg/L	0.684	0.188	0.953	1.000	0.000
	10 ppm Vs. 30 μg/L	0.011	0.570	0.357	0.003	0.000
	20 ppm Vs. 30 μg/L	0.167	0.008	0.141	0.004	0.083
		ALP	**ACP**	**AChE**	**Na^+/K^+ ATPase**	**Ca^{2+}A TPase**
Tukey	**1 day Vs 7 day**	0.058	0.000	0.000	0.000	0.000
	1 day Vs 15 day	0.000	0.000	0.000	0.000	0.003
	7 day Vs 15 day	0.000	0.000	0.007	0.021	0.000

Table.3.17 Multiple Comparison Test for Mercury

		ALP	**ACP**	**AChE**	**Na^+/K^+ ATPase**	**Ca^{2+} ATPase**
Tukey	**Control Vs 10μg/L**	0.985	0.016	0.000	0.917	0.962
	Control Vs 20μg/L	0.857	0.226	0.000	0.000	0.274
	Control Vs 50 μg/L	0.729	0.492	0.996	1.000	0.012
	10 ppm Vs. 20 μg/L	0.664	0.662	0.000	0.000	0.108
	10 ppm Vs. 50 μg/L	0.513	0.355	0.000	0.950	0.003
	20 ppm Vs. 50 μg/L	0.995	0.956	0.000	0.000	0.522
		ALP	**ACP**	**AChE**	**Na^+/K^+ ATPase**	**Ca^{2+} ATPase**
Tukey	**1 day Vs 7 day**	0.000	0.000	0.000	0.000	0.017
	1 day Vs 15 day	0.000	0.000	0.000	0.000	0.000
	7 day Vs 15 day	0.000	0.000	0.040	0.940	0.000

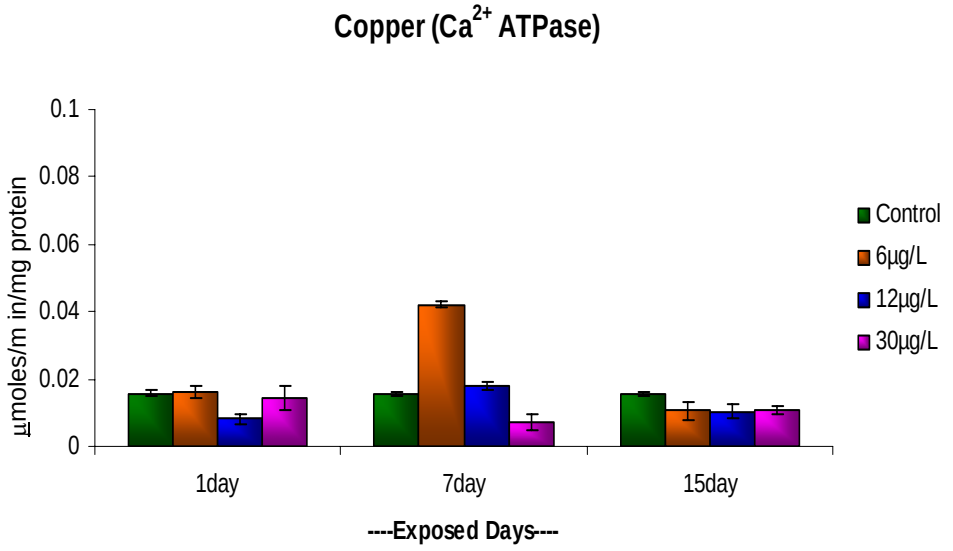


Fig. 3.9. Haemolymph Ca^{2+}ATPase activity in copper exposed *P.viridis*

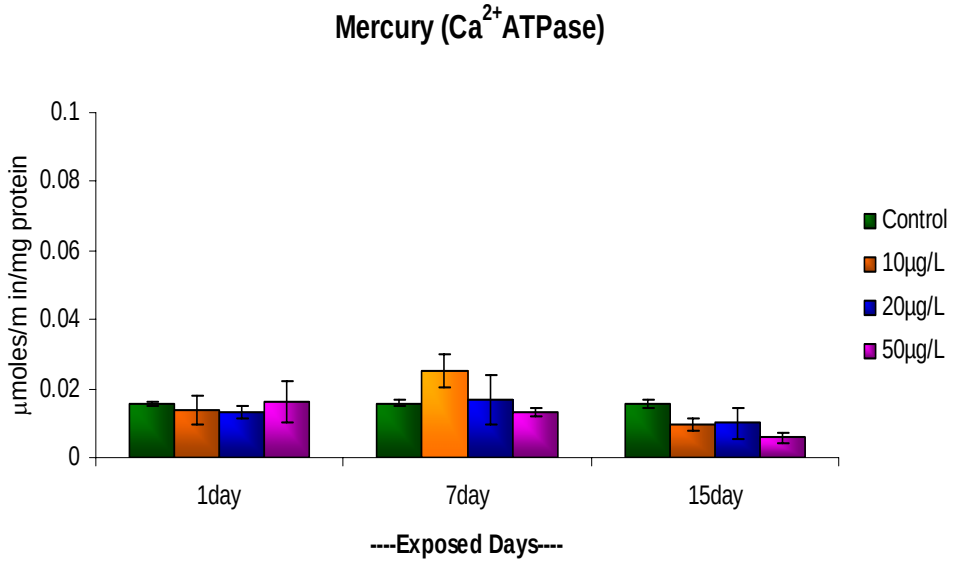


Fig. 3.10. Haemolymph Ca^{2+} ATPase activity in Mercury exposed *P.viridis*

Chapter 4 Metals induced changes in the Haemolymph

A. Superoxide anion production

4.1 Introduction

Molluscs have an innate defense system consisting of both cellular and humoral factors. Phagocytosis, which represents an important means to eliminate foreign particles, has been well described and documented in invertebrate. The blood cells or haemocytes, of bivalve molluscs play a prominent role in the defence against potential pathogens and xenobiotics. Immune function is largely affected by phagocytic haemocytes, and is complemented by an array of detoxifying mechanisms, which include release of degradative and oxidative enzymes, and the generation of highly reactive oxygen and nitrogen species (Wootton and Pipe 2003; Goodall et al., 2004). The production of reactive oxygen species (ROS) by phagocytic haemocytes of bivalves is believed to accompany the release of degradative enzymes for the destruction of foreign material (Pipe, 1992; Noel et al., 1993).

Cellular defense reaction by the production of reactive oxygen metabolites is immunologically vital and has been well characterized in molluscs (Adema et al., 1991; Arumugam et al., 2000). The oxygen dependent defence mechanism consists in the generation of reactive oxygen intermediates (ROIs) with powerful microbicidal activity. An important mechanism for cell killing is the production of reactive oxygen species (ROS). Following haemocyte stimulation with the contact of any foreign element, an increase in oxygen consumption (respiratory burst) is

observed. This is due to an increasing activity of membrane bound NADPH oxidase complex that assemble at either the plasma or granule membranes, and generate H_2O_2 in response to xenobiotics, which catalyses the monovalent reduction of the molecular oxygen, thus providing a variety of cytotoxic ROS. The killing of the invading microorganisms and pathogens occurs by inflicting extensive damage to membranes, DNA and proteins Thannickal and Fanburg, (2000). Since the excess of ROS is dangerous also for host cells and tissues, aerobic organisms have developed antioxidant systems to protect them from oxidative stress damage. Among these antioxidants superoxide dismutase is the first and the most important defense line.

4.1.1 Review of literature

NADPH-oxidase driven superoxide anion generation produces toxic metabolites that are capable of destroying invasive pathogens. In invertebrates, this microbicidal system has been first demonstrated in gastropods (Dikkeboom et al., 1988). It was followed by several others like Larson et al., (1989) in oyster, *C.* v*irginica*, and in several marine bivalves by Bachere et al., (1991a) with special attention to the interaction of the oxidative metabolism with specific intracellular parasites. Changes in the levels of generation of superoxide anion caused by contaminant exposure have been well documented in invertebrates (Adema et al., 1991; Coles et al., 1994, 1995; Pipe and Coles 1995; Cajaraville et al., 1996; Dyrynda et al. 1998; Arumugam et al., 2000; Wootton et al., 2003)

Alteration of ROS production by the haemocytes of bivalves submitted to xenobiotics has been reported in the oyster *C. virginica* (Anderson, 1994), in the mussels *M. galloprovincialis* (Carballal et al., 1997*a*; Pipe et al., 1995) and *M. edulis* (Winston *et al*.,1996) and in *P.viridis* (Thiagarajan et al., 2006)

The intracellular release of superoxide by haemocytes was stimulated in *Vibrio* challenged mussels with no copper pre exposure but was significantly reduced in mussels pre-exposed to 0.2 ppm of copper for 7 weeks. The generation of superoxide radicals for the killing of invading pathogens has also been demonstrated in some bivalves, such as the mussels *M. edulis* (Noël *et al*., 1991; Pipe, 1992) and the oyster *C. virginica* (Fisher *et al*., 1996). However, Nakayama and Maruyama (1998) reported that the haemocytes of the clam *T. crocea* did not produce superoxide anion contrary to the haemocytes of the oyster *C. gigas* and the mussel *Fulvia mutica*(*F.mutica*) In a comparative study *M. edulis* haemocytes were more active in superoxide generation than haemocytes of *C. edule* and *E. siliqua*. The exposure of marine molluscs to Cu can induce oxidative stress through the formation of reactive oxygen species (Viarengo et al., 1990; Isani et al., 2003). Intracellular superoxide anion (O^{2-}) production was the highest in shrimp that were fed with diets supplemented with copper (Lee et al., 2002)

4.1.2 Materials and Method

The maintenance of the target animal, and extraction procedure of haemolymph were the same as explained in detail in Chapter 1. Determination of Respiratory burst activity/super oxide anion production was carried out following the method of Cheng et al. (2000).

About 0.1ml of haemolymph was incubated with NBT (0.2%) for 30 minutes at 10°C. It was then centrifuged at 1200 rpm for 10 minutes at 4°C. To the pellet, 0.1ml of 100% methanol was added and left for incubation for 10 minutes. It was centrifuged again at 3000rpm. The supernatant was removed and the formazan dye formed was dissolved in 120µl of 2M KOH and 140 µl DMSO and diluted with 2ml of water. The

blue color obtained was measured at 620 nm in a UV-Visible HITACHI-U-2001 spectrophotometer

Statistical analysis

Results are presented as means and standard errors. Data obtained were subjected to Analysis of variance (ANOVA) with a Tukey post hoc test. For all statistical tests, results were concluded as significant with a probability (*P*) value of < 0.05.

4.1.3 Result

The reduction of NBT to formazan by the haemocytes of *Perna viridis* suggested the production of superoxide anion by the green mussels when exposed to copper and mercury. Copper- exposed mussel had a decreased superoxide anion production on the first day when compared to controls, which then increased on the 7^{th} and 15^{th} day of observation. In those exposed to mercury, the release of reactive oxygen metabolites from haemocytes decreased well below the control values, irrespective of days and concentrations (Table.4.1, Figure 4.1, 4.2)

Statistical analysis showed all these alterations were found to be significant at P< 0.05 (Table 4.2, 4.3, 4.19, 4.20).

4.1.4 Discussion

In recent years there has been increasing interest in environmental modulation of the release of reactive oxygen intermediates. NADPH-oxidase driven superoxide anion generation produces toxic metabolites that are capable of destroying invasive pathogens. This cellular defense reaction is immunologically vital and has been well characterized in molluscs (Adema et al., 1991; Arumugam et al., 2000). In the present study, mercury had a slightly stronger inhibitory effect on the generation

of ROS by haemocytes, compared to copper. Although both metals had a similar inhibitory effect on the first day of exposure when compared to the controls, O_2 generation increased from that of first day to values that were similar to those of the control on the 7th and 15^{th} days of observation in copper treated mussels. This implicates the ability of mussel to adapt to the heavy metal copper.

Decrease of intra-cellular O_2 generation on exposure to mercury on all days, and on the first day of copper exposure shows that intracellular generation is susceptible to these metals. Hg^{2+} is a toxic metal for oyster haemocytes (Cheng and Sullivan, 1984). Reduction in O_2 generation due to metal exposure (Anderson et al., 1992; Coles et al., 1994), could be attributed to direct or indirect interactions of metals with cytoskeleton (Gomez-Mendikute et al., 2002; Gomez-Mendikute and Cajaraville, 2003). Perhaps, disruption of cytoskeleton proteins could in turn affect the assembly of NADPH-oxidase complex on the plasma membrane, and this could result in non-activation of the complex (Thiagarajan et al., 2006). Such an alteration in haemocyte activation and subsequent inhibition of O_2 generation could cause susceptibility of mussel to metal toxicity over prolonged periods of exposure, and more importantly increased generation of ROS from metabolism that reflects oxidative stress.

The levels of superoxide anion production were strikingly higher than the levels in the control mussels exposed to 12µg/L copper on the 15^{th} day of exposures with a value of 0.107 ±0.011 U/mg protein, but for mercury exposed specimens the activity decreased to almost half to that of the control mussels. The probable reason can be the property of copper as an effective catalyst of many oxidation and reduction reactions and so the significant alterations in NBT reduction could be a direct effect of the elevated copper

levels in the tissues due to prolonged exposure. NBT reduction assay is easy to perform and the formaozan is produced quickly even when low aliquots of haemolymph are taken. NBT reduction uses haemolymph as such helping the measurement of superoxide anion from living cell, as no harsh treatment like centrifugation or ultrsonication is employed that causes cell mortality.

4.1.5 Conclusion

From the results obtained NBT reduction assay appears to be a simple and reliable method for rapid quantification of intracellular superoxide anion production in *P.viridis*. From the results it is clear that mercury disrupts NADP-H respiratory complex leading to the inhibition of enzyme activity in mussels, while copper ions cause increased superoxide anion only on prolonged exposure. The mussels can adapt to copper exposure for a short duration while mercury exposed *P. viridis* has decreased ROS production, which point out to the failure of defense mechanism.

Table 4.1 Haemolymph Superoxide anion production in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	0.063± 0.0014	0.040± 0.0043	0.033± 0.0042	0.025± 0.0023
	7st day	0.061± 0.0033	0.068± 0.0043	0.078± 0.0055	0.053± 0.0022
	15st day	0.060± 0.0023	0.072± 0.0024	0.107± 0.0109	0.067± 0.0013
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	0.063± 0.0017	0.045± 0.0022	0.037± 0.0042	0.042± 0.0013
	7th day	0.063± 0.0013	0.037± 0.0026	0.042± 0.0008	0.035± 0.0039
	15th day	0.062± 0.0020	0.043± 0.0027	0.036± 0.0028	0.033± 0.0025

Table 4.2 ANOVA Table for Superoxide anion production (Copper)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.017	2	.008	428.811	.000
Concentration	.005	3	.002	88.759	.000
Day * Concentration	.009	6	.002	79.988	.000
Error	.001	60	1.96E-005		
Total	.296	72			

Table 4.3 ANOVA Table for Superoxide anion production (Mercury)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.000	2	7.56E-005	11.620	.000
Concentration	.008	3	.003	404.085	.000
Day * Concentration	.001	6	8.52E-005	13.101	.000
Error	.000	60	6.51E-006		
Total	.153	72			

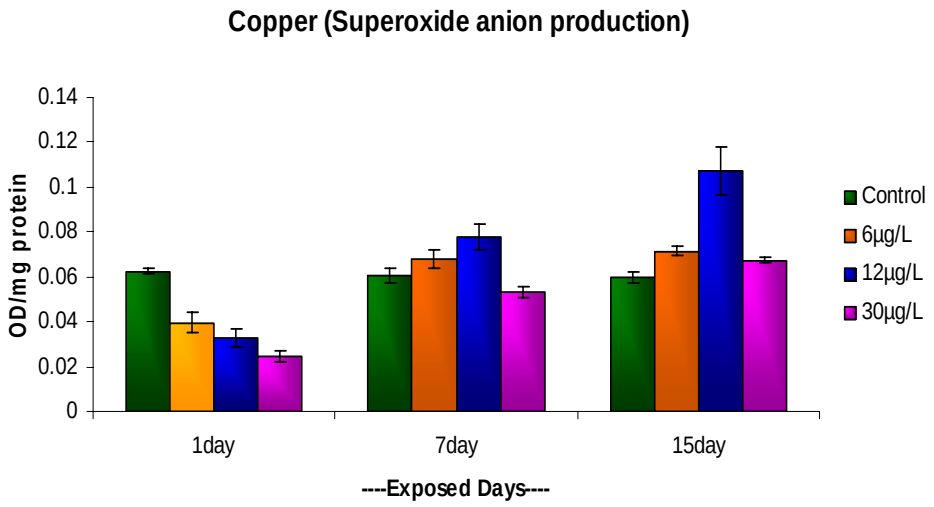


Fig. 4.1 Haemolymph Superoxide anion production in copper exposed *P.viridis*

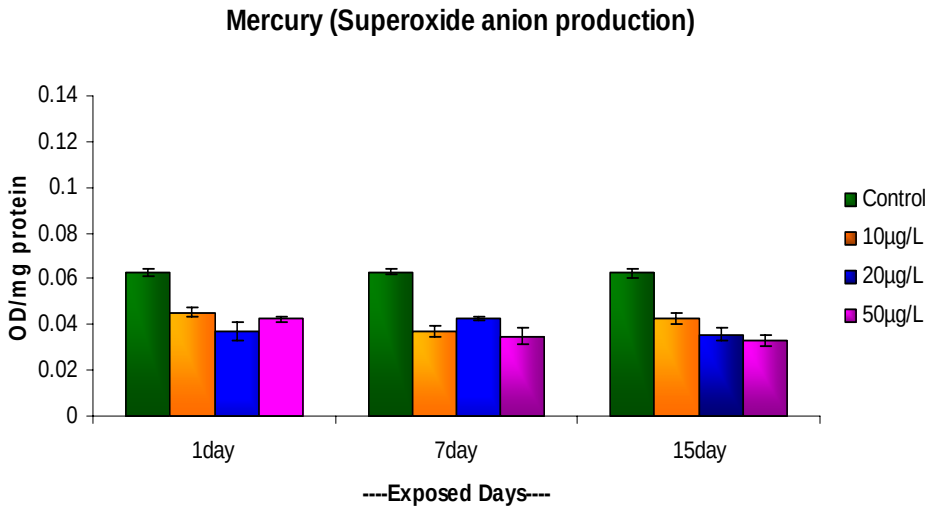


Fig. 4.2 Haemolymph Superoxide anionproduction in mercury exposed *P.viridis*

B. Antioxidant activities of selected haemolymph enzymes

4.2 Introduction

All aerobic organisms generate free radicals such as reactive oxygen species (ROS) in the process of their oxidative metabolism (Felton, 1995; Felton and Summers, 1995; Michiels et al., 1994; Pardini, 1995).These reactive oxygen species include the superoxide anion, $O_2•^-$, the hydroperoxyl radical, $HO_2•$, hydrogen peroxide, H_2O_2, and the hydroxyl radical, OH•, all intermediates in the reduction of O_2 to H_2O and derived from enzymatic reactions, and auto oxidation of redox-active chemicals occurring in living cells. These free radicals are highly reactive unstable molecules that have an unpaired electron in their outer shell. They react with (oxidize) various cellular components including DNA, proteins, lipids and fatty acids. These reactions between cellular components and free radicals lead to DNA damage, mitochondrial malfunction and cell membrane damage. These destructive reactions contribute to the processes of aging, carcinogenesis and cell death. Free radical formation and the effect of these toxic molecules on cell function (which can result in cell death) are collectively called “oxidative stress”.

Antioxidants are molecules or compounds that act as free radical scavengers. Most antioxidants are electron donors and react with the free radicals to form innocuous end products such as water. These antioxidants bind and inactivate the free radicals. Thus, antioxidants protect against oxidative stress and prevent damage to cells. By definition oxidative stress results when formation of pro oxidants (free radicals) is unbalanced by similar rate of consumption by antioxidants.

Oxidative stress to marine organisms, including various invertebrate species, has been causally associated with natural abiotic factors and

chemical contamination from anthropogenic sources. Environmental pollutants, including trace metals and organic compounds, are known to enhance the formation of ROS and variations in the endogenous levels of antioxidants in marine organisms have been proposed as useful biomarkers in monitoring studies (Winston and Di Giulio, 1991).

To protect against the effects of "oxidative stress", organisms have a variety of detoxifying enzymes at their disposal, such as superoxide dismutase, catalase, glutathione *S*-transferase, glutathione peroxidase and glutathione reductase.

Superoxide dismutase (SOD) (SOD; E.C.1.15.1.1) constitutes the first most important defense line. These virtually exist in all organisms, and its major function is to scavenge superoxide anion. SOD catalyses the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (McCord and Fridovich, 1969).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

SOD is a metalloenzyme characterized by redox active metals at the catalytic sites, and at least three different cellular types of SOD have been described: a Cu/Zn-SOD (SOD1) localized in cytosol, nucleus and peroxisomes (Fridovich, 1995), a mitochondrial Mn-SOD (SOD2) and a Fe-SOD inhibited by H_2O_2 which is eliminated by the action of catalase (Aebi, 1984), and glutathione peroxidase .

Catalase (CAT; EC 1.11.1.6) is a commonly studied antioxidant enzyme involved in the initial antioxidative mechanism and widely used as a biomarker in mussel (Cajaraville et al., 2000; Khessiba et al., 2001; Nasci et al., 2002; Lau and Wong, 2003; Rome`o et al., 2003). Moreover, high catalase activity is found in invertebrates, confirming its important

role in antioxidant defense in aquatic invertebrates (Livingstone et al., 1992). It reduces H_2O_2, produced by the superoxide dismutase enzyme (SOD), to water and oxygen.

$2H_2O_2 \rightarrow O_2 + 2H_2O$

$2GSH + 2\ H_2O_2 \rightarrow GSSG + 2H_2O$ (catalyzed by glutathione peroxidase).

Glutathione-S-Transferase (GST; EC 2.5.1.18) is involved in the detoxification of a wide variety of toxic compounds by conjugating them to glutathione and thus facilitating their removal from the organism. Some GSTs also have glutathione peroxidase activity, thus providing an oxygen-detoxification function i.e., reduction of lipid peroxides. This activity is particularly important in invertebrates because they are deficient in the other (vertebrate-type, selenium-dependent) glutathione peroxidases .

Lipid peroxidation (LPO) is a well-known mechanism of cellular injury in vertebrates and invertebrates. Malonaldehyde (MDA) is a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis, which is mutagenic and carcinogenic. It reacts with DNA to form DNA adducts (Marnett, 1999). Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation. MDA can react readily with amino groups on proteins and other molecule to form a variety of adducts.

4.2.1 Review of literature

Antioxidants represent the cellular defense mechanisms, which counteract toxicity of reactive oxygen species (ROS), and these mechanisms have been extensively investigated in sentinel organisms

such as marine mussels (Winston et al., 1990; Viarengo et al., 1991a, b; Livingstone et al., 1992; Regoli, 1998; Camus et al., 2004). Responsiveness of antioxidants to pollutants is difficult to predict and a high degree of variability has been reported as a function of class of chemicals, kind of exposure, and phase of the biological cycle (Livingstone, 2001). Antioxidants as potential indicators of oxidative stress in marine organisms is suggested by Verlecar et al. (2008). Laboratory and field studies have indicated that variations in the levels or activities of antioxidants are potential biomarkers revealing a contaminant-mediated biological effect on the organisms (Porte et al., 1991; Ribera et al., 1991; Livingstone, 1991; Regoli and Principato, 1995; Sole` et al., 1996; Camus et al., 2004). Numerous components of the detoxification and antioxidant system in mollusc species have been shown to be specifically induced by metals or PAH in controlled laboratory conditions (Regoli and Principato, 1995. Environmental factors were found to play a crucial role in regulating the oxidative stress capacity of tissues of *P. viridis* (Verlecar et al., 2008).

SOD, has been reported to correlate well with immune competence of molluscs (Liu et al., 2004). As a free radical elimination enzyme, SOD is essential to minimize the oxidative damage to host cells in the immune defense (Zhang et al., 2005). Study of Geret et al. (2002) indicates that Cu induces an imbalance in the oxygen metabolism during the first week of exposure due to a decrease in mitochondrial SOD and CAT, selenium dependent, and total glutathione peroxidase activities. Superoxide dismutase and Se-dependent glutathione peroxidase are two main antioxidants in organisms (Orbea et al., 2000; Dautremepuits et al., 2004; Chandran et al., 2005).Catalase (CAT) is a commonly studied antioxidant enzyme involved in the initial anti-oxidative mechanism and widely used

as a biomarker in mussel (Cajaraville et al., 2000; Khessiba et al., 2001; Nasci et al., 2002; Lau and Wong, 2003; Rome`o et al., 2003). Almeida et al. (2003) studied on the levels of lipid peroxidation in digestive gland, mantle tissue and gills of the mussel from the contaminated site. Alteration in antioxidant system due to copper stress was investigated by (Isani et al., 2003;Das et al., 2004; Rajalakshmi and Mohandas, 2005; Munoz et al., 2006). Significant reductions in the activities of antioxidants along with increased lipid peroxidation in WSSV-infected shrimp confirm increased oxidative stress (Mohanakumar and Ramasamy, 2006). Increased lipid peroxidation was observed when the clam *R. decussatus* and mussel *M. galloprovincialis* were exposed to Cu (Geret, et. al., 2002; Romeo and Gnassia-Barelli, 1997; Viarengo et al., 1990). Lipid peroxidation is considered a biomarker of damage (Geffard 2001). Variations in SOD, CAT, GPx and MDA activities suggest their potential use as biomarkers of effects, such as oxidative stress, resulting from Cd contamination in the mollusc *R. decussates* (Geret, et. al., 2002)

4.2.2 Materials and Methods

Estimation of superoxide dismutase (SOD) The maintenance of the target animal, and extraction procedure of haemolymph were the as explained in details in Chapter 1

Superoxide dismutase activity in the haemolymph/haemocyte was determined following the method of Kakkar et al. (1984)

Haemolymph sample was extracted and diluted as explained in chapter1. The extracted haemolymph was centrifuged at 700rpm, and the cell- free haemolymph was used to analyse SOD activity. The assay mixture contained 1.2ml of 0.052M sodium pyrophosphate buffer (pH 8.3), 0.1ml of 186 µM phenazine methosulphate, 0.3ml of 300µM

nitroblue tetrazolium, and 1.3ml of distilled water and 0.1 ml of enzyme source (haemolymph). The tubes were kept at 30^0C for one minute and then 0.2 ml of 780µM NADH was added and incubated at 30^0C for 90 seconds. The reaction was stopped by the addition of 1ml of glacial acetic acid. Reaction mixture was shaken vigorously with 4ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. The upper butanol layer was removed. Absorbance of the chromogen in butanol was measured at 560nm against n-butanol blank UV- Vis HITACHI-U-2001 spectrophotometer. A system devoid of enzyme served as control. One Unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in one minute, under the assay conditions, and specific activity is expressed as units/mg protein.

Estimation of Catalase (CAT) Catalase activity was determined following the method of Maehly and Chance (1955).

The activity was estimated spectrophotometrically following a decrease in absorbance at 230 nm. The reaction mixture contained 0.01M phosphate buffer (pH 7), 30µM H_2O_2 and enzyme source (Haemolymph). Specific activity was expressed as International units/mg protein.1U=change in absorbance / min / extinction coefficient (0.021). Protein was estimated following the method of Lowry et al. (1951).

Estimation of glutathione peroxidase (GPx)

Glutathione peroxidase was estimated following the method of Rotruck (1973).

To 0.2ml of 0.4M tris buffer (pH- 7), 0.2ml 0.4mM EDTA, 0.1ml 10mMsodium azide and 0.5 ml enzyme source (haemolymph) were added and mixed well. To this mixture, 0.2ml of 2mM GSH followed by

0.1ml of 0.2mM H_2O_2 solution was added. The contents were mixed well and incubated at 37^0C for 10 minutes along with a control containing all reagents except haemolymph. After 10 minutes, the reaction was arrested by the addition of 1.5 ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH. Values were expressed as µg of GSH/min/mg protein. Protein was estimated following the method of Lowry, et al. (1951).

Estimation of Glutathione S-transferase (GST)

Glutathione S- transferase was estimated following the method of Habig et.al., 1974.

The reaction mixture containing 200µl phosphate buffer (pH 6.5) , 20 µl 1-chloro-2,4-dinitrobenzene (CDNB) in 95% ethanol, and 730 µl distilled water was taken in the control tube, and 200 µl 0.5 M phosphate buffer,20 µl 25mM CDNB, and 680 µl distilled water were taken in the test sample tubes. Tubes were incubated at 37^0C for 10 minutes. After the incubation, added 50 µl of 20mM GSH in both sets of tubes. After through mixing, added 50 µl of haemolymph in the test sample tubes. Increase in absorbance was noted at 340nm for 5 minutes using an UV-Visible spectrophotometer (HITACHI-U-2001). Values are expressed in n moles of CDNB conjugated/min/mg protein. Extinction coefficient between CDNB-GSH conjugate and CDNB is 9.6mM-1cm-1. Protein was estimated by the method of Lowry et al. (1951).

Estimation of Lipid peroxidation (LPO)

Malonaldehyde content was estimated following the method of Niehaus and Samuelson (1968).

Haemolymph was added to 15% thiobarbituric acid, mixed well and heated for 15 minutes in a boiling water bath. It was then cooled and centrifuged for 10 minutes at 600*g*. The absorbance was read at 535 nm, spectrophotometrically in a UV- Vis HITACHI-U-2001 spectrophotometer against a reagent blank that contained no haemolymph. The extinction coefficient of malonaldehyde is 1.56×10^5 M^{-1} cm^{-1}. Protein was estimated by the method of Lowry et al.(1951).

Statistical analysis

All data are presented as means and standard errors. Data were analyzed by two way analysis of variance and significance was concluded at $P < 0.05$ probability level. Multiple comparison was done by Tukeys test.

4.2.3 Results

SOD showed a significant increased activity with increasing concentrations of copper. Increase in activity was proportional to days of exposure as well, with highest SOD activity on the 15^{th} day. Mercury exposed mussel too exhibited the same trend with concentrations and exposure days (Table. 4.4, Figure 4.3, 4.4).

CAT activity remained slightly lower than that of the control mussels on the first day of observation in mussels exposed to 6 and 12 µg/L of copper. On the 7^{th} and 15^{th} days of observation, CAT activity showed dose and time dependent elevation.

Increased level of CAT activity was exhibited by *P.viridis* specimens exposed to mercury at concentrations 10 and 20µg/L on the first day , which increased on day 7. By the end of the experimental period, CAT activities in 10 µg/L and 20µg/L mercury exposed mussels were found decreased. The decreased enzyme levels still remained higher

than that of the control values. Green mussels exposed to the highest concentration of mercury had an inhibitory effect on CAT activity on the 1st and 15th days of exposure (Table. 4.7, Figure 4.5, 4.6).

Except for a marginal decrease at 6 µg/L Cu on the first day, GPx activity increased with concentration and period of exposure in organisms treated with copper. On analysis, mussels exposed to all concentrations of Hg exhibited concentration and time dependent increase in GPx activity (Table. 4.10, Figure 4.7, 4.8).

In copper treated *P.viridis*, GST activity was found to be higher for those mussels exposed to 6 and 12 µg/L copper. Mussels exposed to 30 µg/L showed an increased activity only on the 1st day of observation with activity decreasing below control level on the 7th and 15th day. Mercury treated *P.viridis* gave similar results but in this case GST activity decreased only on the15th day at 20 µg/L and 50 µg/L mercury exposed mussels (Table. 4.13 Figure 4.9, 4.10).

P.viridis exposed to copper showed interesting result for lipid peroxidation. The increased lipid peroxidation activities that occurred in the animals on the 1st and 7th day were seen to decrease. In mussels exposed to mercury, lipid peroxidation was observed to exhibit a time and concentration dependent increase. Mussels exposed to 50µg/L Hg had the highest MDA produced on the 15th day of observation, which is a chronic effect of mercury (Table, 4.16 Figure 4.11, 4.12)

The changes in activity for both the toxicants were found to be statistically significant at $P<0.05$ lelvel in terms of days and concentration for all the above parameters analysed (Table.4.5, 4.6, 4.8, 4.9, 4.11, 4.12, 4.14, 4.15, 4.17-4.20).

4.2.4 Discussion

Increased oxidative stress has been suggested to enhance the antioxidant enzymes activities in animals, as a protective action towards oxidative stress. Superoxide dismutase (SOD) is one of the main antioxidant defence enzymes generated in response to oxidative stress. It converts the highly toxic superoxide anions into hydrogen peroxide. In the present study, the activity of SOD was significantly increased in the haemolymph. On the15th day, *P.viridis* exposed to the highest concentration of mercury had a two-fold increase in SOD activity while copper exposed ones had 3.5 fold increase. It is interesting to note that at this time period the production of superoxide anion in Cu stressed clams was higher while in mercury exposed ones it was lower in all concentrations and time periods. Obviously, SOD could nullify the superoxide anion stress caused by mercury faster, while in cu stressed ones, higher production of superoxide anions is countered by higher production of SOD to neutralize the adverse effect. This explains the higher antioxidant activity in mussels exposed to heavy metals. Increased SOD activity on 15th day of 0.45mg/L Hg exposure was reported by Verlecar et al. (2008) indicating protective behaviour of the cell against super oxide radicals. Increase in SOD activity has been reported by Prakash and Rao (1995) in mussels exposed to Al, Pb and Cd.

Hydrogen peroxide is toxic to cells. CAT and GPX are the major primary antioxidant defense component that catalyses the decomposition of H_2O_2 which is produced by the action of superoxide dismutase to H_2O. Glutathione peroxidase catalyses the reduction of hydroperoxides, with the conversion of reduced glutathione (GSH) to glutathione disulfide (GSSG). At high H_2O_2 concentrations, organic peroxides are metabolized by Catalase. The inhibition of CAT activity at the highest concentration

of mercury by the 15th day confirms the presence of oxidative stress, and interestingly the reverse was true in the case of Cu exposure. This observation, i.e. decreased CAT activity on the 15th day, concurs with the observations of Regoli and Principato, (1995), that as the stress increases, an inhibition of enzyme activity such as CAT occurs. Reduced capability in neutralizing various ROS and simultaneous inhibition of CAT, characterizes an increased susceptibility to oxidative stress. The inhibition of CAT activity, especially for the highest concentration of mercury, can be correlated either due to an increase of the superoxide anion as suggested by Kono and Fridovich (1982) or to a decrease of NADPH, since this coenzyme is required for the full activity of CAT.

Geret et al. (2002) observed copper to have a significant inhibitory effect on the activity of CAT and glutathione peroxidase for the first day at concentration 25 µg/L. In the present study slightly reduced CAT and GPx activities were noted on the first day for lower copper concentrations. According to Geret (2002) this inhibition is Cu dependent. Copper is known to be involved in redox reactions (Fenton reactions), which result in the production of oxyradicals. Cu^{2+} ions also react with ROOH leading to the formation of ROO• and Cu^{+}. Cu^{+} can react with H_2O_2 leading to the formation of OH•. H_2O_2 is the substrate for CAT and glutathione peroxidase. However, Cu^{+} in the redox reaction use H_2O_2, which is no longer available for CAT and GPX. This could explain the decrease in the activity of these enzymes on the 1st day when exposed to copper. A similar variation in activity was observed in the gills of the freshwater bivalve *Unio tumidus* after 3 days of Cu exposure (30 µg/L) (Doyotte, et al., 1997). The increased CAT activities on the 7th and 15th days in all concentrations of Cu are to counter the increased activity of superoxide anions at these time periods.

GPx is inactivated with increased levels of hydrogen peroxide, which in turn is due to the lower activity of catalase . Though 50 µg/L mercury treated mussel had decreased CAT activity, their GPx activity did not decrease. It is interesting to note that the activity of superoxide anions also decreased at this concentration. Obviously there was no need for extra production and release of CAT, and hence decreased activity value. GPx activity was elevated as a response to chronic toxicity for both copper and mercury exposed *P.viridis.* Other studies in which increased CAT (Prakash and Rao, 1995) and GPX activities were reported are in the digestive gland of *P. viridis* in response to metals, seasonal changes in environmental parameters and pollutants (Verlecar et al., 2007, 2008).

Besides participating as a hydrogen donor in the glutathione peroxidation reaction, reduced glutathione has a direct antioxidant function by reaction with superoxide, hydroxyl radical and singlet oxygen, leading to the formation of oxidized glutathione, and in the present study, the activity of glutathione-*S*-transferase was also significantly increased when compared with the activity in the controls except for a few observation. Significant increase in GST activity as observed on the 1^{st} and 7^{th} day in copper and mercury exposed specimen suggests the protective action against reactive oxygen radicals.

An effective antioxidant control will end up with low MDA level and vice versa (Lau and Wong, 2003). CAT activity in mussels exposed to mercury at 50µg/L had a maximum inhibition and as a consequence they had the maximum lipid peroxidation. CAT activity was well evident in copper exposed mussel on chronic exposure of 15 days, and hence MDA produced was almost similar to that of control mussels or below. This shows animals can adapt themselves to copper stress, whereas

mercury is highly toxic and could not be regulated by mussels on prolonged exposure. Elevated SOD and CAT activities along with decreased lipid peroxidation prove mussels' efforts to curtail oxidative damage caused by copper. An enhanced level of thiobarbituric acid reactive substances (TBARS) was observed in the digestive gland of the mussel, *P.viridis* in response to mercury (Geret et al., 2002; Almeida et al., 2004; Verlecar 2008).

All marine organisms are known to contain high levels of polyunsaturated fatty acids, which are the substrates for lipid peroxidation, and the presence of antioxidants elevates the resistance of their cell membranes to oxidative stress. The present study has shown that the level of lipid peroxidation was significantly increased in mercury exposed *P.viridis* when compared with controls. This result is similar to those obtained in gills and digestive gland of *Mytilus edulis* on mercury exposure by Geret et al. (2002). Mercury have high affinities for glutathione(GSH), which is the primary intracellular antioxidant agent, and can bind and cause the irreversible excretion of GSH leading to depletion of GSH and increase of MDA.

In present study LPO was high on 1^{st} and 7^{th} day of copper exposure, which decreased by the 15^{th} day well below that of the control value. LPO in the gills of *Ruditapes decussatus* was stimulated after 3 days of exposure to 25 µg Cu L^{-1}, and at 28 th day for 0.5 and 2.5 µg Cu L^{-1} (Geret et al., 2002), An increase in basal peroxidation was observed in the gills of clams (*R. decussatus*) exposed to 30 µgCuL^{-1} for 9 days (Romeo and Gnassia-Barelli, 1997) in the gills of the mussel *M. galloprovincialis* after exposure to 40 µgCuL^{-1} for 6 days (Viarengo et al., 1990) . Cu ions can induce the production of

reactive oxygen species through a Fenton-like redox cycling mechanism (Halliwell and Gutteridge, 1984), and participate in the initiation and propagation of lipid peroxidation (Viarengo et al., 1990). Present study showed decreased MDA production by 15 days when exposed to copper. Similar decrease in MDA level was obtained in gills of *Mytilus edulis* on exposure to 40 µg/L^{-1} Cu for 21 days (Geret et al., 2002). The probable reason as cited by Geret et al., (2002) is the pairing of copper with metallothionein resulting in an intensification of antioxidant systems.

4.2.5 Conclusion

In the present study, significant differences have been recorded in the activities of antioxidant enzymes in the haemolymph of *P.viridis* exposed to copper and mercury as compared with the control mussels. This indicates that there is an increased level of oxidative stress due to the presence of heavy metals, and that an imbalance is generated between pro-oxidants and antioxidants. The study made in *P.viridis* haemolymph can help to understand mechanism through which metals exert their toxicity in organisms and hence the results can be used to explain the impact of heavy metal toxicity on organisms in fields. Copper exposed mussels are likely to adapt themselves even to the highest concentration of copper but mercury exposed mussels tend to move to degradation with its antioxidant system overwhelmed by lipid peroxidation, which can lead to cell damage beyond control, ultimately resulting in death.

This study puts forward certain interesting questions, which need to be addressed sooner or later. The sub-lethal concentrations of the two metals used to study the activity pattern of acid and alkaline phosphatases, superoxide ions, super oxide dismutase, catalase and

other antioxidant enzymes, were the same for all enzyme studied, so also the time- periods of exposure of the organism. While certain enzymes showed increased activities at certain time periods others showed decreased activities. At this stage it is difficult to ascertain if this bivalve species has functionally different sub-populations of granulocytes, and if so whether the different functions attributed to haemocytes in defence mechanism are being performed by different sub-populations of haemocytes as opined by Mohandas (1985) in his studies on granulocytes of *M.mercenaria*. It has to be ascertained, in that case, whether the different sub-populations perform different enzyme activities or whether all the enzyme activities are performed by all sub-populations of haemocytes with a 'switch on-switch off' mechanism. From the present study using two different metals as challenges, it is assumed that each metal evokes different stimuli in the sub-populations for the synthesis and release of enzymes into the haemolymph. The fluctuations in the activity levels of different enzymes on exposure of the organism to the two different metals make one assume that the granulocytes are functionally a hetergenous population with different sub-populations. Using the freshwater snail, *Lymnaea stagnalis*, Adema et al.(1994) confirmed the postulation of Mohandas (1985) about the existence of functionally different sub-population by demonstrating through separation of haemocytes by density gradient centrifugation that in *L.stagnalis* there are functionally different five sub-populations. So, it is possible that bivalves may also be having functionally different sub-populations.

Table 4.4 Haemolymph Superoxide dismutase activity in metals exposed *P.viridis*

TOXICANT	Exposed day	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	1st day	2.927± 0.0745	3.5033± 0.1046	4.187± 0.2066	5.437± 0.3314
	7th day	2.907± 0.0450	4.460± 0.2871	5.497± 0.2525	6.280± 0.0980
	15th day	2.940± 0.0335	6.773± 0.3652	8.840± 0.5413	10.390± 0.1457
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	1st day	2.930± 0.0486	3.810± 0.1270	3.980± 0.1268	4.330± 0.0808
	7th day	2.917± 0.0572	4.426± 0.2435	4.97± 0.1211	5.620± 0.1213
	15th day	2.907± 0.0450	4.390± 0.1511	5.810± 0.1231	6.197± 0.0889

Table 4.5 ANOVA Table for Superoxide dismutase activity in copper exposed *P. viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	135.870	2	67.935	1051.265	.000
Concentration	194.949	3	64.983	1005.582	.000
Day * Concentration	51.425	6	8.571	132.630	.000
Error	3.877	60	.065		
Total	2443.091	72			

Table 4.6 ANOVA Table for Superoxide dismutase activity in mercury exposed *P. viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	14.112	2	7.056	467.969	.000
Concentration	62.333	3	20.778	1378.027	.000
Day * Concentration	8.346	6	1.391	92.257	.000
Error	.905	60	.015		
Total	1452.643	72			

Table 4.7 Haemolymph Catalase activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6µg/L	12µg/L	30µg/L
COPPER	**1st day**	1.137± 0.0502	1.133± 0.0183	1.095± 0.1768	2.055± 0.0071
	7th day	1.116± 0.0373	2.645± 0.1202	2.840± 0.0141	3.415± 0.0212
	15th day	1.108± 0.0160	2.850± 0.0424	10.640± 0.0141	14.235± 0.0636
		Control	**10µg/L**	**20µg/L**	**50µg/L**
MERCURY	**1st day**	1.137± 0.0502	3.510± 0.0424	2.170± 0.0531	1.025± 0.0212
	7th day	1.128± 0.0476	4.623± 0.1524	5.255± 0.0925	2.807± 0.1232
	15th day	1.134± 0.0442	1.737± 0.1023	3.642± 0.1589	0.886± 0.0166

Table 4.8 ANOVA Table for Catalase activity (Copper)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	445.124	2	222.562	14075.558	.000
Concentration	325.838	3	108.613	6869.028	.000
Day * Concentration	386.188	6	64.365	4070.630	.000
Error	.949	60	.016		
Total	2125.630	72			

Table 4.9 ANOVA Table for Catalase activity in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	38.186	2	19.093	1844.013	.000
Concentration	84.991	3	28.330	2736.165	.000
Day * Concentration	29.353	6	4.892	472.481	.000
Error	.621	60	.010		
Total	576.901	72			

Table 4.10 Haemolymph Glutathione peroxidase activity metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	**1st day**	1.347± 0.0281	1.282± 0.0331	3.468± 0.0549	3.900± 0.0751
	7th day	1.348± 0.0299	1.797± 0.0459	4.135± 0.0907	3.848± 0.0313
	15th day	1.343± 0.0163	3.460± 0.0555	4.392± 0.0656	3.488± 0.0371
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	**1st day**	1.353± 0.0273	1.508± 0.0331	3.038± 0.0355	3.445± 0.0274
	7th day	1.335± 0.0235	1.728± 0.0407	3.235± 0.0207	3.815± 0.0524
	15th day	1.355± 0.0308	2.257± 0.0612	3.458± 0.0248	4.295± 0.0836

Table 4.11 ANOVA Table for Glutathione peroxidase activity in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	5.458	2	2.729	1030.866	.000
Concentration	86.900	3	28.967	10941.087	.000
Day * Concentration	13.425	6	2.237	845.133	.000
Error	.159	60	.003		
Total	677.444	72			

Table 4.12 ANOVA Table for Glutathione peroxidase activity in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	3.119	2	1.559	869.093	.000
Concentration	74.453	3	24.818	13831.251	.000
Day * Concentration	1.367	6	.228	127.007	.000
Error	.108	60	.002		
Total	554.076	72			

Table 4.13 Haemolymph glutathione-S-transferase activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	**1st day**	6.033± 0.175119	10.099± 0.2760	15.044± 0.8185	11.566± 0.9728
	7th day	6.017± 0.1941	13.825± 1.4563	12.524± 0.8718	6.090± 1.0643
	15th day	5.967± 0.2066	10.237± 0.0909	11.235± 1.5414	3.320± 0.4038
		Control	**10 µg/L**	**20 µg/L**	**50 µg/L**
MERCURY	**1st day**	6.033± 0.1506	10.240± 0.1928	17.120± 1.0726	13.868± 0.4500
	7th day	6.033± 0.1862	14.769± 0.9586	14.745± 0.2556	7.737± 0.3386
	15th day	5.983± 0.1329	11.021± 0.4233	3.314± 0.1689	3.150± 0.1217

Table 4.14 ANOVA Table for glutathione S transferase activity in copper exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	110.583	2	55.291	79.326	.000
Concentration	607.300	3	202.433	290.431	.000
Day * Concentration	199.313	6	33.219	47.659	.000
Error	41.821	60	.697		
Total	7226.127	72			

Table 4.15 ANOVA Table for glutathione S transferase activity in mercury exposed *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	23.784	2	11.892	1801.281	.000
Concentration	16.785	3	5.595	847.466	.000
Day * Concentration	13.635	6	2.272	344.214	.000
Error	.396	60	.007		
Total	511.587	72			

Table 4.16 Haemolymph Lipid Peroxidation activity in metals exposed *P.viridis*

TOXICANT	Exposed days	Control	6µg/L	12µg/L	30µg/L
COPPER	**1st day**	0.079± 0.001472	0.082± 0.000896	0.097± 0.003033	0.170± 0.001941
	7th day	0.081± 0.0020	0.133± 0.0027	0.144± 0.0018	0.142± 0.0043
	15th day	0.079± 0.0017	0.045± 0.0033	0.063± 0.0023	0.073± 0.0031
		Control	**10µg/L**	**20µg/L**	**50µg/L**
MERCURY	**1st day**	0.080± 0.0020	0.067± 0.0021	0.061± 0.0054	0.086± 0.0029
	7th day	0.0703± 0.0018	0.095± 0.0025	0.105± 0.0045	0.135± 0.0037
	15th day	0.079± 0.0014	0.141± 0.0024	0.155± 0.0035	0.200± 0.0059

Table 4.17 ANOVA Table for Lipid Peroxidation (Copper)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.045	2	.023	3541.200	.000
Concentration	.025	3	.008	1294.093	.000
Day * Concentration	.028	6	.005	727.087	.000
Error	.000	60	6.41E-006		
Total	.803	72			

Table 4.18 ANOVA Table for Lipid Peroxidation (Mercury)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day	.060	2	.030	2506.818	.000
Concentration	.034	3	.011	946.089	.000
Day * Concentration	.023	6	.004	316.839	.000
Error	.001	60	1.20E-005		
Total	.941	72			

Table 4.19 Multiple Comparison Test for Copper

		Super oxide anion production	SOD	CAT	GPX	GST	LPO
Tukey	**Control Vs 6 µg/L**	0.762	0.000	0.000	0.000	0.000	0.000
	Control Vs 12 µg/L	0.000	0.000	0.000	0.000	0.000	0.000
	Control Vs 30 µg/L	0.000	0.000	0.000	0.000	0.004	0.000
	6 ppm Vs. 12 µg/L	0.000	0.000	0.000	0.000	0.000	0.000
	6 ppm Vs. 30 µg/L	0.000	0.000	0.000	0.000	0.000	0.000
	12 ppm Vs. 30 µg/L	0.000	0.000	0.000	0.000	0.000	0.000
		Super oxide anion production	**SOD**	**CAT**	**GPX**	**GST**	**LPO**
Tukey	**1 day Vs 7 day**	.000	.000	0.000	0.000	0.000	0.000
	1 day Vs 15 day	.000	.000	0.000	0.000	0.000	0.000
	7 day Vs 15 day	.000	.000	0.000	0.000	0.000	0.000

Table 4.20 Multiple Comparison Test for Mercury

		Super oxide anion production	SOD	CAT	GPX	GST	LPO
Tukey	**Control Vs 10 µg g/L**	0.000	0.000	0.000	0.000	0.000	0.000
	Control Vs 20 µg g/L	0.000	0.000	0.000	0.000	0.000	0.000
	Control Vs 50 µg g/L	0.000	0.000	0.000	0.000	0.000	0.000
	10 ppm Vs. 20 µg g/L	0.001	0.000	0.000	0.000	0.905	0.000
	10 ppm Vs. 50 µg g/L	0.000	0.000	0.000	0.000	0.000	0.000
	20 ppm Vs. 50 µg g/L	0.404	0.000	0.000	0.000	0.000	0.000
		Super oxide anion production	**SOD**	**CAT**	**GPX**	**GST**	**LPO**
Tukey	**1 day Vs 7 day**	0.003	0.000	0.000	0.000	0.258	0.000
	1 day Vs 15 day	0.000	0.000	0.000	0.000	0.000	0.000
	7 day Vs 15 day	0.465	0.000	0.000	0.000	0.000	0.000

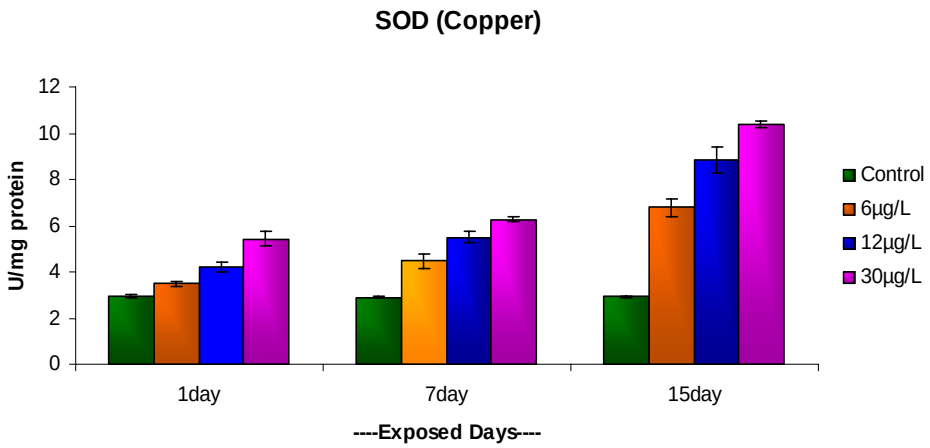


Fig.4.3 Superoxide dismutase activity in copper exposed *P.viridis*

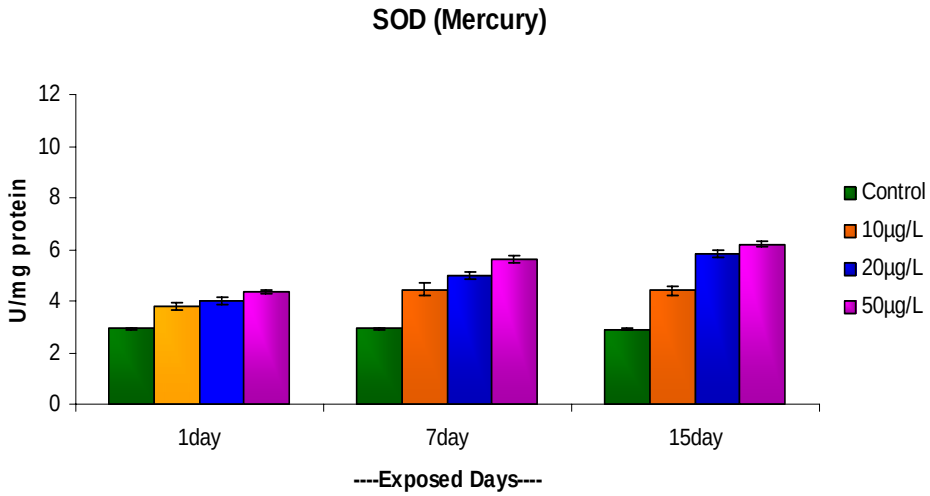


Fig. 4.4 Superoxide dismutase activity in mercury exposed *P.viridis*

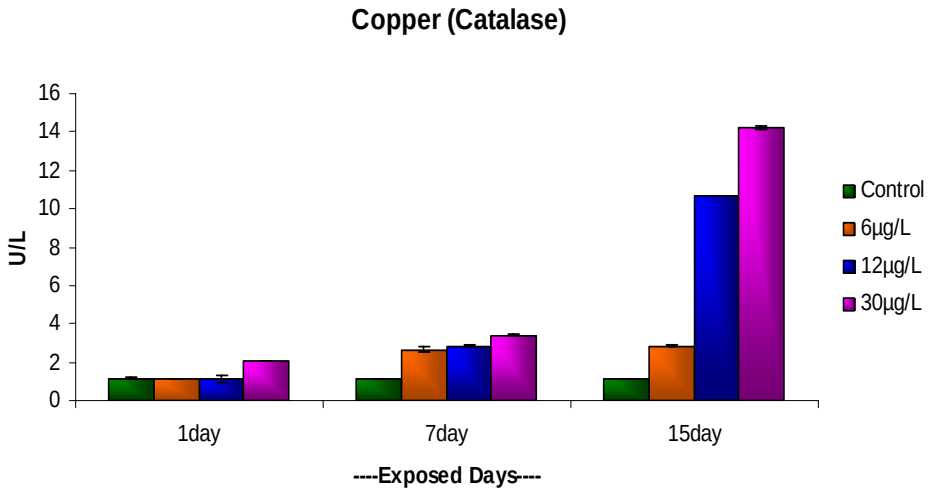


Fig. 4. 5 Catalase activity in copper exposed *P.viridis*

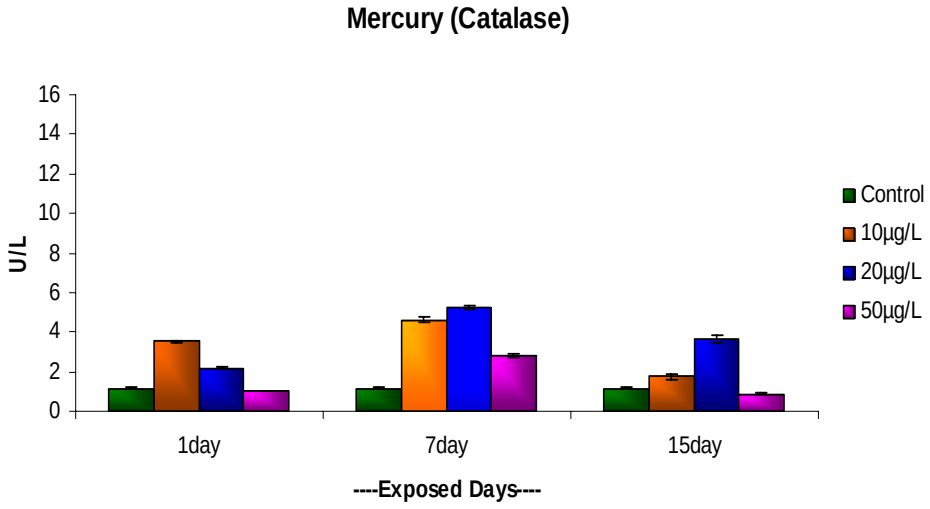


Fig. 4.6 Catalase activity in mercury exposed *P.viridis*

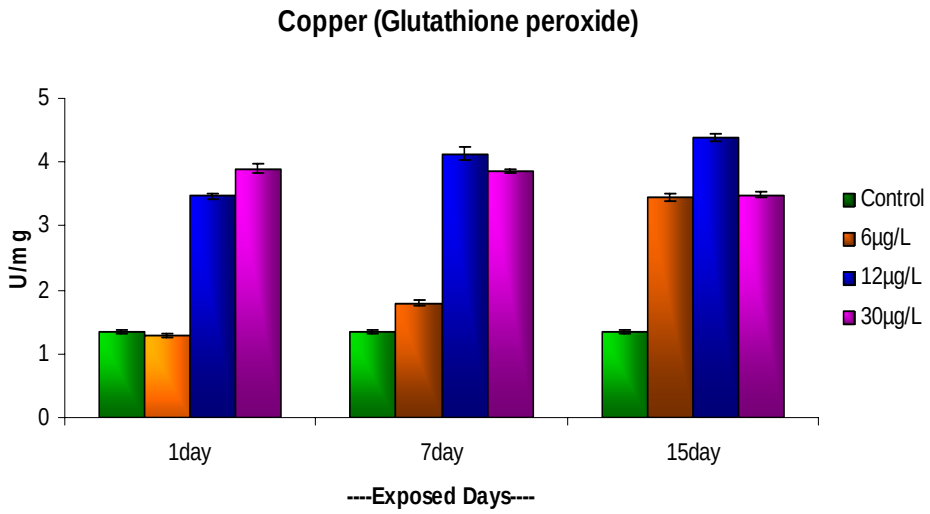


Fig. 4.7 Glutathione peroxidase activity in copper exposed *P.viridis*

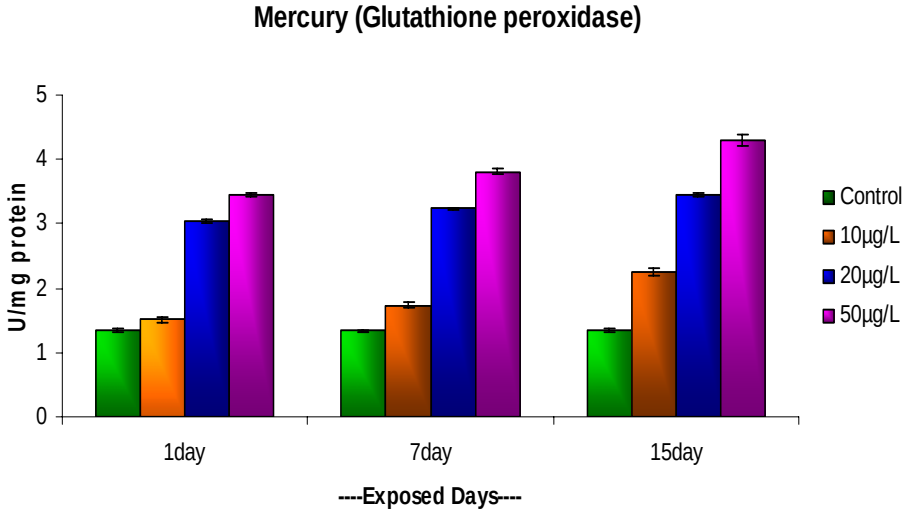


Fig. 4.8 Glutathione peroxidase activity in mercury exposed *P.viridis*

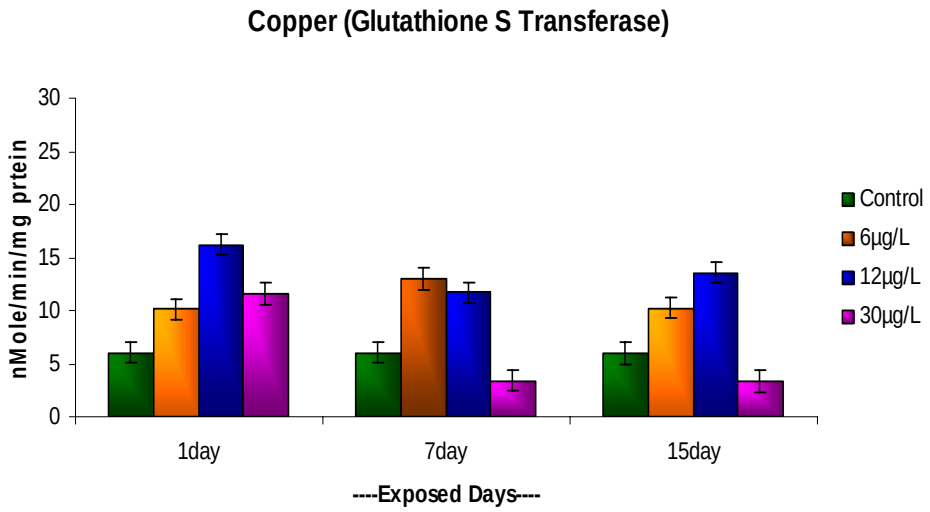


Fig. 4.9 Glutathione S Transferase activity in copper exposed *P.viridis*

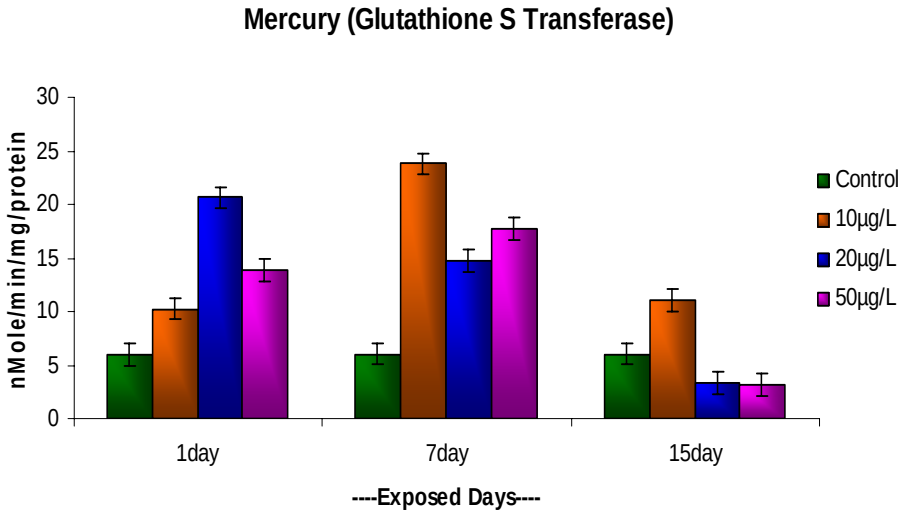


Fig. 4.10 Glutathione S Transferase activity in mercury exposed *P.viridis*

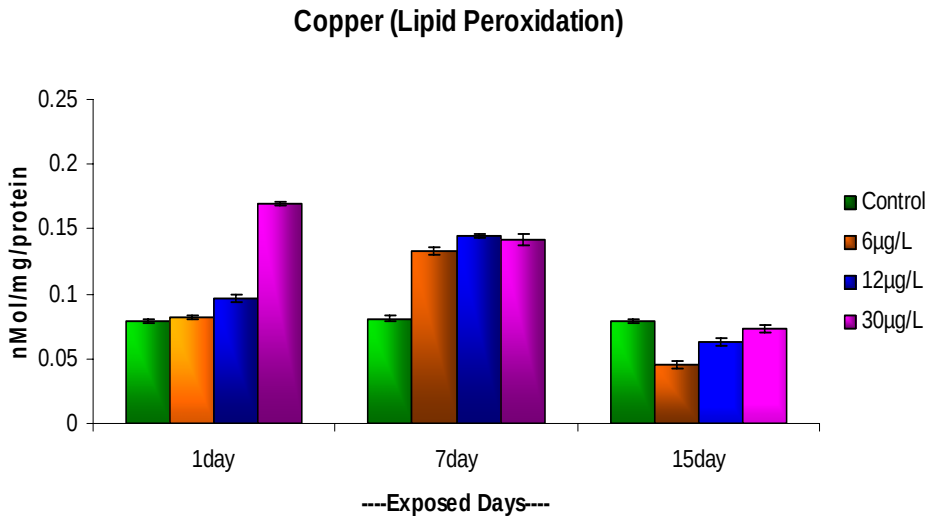


Fig. 4.11 Lipid peroxidation in copper exposed *P.viridis*

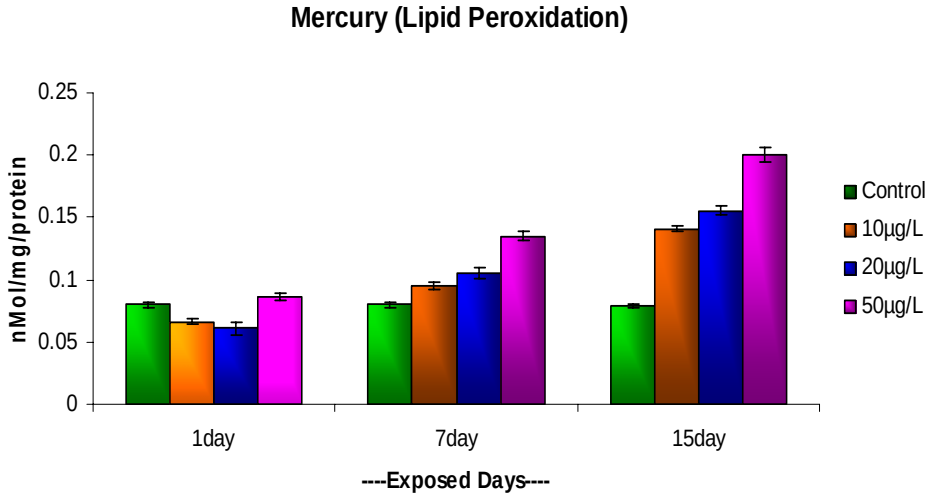


Fig. 4.12 Lipid peroxidation in mercury exposed *P.viridis*

Chapter 5 Histology

5.1 Introduction

There are various methods adopted to understand and explain the toxic effects in marine organisms. Examining the morphological changes and histological alterations are among the important ready reckoners adopted by researchers. The English microscopist Quekett in 1852 defined histology as the science of the microscopic anatomy of animals and plants. Animal histology deals with the minute structures of the tissues and organs of the animal body. Histology can well be correlated to physiology and biochemistry as a thorough investigation on any of these gives insight into the functioning of tissues and organs.

5.2 Review of Literature

With the "mussel watch" program, studies on the toxic effects of heavy metal to marine invertebrates have gained momentum. Heavy metal uptakes in the gills of different bivalve have been reported by Bebianno et al (1993), Jana and Das (1997), Holwerda et al. (1989) and Everats (1990). Pathology, as a standard part of environmental monitoring programmes on effects of pollution, was approved by Balouet and Poder (1981), Couch (1985), and Moore (1980). Histopathology of gill on exposure to copper and diesel oil was studied by Auffret (1988). .Heavy metal mediated changes in soft tissues, especially those associated with gill filament morphology were studied by Sunila(1986,1987,1988); and Gregory et al.,(1999) . Gómez-Mendikute et al. (2005) characterised mussel gill cells *in vivo* and *in vitro* using morphological, histochemical

and functional endpoints. In bivalves, such as green mussels, gill tissues are attractive models in ecotoxicological studies because gills are the first uptake site for many toxicants in the aquatic environment. Gill filaments with their large surface area are the main interface between the organism and its environment. Gills are thus continuously affected by exposure to pollutants.

There will be structural and functional alterations in individual cell types or groups of cells at an early stage of response before alteration in cellular structure could manifest at organismic level (Moore, 1980). In the present study gill tissues were analysed by histopathologic techniques to understand alterations and damages, if any, on exposure to two heavy metals, copper and mercury.

5.3 Materials and methods

Green mussels were exposed to 6, 12 and 30µg/L Copper, and 10, 20 and 50 µg/L mercury for 15 days. At the end of exposure period, gills were gently cut out and fixed in Davidson's fixative for 24 hours. (Davidson fixative composition- 95 % ethyl alcohol -350 ml, Formalin -200 ml, Glacial Acetic acid -115 ml and Distilled Water- 335 ml). Further processing of the fixed tissue involved the standard procedures of dehydration, clearing of tissue, infiltration with paraffin wax, embedding and sectioning of tissues. The procedure is as follows:

Dehydration

Dehydration of tissues was done with progressive higher grades of alcohol series as- (50%, 70 % and 90 % alcohol for 1h, and 100 % alcohol for 30min.

Clearing

For clearing, tissues were changed to Alcohol-Acetone solution (1:1) for 30 min, and then to acetone (45min – 1hour), followed by acetone- xylene (1:1) for 30 min, and to xylene alone (45min – 1hour).Tissues were then transferred to xylene: wax (30 min).

Infiltration and Embedding

Tissues were infiltered in 2-3 changes of molten paraffin of melting point 58-62^{0}C, and then embedded in wax at 58-60^{0}C, made into blocks which were labeled, and stored in polythene cover.

Sectioning

Paraffin blocks were trimmed to suitable size and sections of tissue were cut using a microtome at 5 - 7 µm thickness. The resulting ribbons containing tissue sections were fixed on to glass slides using Mayer's egg albumin glycerol (1.1v/v) as an adhesive.

Staining

Before the tissue sections are subjected to staining, the sections were deparaffinized thoroughly and hydrated. Slides were placed in xylene to de-paraffinise, and were giving brief dips in grades of alcohol (100%, 90%, 70% -20 minutes each).Following hydration slides were stained in hematoxylin (2-5 min). Harris' Hematoxylin was prepared using Hematoxylin Crystals-2.5 g, 100% Ethanol-25 ml, Ammonium or Potassium Alum -50 g, Distilled Water-500 ml, and Mercuric oxide (red)-1.25 g

Sections were de-stained in acid alcohol, and counter stained in Eosin (1min).(1% Stock Alcoholic Eosin had Eosin Y water soluble-1 g, Distilled Water-20 ml, Alcohol, 95%-80 ml).Slides were further dehydrated in alcohol and were then transferred to xylene (30min).Slides

were Mounted in DPX and labeled. Sections were observed and photographed under a light microscope (OLYMPUS BX41TF, Japan).

5.4 Result

By comparing the histological structure of the gills of the control and of metals- exposed mussels, the following observations were made.

Control: A healthy untreated gill showed two pairs of demibranchs that are suspended from the ctenidial axis that is fused along the dorsal margin of the mantle. Each arm of a demibranch is made up of ascending and descending lamellae, which in turn is made of many gill filaments. The neighbouring gill filaments were attached to one another through interlocking clumps of cilia providing gills a sheet like consistency, and each lamellae are joined with each other by interlamellar junction (Plate I).

Copper 6µg/L – Gill filament showed only a slight loss of cilia but the tip calyx was seen to be lost. Slight detachment and degeneration of epithelial cells were observed. Epithelial cells became more eosinophilic**,** swollen with increased inter spacing of lumen. Cytoplasm of epithelial cells became more granular.

Copper12µg/L ppm: Major alterations at this concentration were appearance of mucous cells, loss of cilia, desquamation of epithelial cells, enlargement and infiltration of haemocytes (not extensive). Cells had a vacuolated appearance and some cells, especially those at the base, were eosinophilic and granular. Adhesion between gill filaments was observed which might be due to hyperplasia.

Copper 30µg/L ppm: Gill Filament was highly elongated with a swollen lumen. Loss of original shape was obviously due to high

necrosis. Severe loss of epithelium could be observed. In spite of severe denudation, small tufts of cilia were seen on the lateral sides.

Mercury 10µg/L ppm - Loss of cilia were observed at many places. Sloughing of frontal cilia was evident but lateral cilia still seen to be present in a ruffled condition. Mucous cell formation, desquamations of cells, eosinophilic conversion of cells and appearance haemocytes in the slightly dilated lumen were other histological alterations.

Mercury 20µg/L ppm: The main pathological changes were loss of cilia at many places, elongation of gill filaments, complete sloughing of epithelial cells, eosinophilic conversion of cells, hyperchromatia of the nucleus, and haemocytic infiltration into the much dilated lumen. In short, mussel gills at this concentration depicted the same pathological condition as those seen in lowest selected concentration of 10µg/L of mercury but with more and severe denudation of cells.

Mercury 50µg/L: At this concentration, total loss of gill architecture was observed. Complete desquamation of epithelial cells with loss of cilia, dialation of gill tip and clubbing of cells were the other striking alterations brought about by mercuric exposure.

5.5 Discussion

A gill filament has three zones: Frontal, intermediate and abfrontal (Sunila, 1986, Gómez-Mendikute, 2005). Frontal cell possess ciliated cells on them.

Cilia on the gill filaments have specific arrangements and functions (Gosling, 2003).Frontal cilia, which are abundantly distributed on the free outer surface of the gill move particles along surface of the gill to the food grooves. Thus, the transport and uptake of nutrients and of

contaminants occur in the frontal zone of the gill filaments Owen, 1974, 1978; Owen and McCrae, 1976). Lateral cilia are set along the sides of the filament and these cilia are responsible for generating the feeding or respiratory current. Lying between frontal and lateral cilia is the eulatero-frontal cilia that flick particles from the water and convey them to the frontal cilia.

Loss of cilia was a general observation and the degree of loss was proportional to the concentration of heavy metal tested. The first cilia to be denuded are the frontal cilia. Due to denudation of cilia all the aforementioned functions such as intake of water, collection of food material and its transportation to palp are impaired. This can adversely affect the growth of mussel, an important aquaculture candidate (Qasim et al., 1977; Sivalingam, 1977; Sreenivasan et al., 1989) that fetches a good market value in shellfish economy. Brown and Newell (1972) observed reduced rates of oxygen consumption in solutions contaminated with copper and zinc and concluded that a reduced energy demand was caused by a suppression of ciliary activity rather than by a direct effect on respiratory enzymes. Even after a long term exposure of 15 days the lateral cilia were still seen to be present. Earlier studies by Gregory et al., (1999, 2000) on the morphology of *P. perna* gill filaments exposed to different concentrations of mercury showed alteration by 16 days that included loss of abfrontal cilia and a marked increase in the number of lateral cilia. Extended exposure to mercury may deleteriously affect respiration. The observed increase in lateral cilia may be a response by the animal to increase flow of oxygenated water over the epithelium thereby improving respiration. The “endothelial” cells are flattened non-ciliated cells in the intermediate zone of the gill filament in which gas exchange and interactions between the external medium and haemolymph

occur. The destruction of endothelial cells observed here can harm the well being of animals in natural conditions.

Toxicants come to contact with gills first, and animal responds by secreting excess quantities of mucus. The increased mucus production may serve as a protective barrier to pollutants, as a binding site to capture heavy metals before they can damage the tissues, or as a means of expelling pollutants absorbed by secretory and other cells. (Jose et.al., 2005). Appearance of mucous cell at lower concentration of both heavy metals can hence be considered as animal's effort to prevent toxicant entry. Excess mucus secretion and low byssus production were the immediate physiological alterations that were observed in heavy metal treated animals. Increased mucous secretion and its deposition on gills are known effects of heavy metal toxicity (Cusimano et al., 1986).

Gill damage will reduce the surface area leading to hypoxia under heavy metal stress. A drop in oxygen consumption also appears to be a protective mechanism to ensure that there is a low intake of toxicants. Hyperplasia was found to be a common response to irritant as a form of protection.So, by these alterations animals are trying to defend themselves and survive.

When specimens of *P.viridis* were exposed to copper and mercury, dilation of lumen occurred giving gill filament a swollen appearance. In addition the frontal cells were seen enlarged (hypertrophic) at the highest concentration of copper and mercury. Increased infiltration of haemocytes was observed in all group of treated mussel except in the 6µg/L copper- treated ones, the lowest copper concentration employed. Presence of large number of haemocytes in the gill indicates internal haemorrhage. Choi et al, (2003) suggested that enlargement of gill cells is

often accompanied by the presence of haemocytes, indicating tissue damage as well, mainly in the form of inflammation.

Detachment and desquamation of epithelial cells were common in all the samples with severity increasing proportional to concentration. This indicates epithelial cells to be a target site of copper and mercury toxicity. This finding is in concert with previous report that epithelial tissues are the major sites for metal accumulation in molluscan tissue (George et al.,1986; Marigomez et al.1990; Nigro et al. 1992; Abd Allah and Moustafa, 2002), and they are most susceptible for histological alterations resulting from the toxic effects of metals (Moore 1985). Choi et al., (2003) observed epithelial cell layers of gill to be detached from the remaining tissue parts of *L.elliptica* and Scanning Electron Micrographs showed that the detachment of the epithelial layers was largely due to shell shrinkage. Cell necrosis, rupture of cells, denudation of cilia, which were some of the other morphological alteration as a consequence of toxic insult. Gill epithelial cells play an important role in respiratory gas exchange and therefore, necrotic damage in the epithelium may result in serious dysfunction of the tissues, consequently leading to deleterious effects at higher biological organization levels.

5.6 Conclusion

Histology yields basic information on tissue disorders related to the general state of the animal. In the present investigation, the observed severe pathological changes in the gill tissue obviously reflect a poor health condition of mussels induced by prolonged exposure of copper and mercury. Furthermore, it confirms gills as the target tissue for toxicity of xenobiotics. It is concluded that though this type of histopathological analysis requires sacrificing of the target animal, is time consuming, and

requires great expertise in tissue sectioning and interpretation, standard histology provides useful and reliable information on the health of mussels.

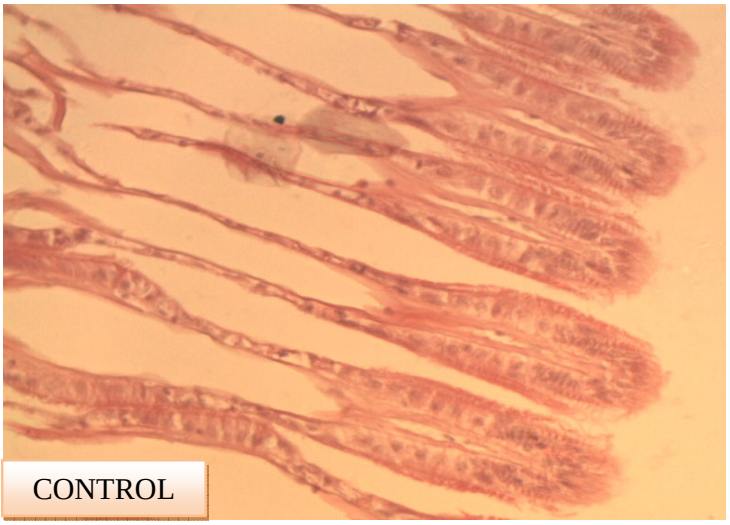


Plate I

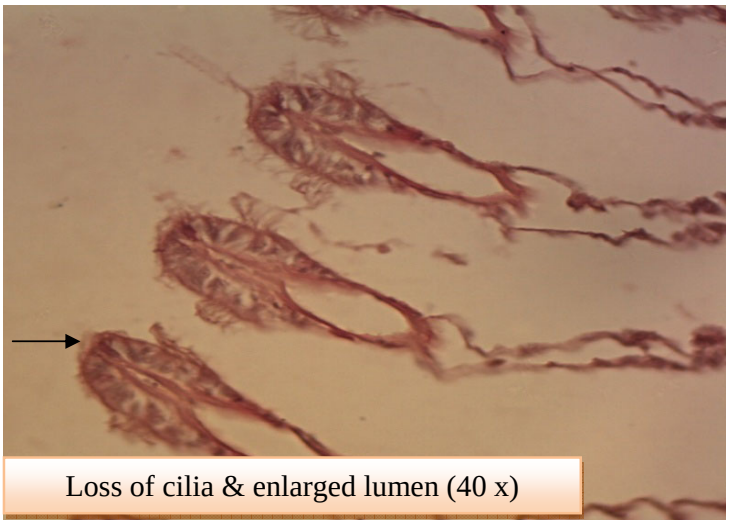
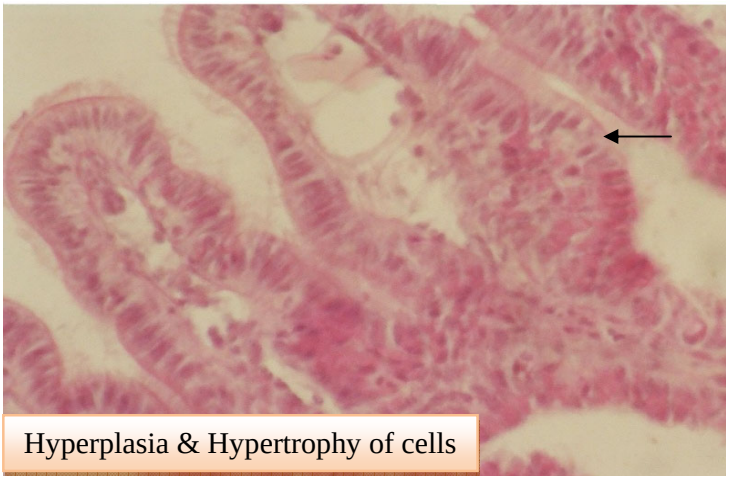
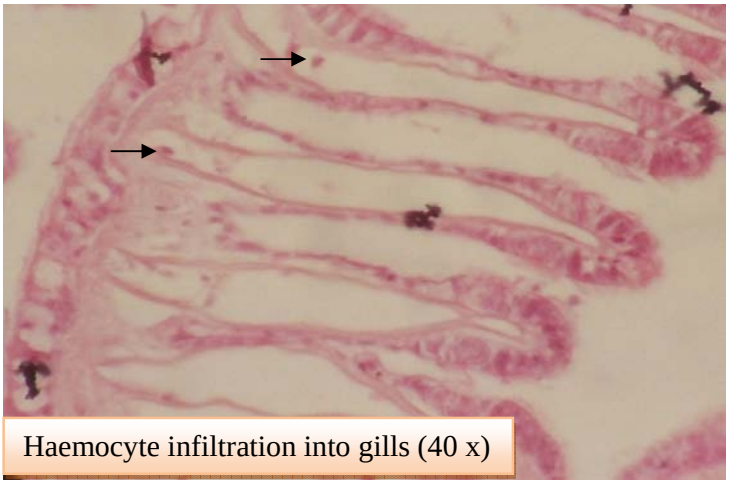


Plate II



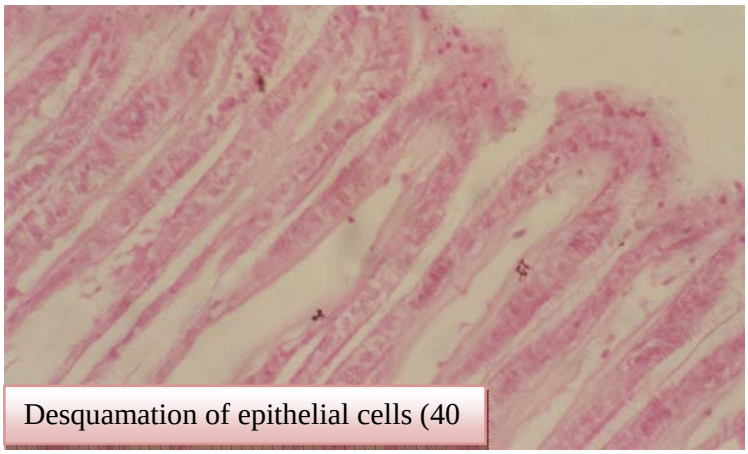
Hyperplasia & Hypertrophy of cells

Plate III



Haemocyte infiltration into gills (40 x)

Plate IV



Desquamation of epithelial cells (40

Plate V

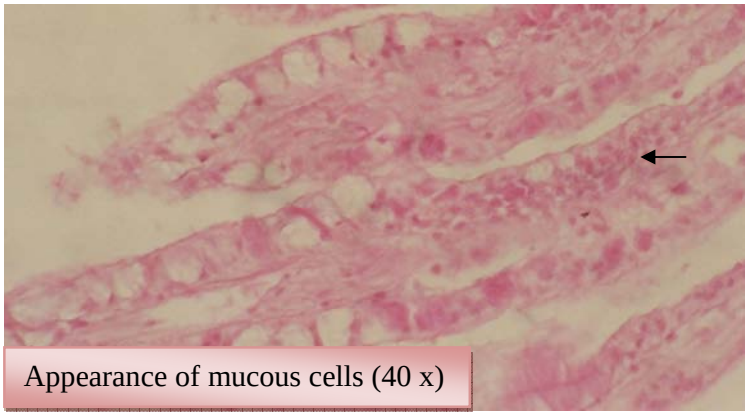


Plate VI

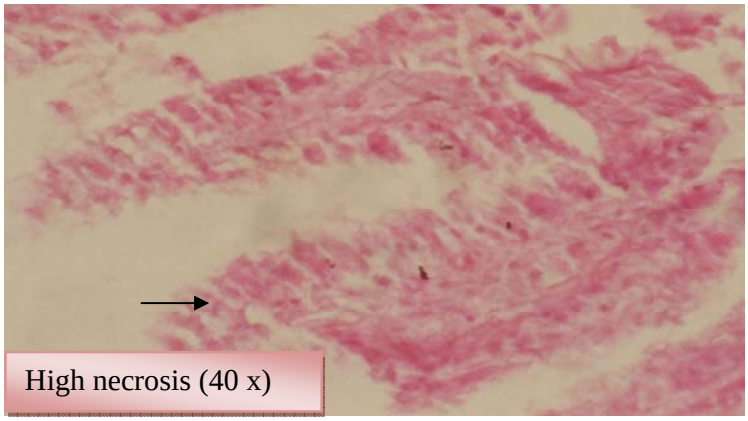


Plate VII

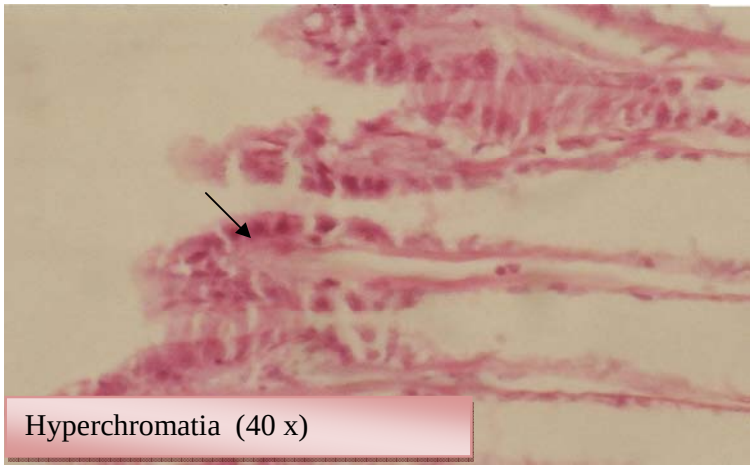


Plate VIII

Place I Histological section of gills of control P. viridis

Place II – VIII Pathological alteration in gills of *P. viridis* on exposure to sub lethal concentrations of copper & mercury.

Chapter 6 Bioaccumulation of heavy metals

6.1 Introduction

The key objectives addressed in the previous chapters were to ascertain the functional ability of immunological biomarkers to detect heavy metal stress even when metal concentrations were comparatively low. For that purpose, a standardized set of immunopathological and biochemical parameters was measured in the green mussel *P.viridis.* The last objective of this investigation was to evaluate the possibility of bioaccumulation of metals in the haemolymph in comparison to other tissues. In this chapter bioaccumulation of copper and mercury in the haemolymph was studied to confirm if the immunological alterations observed in previous chapters were the result of bioconcentration of heavy metals.

Bioaccumulation is the general term describing the net uptake of chemicals, usually non essential ones, from the environment by any or all of the possible routes i.e., respiration and diet from any source in the aquatic environment, where chemicals are present in water, sediment or in other organism. Bioaccumulations, which are precursors of cytotoxicity is the net result of uptake and excretion, and when uptake exceeds excretion, a net bioaccumulation of metal occurs.

Most studies on marine environment, which have attempted to determine metals in animals, have focused on animals nearer the lower end of the food web. This is because these animals can be directly harvested for human or livestock consumption, can serve to transfer

metals trophically to carnivores, and can modify the speciation, cycling, and transport of metals in marine systems. Lying in the second trophic level in the waters' ecosystem, mollusks have long been known to accumulate essential and non-essential elements in aquatic ecosystems (Dallinger and Rainbow, 1993). Studies concerning the accumulation and toxic effects of metals in bivalve mollusks were mostly focused on mussels in view of their wide geographic distribution, sedentary way of life, filter feeding behaviour, convenient size, ease of laboratory experimentation, considerable capacity for accumulation of a wide variety of toxic compounds in their tissues as well as high response to pollutants. One fundamental assumption and basis of biomonitoring programmes is that the concentration in the bivalves reflects the available levels of metals in the ambient environment. Mussels accumulate many chemicals due to their great filtration capacity and their contact with sediments, and concentrate metals in soft tissues, and hence serve as bioindicators of metal contamination (Nicholson, 2003a, b). But, this is of great concern as bivalves that are loaded with harmful pollutants enter the food chain on consumption resulting in biomagnifications at higher trophic levels. The ability of mollusc to concentrate high amount of heavy metals without any apparent bad effects could make these animals very dangerous to their predators (Carpene, 1993).

Metal contamination of aquatic ecosystems is a matter of concern as many metals are persistent and potentially deleterious to aquatic life. All animals can accumulate metal dissolved in the ambient sea water and/or from the food. Environmental pollution by metals has become one of the most important problems in the world (Chandran et al., 2005). In aquatic systems, the heavy metals of greatest concern are copper, zinc, cadmium,

mercury, and lead. These elements are toxic to organisms above specific threshold concentrations but many of them (e.g., copper and zinc) are essential for metabolism at lower concentrations. Lead, cadmium and mercury have no known biological function. Other elements of concern are aluminium, chromium, selenium, silver, arsenic, and antimony, which have contributed to serious problems in freshwater, estuarine, and coastal ecosystems. Most of the studies on marine environment, which have attempted to determine environmental poisoning by metals, have boosted up in the last decades due to concern on the growing use of metals in agricultural, chemical and industrial processes posing threats to lives of organisms. Heavy metals investigated in the present study are copper and mercury.

Copper is an essential element for bivalve's development, and is present in many enzymes and respiratory pigments (White and Rainbow, 1985). Copper is a cofactor of the prion protein and many metalloenzymes, such as cytochrome C-oxidase and Cu/Zn superoxide dismutase, and is involved in byssus secretion.Copper, at elevated concentrations, is toxic to organisms (Nicholson, 2003a). It can generate reactive oxygen species (ROS) and because of its high affinity for thiol groups, copper is also able to bind to cysteine, with consequent inactivation of the proteins themselves. Mussels exposed to cupric ions were shown to cause respiratory and cardiovascular depressions (Scott and Major, 1972), reduced rates of oxygen consumption (Brown and Newell, 1972), inhibited filtration (Abel, 1976), bradycardia (Scott and Major,1972), retarded growth(D'Silva and Kureishy,1978), reduced production of byssus and increased mucus production.(Scott and Major, 1972). Copper affects enzyme activities, electron transport reactions, membrane permeability and cell division (Davenport and Redpath, 1984).

The concentration of total copper in the clean oceanic seawater falls in the range of about 0.05 to 0.35µg/L at the surface. Highest concentrations occur in the low salinity regions of estuaries, and concentrations decrease with increasing salinity.

Mercury is a trace metal normally present at very low concentrations in marine environment. It is a non-essential metal that consistently biomagnifies through the food chain. Mercury can exist in various oxidation states that are highly soluble, reactive and toxic to bivalves at low concentrations. Minamata bay incident in southwestern Kyushu, Japan, the most extensively documented case of mercury poisoning, reveals the deleterious effects on man, of chronic discharges of low-level methyl mercury wastes into coastal waters. In India, Chloroalkali plants are the main source for mercury release to the atmosphere and surface waters. Besides, these coal-fired plants such as thermal power, steel, and cement plants also contribute significantly to mercury pollution (Nanda, 1993). Toxic effects of mercury to bivalve mollusks studied so far include imbalance of Ca^{2+} signaling pathway (Burlando et al., 2004), immunotoxicity (Gagnaire et al., 2004), ROS generation, genetic abnormalities (Yap et al., 2004), reduction in filtration rate, alteration in gill structure (Gregory et al., 2002), and inhibition of Ca^{2+}-ATPase activity (Pattnaik et al., 2007).

The aim of the present study is to understand the accumulation pattern of copper and mercury in *P. viridis* haemolymph, and in tissues such as gills, digestive gland, and other soft body parts under chronic exposure of metals. *P. viridis* has an open circulatory system, and any change in their ambient environment will be reflected in the haemolymph as soon as water bathes gills. Hence, metal in ambient

water will be reflected in haemolymph as well. A life in water requires that gills and other body surfaces be designed for the efficient exchange of oxygen and other essential molecules. Unfortunately, the same physiological designs that make aquatic life so successful also lead to the efficient uptake of many other nonessential chemicals resulting in metal accumulation. The digestive gland represents the major site of metal accumulation in bivalves (Pipe et al., 1999).

6.2 Review of literature

Transfer of metals through food chains has been considered in studies way back to the 1960s (Bryan, 1964; Hoss, 1964). A large body of field and laboratory studies have since indicated the concentration of metals in bivalves. Many researchers have reported the potentiality of using mollusks, especially mussels and oysters, as bioindicators or biomarkers for monitoring heavy metal contaminations in aquatic system (Philips, 1980; Philips and Rainbow, 1994; Gagnaire et al., 2004). Estuarine and coastal waters are often at the risk of pollution of toxic metals such as mercury from natural as well as anthropogenic sources (Nanda, 1993; EPA, 1997; Das et al., 2001). As a major marine pollutant, copper induces different responses at different levels in the bivalves (Regoli et al., 1998).

Concentrations of heavy metal in soft tissue of *P.viridis* had already been studied (Eislet 1981; Philips and Rainbow, 1988; Chan 1988; Yap et al., 2002; Shi and Wang 2004; Verlecar et al., 2008) Laporte et al., (1997) showed mercury accumulation in the gill tissue, at smaller levels in the carapace and internal organs, and at very low concentration in circulating haemolymph of the crab *Carcinus maenus*. Mercury and other metals, such as lead and cadmium, have been shown to bioaccumulate in

small fish and other living organisms in marine and freshwater ecosystems (e.g. Great Lakes) (Bernier et al., 1995). Cu^{2+} concentrations in the mussel haemolymph was examined by Pickwell and Steinert (1984).

6.3 Materials and methods

For bioaccumulation studies, specimens of *P.viridis* were exposed to the required sublethal concentrations of copper and mercury for a period of 15 days . Specimens of *P.viridis* were maintained in clean FRP tank containing 5L of sea water diluted with the required toxicants. Water was changed once in 24 hours without disturbing the organism, and fresh toxicants in the required concentrations added daily. At the end of 15 days, haemolymph samples were extracted from the adductor muscle sinuses and stored in acid washed plastic vials. *P.viridis* was cut open and dissected to separate gills, digestive gland and remaining body parts as soft tissue. They were weighed and dried at 80°C for 48 h in hot air oven before digestion.

For determination of copper about 0.5 g of dry tissue, and 0.5ml of haemolymph samples were digested in concentrated nitric acid (70%), and hydrochloric acid (70%) in 4:1 proportion. They were placed in a hot block digester and fully digested, first at low temperature of 40°C for 1 hour, and later at 140°C for 3 hours. For mercury analysis the procedure was slightly different. Tissue and haemolymph were digested in a teflon tube. Digestion was done in a microwave unit for 3 min at an intensity of 100%, and for 40 min at an intensity of 60%. The digested samples were then diluted to a certain volume with deionised water. The samples were then filtered, and the filtrates stored until analysis.

The concentrations of Cu and Hg were analyzed by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) (Perkin – Elmer, Elan 6000). The stable metal tissue was expressed as µg/g DW (dry weight) for tissue concentration, and µg/ml as haemolymph content.

Statistical analysis

Results are presented as means and standard errors. Data obtained were subjected to Analysis of variance (ANOVA) with a Tukey post hoc test. For all statistical tests, results were concluded as significant at (P) value of < 0.05.

6.4 Results

Supporting the vast array of existing literature, bioaccumulation of copper and mercury occurred in different tissue parts of *P.viridis*. The ability of the mussel to carry elevated concentrations of copper and mercury was remarkably great.

When haemolymph of *P. viridis* was analysed there was an increase in the accumulation of copper, with increasing test concentration. Copper content was low, 0.03 ± 0.01 µg/ml, in control mussels, while accumulated mercury content was 0.1 µg/ml in control organisms. Mercury accumulated in haemolymph in mussels exposed to higher concentrations of 20µg/L and 50µg/L.

Total copper concentrations in various tissues of *P.viridis* increased significantly with increasing exposure concentrations. Accumulation of copper in tissue was the highest in the digestive gland, followed by soft tissue and then gills. Accumulation of mercury was found to be several folds higher in mussels in all concentrations, and the highest value was obtained in mussels exposed to the highest concentration of mercury.

Highest accumulation of mercury was observed in gills followed by digestive gland and soft tissue. The accumulation pattern of copper and mercury is illustrated (Table. 6.1)

Statistical analysis were found to be significant at $P< 0.05$ probability level (Table. 6.2 to 6.5).

6.5 Discussion

The results obtained have indicated that bioaccumulation in tissues increased with increasing concentration, and also mussel's ability to limit the bioaccumulation of copper and mercury varied from organ to organ. Haemolymph of *P.viridis* exposed to copper and mercury was found to contain higher concentration of these metals. Mussels exposed to 30 µg/L of copper had haemolymph copper content four times higher than that in the control mussels. Mercury could be detected in the haemolymph of those mussels exposed to 20µg/L and 50 µg/L of mercury. The observed results are in agreement with those t of George et al. (1978), and Pickwell and Steinert (1984), who found elevated copper in oyster and mussel serum, respectively, and Laporte et al. (1997) observed increased mercury content in the haemolymph of *C.maenus* .This elevated copper concentration in haemolymph might be due to continuous secretion of copper-binding molecules into the haemolymph as suggested by Pickwell and Steinert (1984). Haemocytes are found to have an active role in heavy metal metabolism, i.e., in the actual uptake (Galtsoff, 1964), distribution to various tissues (Cunningham, 1979), intralysosomal storage (George et al., 1978), and its final elimination. This uptake of heavy metals by granular haemocytes can result in high concentrations of copper and mercury. However, when compared to the metal contents in the gills, metallic load in the digestive gland and soft tissues was low in haemolymph.

Generally, tissues, where absorption takes place (like gills), contained more metals than in other tissues (Bebianno et al., 1993). The accumulation of heavy metals in gills, and digestive gland could be related to the function of these organs. The gills are in continuous contact with the external medium, and are considered responsible of the metal transfer to organism. The digestive gland plays an important role in heavy metal metabolism and contributes to their detoxification. This could explain the high metal content in these organs of mussels. Metal detoxification is achieved by employing physiological and biochemical processes which may, for example, include the induction of metal-binding proteins such as metallothioneins and low molecular weight ligands, such as glutathione.

Because copper is an essential trace nutrient, most marine organisms have evolved mechanisms to control concentrations of the free ion in tissues in the presence of variable concentrations in the ambient water, sediments, and food. This might be the reason for low copper concentration in contrast to mercury content. Copper must have sequestered as Cu^{+} in lysosomes in the digestive gland which can be the reason for the increased level of copper in the digestive gland as observed in present study. Even at the highest sublethal concentration used, the mussels accumulated copper in the tissues only to about 5times the concentration in unexposed reference animals.

Mussel can accumulate high levels of mercury in their tissues. Haemolymph contained comparatively lower levels of mercury. Lower concentration reveals a great lability of the metal in haemolymph, which should not be considered as a storage compartment but more as a transport and distribution system. Although the concentrations of mercury

in water column and sediments may be low, it has been demonstrated that adult bivalves are capable of accumulating heavy metals in their tissues to several orders of magnitude above the concentrations found in the water and sediments. The high levels of heavy metals in the haemolymph give evidence for the presence of metal-binding factors in mussel. In previous investigations, accumulation of mercury was found to take place mainly in the gills (Laporte et al., 1997), which is similar to the present findings, where gills have the maximum accumulation**.**

6.6 Conclusion

High metal content occurs in haemolymph presumably in the granules of haemocytes at their increased concentrations. Levels of copper and mercury in the haemolymph would give an indication to the magnitude of impact on haemocytes and various tissues. This study has also revealed that concentrations of heavy metals in various other tissues are also high suggesting, their temporary storage in haemolymph before the metals are transported to various organs of storage and detoxification. In essence, the bioaccumulation assessment confirms that the haemolymph is inevitably an important tissue in bioaccumulation tests for heavy metals in bivalves.

Table. 6.1 Bioaccumulation of copper and mercury in different tissues of *P.viridis*

TOXICANT	Exposed Tissue	Control	6 µg/L	12 µg/L	30 µg/L
COPPER	Haemolymph (µg/ml)	0.027±	0.045±	0.064±	0.122±
		0.010	0.016	0.004	0.003
	Gill (µg/g DW)	2.334±	3.662±	5.792±	10.830±
		0.023	0.113	0.074	0.063
	Digestive Glands (µg/g DW)	2.763±	5.792±	10.830±	27.473±
		0.067	0.074	0.063	0.058
	Soft Tissues (µg/g DW)	1.215±	4.923±	9.928±	27.287±
		0.010	0.139	0.124	0.083
MERCURY		Control	10 µg/L	20 µg/L	50 µg/L
	Haemolymph (µg/ml)	0.100±	0.100±	0.101±	0.210±
		0.000	0.000	0.001	0.020
	Gill (µg/g DW)	0.102±	54.175±	90.727±	178.847 ±
		0.003	0.114	0.110	0.104
	Digestive Glands (µg/g DW)	0.100±	20.138±	38.106±	76.908±
		0.000	0.073	0.129	0.081
	Soft Tissues (µg/g DW)	0.100±	12.477±	28.173±	45.862±
		0.000	0.138	0.110	0.100

Table. 6.2 ANOVA Table for Bioaccumulation of copper in different tissues of *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	2084.315	3	694.772	135213.597	.000
Concentration	3116.234	3	1038.745	202156.210	.000
Tissue * Concentration	1714.378	9	190.486	37071.681	.000
Error	.411	80	.005		
Total	6915.338	95			

Table 6.3 ANOVA Table for Bioaccumulation of mercury in different tissues of *P.viridis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	84125.666	3	28041.889	4234528.945	.000
Concentration	73112.177	3	24370.726	3680156.656	.000
Tissue * Concentration	54743.172	9	6082.575	918512.961	.000
Error	.530	80	.007		
Total	211981.545	95			

Table 6.4 Results of Multiple Comparison Test for Bioaccumulation in Copper exposed *P. viridis*

		p-value
Tukey	**Control Vs 6 µg/L**	0.0000
	Control Vs 12 µg/L	0.0000
	Control Vs 30 µg/L	0.0000
	6 ppm Vs. 12 µg/L	0.0000
	6 ppm Vs. 30 µg/L	0.0000
	12 ppm Vs. 30 µg/L	0.0000

Table. 6.5 Results of Multiple Comparison Test for bioaccumulation in Mercury exposed *P.viridis*

		p-value
Tukey	**Control Vs 10 µg/L**	0.0000
	Control Vs 20 µg/L	0.0000
	Control Vs 50 µg/L	0.0000
	6 ppm Vs. 20 µg/L	0.0000
	6 ppm Vs. 50 µg/L	0.0000
	12 ppm Vs. 50 µg/L	0.0000

Conclusions

Aquatic toxicology is relatively a new and still evolving discipline, originating from the concern for the safety, conservation, and protection of aquatic environments, and also as an off shoot of toxicology, since degradation of water mass is caused mainly by anthropogenic toxicants dumped into the water bodies by human activities, directly or indirectly. Intent of the present study was to understand and ascertain how best haemolymph parameters, and activity patterns of selected haemolymph organic components and enzymes activity patterns of the green mussel *P.viridis* can be employed as useful and sensitive immunomarker tools for laboratory studies, and to some extent field applications. Various parameters tested in the present have proved that those can be essentially useful in expanding our existing knowledge on bivalve immunity, and immunotoxicology, particularly in relation to the increasing disease susceptibility being documented in shellfishes. This study is all the more important considering the fact that not too many studies have been made in India on molluscan immunology and immunotoxicology, except perhaps the contributions of a few.

It was found that haemolymph could be easily sampled from individual mussels in sufficient quantities from the sinuses of adductor muscles for analytical purpose without sacrificing the test specimens. The first chapter shows that the highly regulated nature of bivalve immune system renders it quite vulnerable to toxicants. A decreased cellular defense mechanism, assessed through investigating the various haemolymph parameters such as total haemocytes counts, differential

counts, cell viability, and lysosomal stability on prolonged exposure to heavy metals, was observed. Changes could easily be analysed and quantified as these tests were simple but reliable, and they are suggested as good immunomarker tools for screening purposes, prior to measuring higher order physiological dysfunction.

Biochemical changes in organic constituents of haemolymph content and aspartate amino transaminase, and alanine amino transaminase activities in metals exposed mussels showed significant variations from the controls. But energy reserves such as glucose, glycogen and protein vary according to season, age and reproductive stage also. But considering the fact that the specimens used in the present study were of uniform size and not fully matured gives an indication that the fluctuations observed in the activity patterns cannot be taken lightly or ignored totally, though additional information on these parameters would always be useful for better understanding of the immune mechanisms. However, haemolymph ALT and AST activities are recommended as useful and purposive immunomarkers.

The apparent sensitivity of ACP and ALP exhibited through fluctuating activity patterns suggests that analysis of these enzymes at different time-periods can be used as biomarkers in metal pollution. In agreement with the observations of other workers, the data I have obtained once again confirm the reliability of choosing AChE activity pattern as a sensitive haemolymph enzyme marker to assess metal stress. The observed inhibitory effects of Na^+/K^+ ATPase and Ca^{2+}ATPase in their activities point to the degrading health of *P.viridis* and hence can be used as a biomarker. Super oxide anion production quantified by NBT assay was the most easily carried out assay among the antioxidant mechanism tested in *P.viridis*..

There is a shortage of readily available data on bivalve haemolymph from our country for comparison purpose, more importantly on immunity and immunotoxicity. One major reason is that molluscan haemolymph was not considered as a tissue until recently, and the significant role haemolymph plays in defence mechanisms was under estimated. Interestingly, the picture on molluscan defence mechanisms is still vague.

An increased metal content in haemolymph was evident from the data obtained postulating haemolymph as an organ of bioaccumulation. From various immune parameters and haemolymph enzyme activity patterns analysed, copper and mercury showed toxic effects on haemolymph. But the impact was more on those exposed to mercury than those exposed to copper. This suggests a better detoxifying mechanism *in P*.viridis for copper as it is also an essential element.

In conclusion, it can be stated that the study of immunotoxicity of invertebrates is still very much in its infancy in our country. Full evaluation of the immunotoxic potential of the contaminants requires a wide range of assays to assess cellular and humoral aspects in immune mechanisms. The significance of laboratory based assessments needs further evaluation under field conditions. The data presented above, showing significant alterations in mussel haemolymph components following copper and mercury exposure, demonstrate clear immune suppression, and provide a clear indication that the immune system of bivalve molluscs is modulated by environmental stressors. More work to establish the link between the assays used to measure immune competence, and the importance of their relationship to the observed suppression of the immune mechanism is needed.

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