HEAVY METAL TOXICITY STUDIES ON THE ESTUARINE CLAM *VILLORITA CYPRINOIDES* (HANLEY)

THESIS

SUBMITTED TO

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

UNDER THE FACULTY OF MARINE SCIENCES

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COCHIN - 682 016, INDIA

1992

CERTIFICATE

This is to certify that this thesis is an authentic record of the research work carried out by Mr. B. SATHYANATHAN, under my scientific supervision and guidance in the School of Environmental Studies, Cochin University of Science and Technology under the Faculty of Marine Sciences, and no part thereof has been presented for the award of any other degree, diploma, or associateship in any University.

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CHAPTER 1

INTRODUCTION

Since environmental awareness has become a political issue and a popular movement, great efforts have been expended by many to evaluate the anthropogenic impact on marine and estuarine ecosystem. This is especially true with respect to introduction of potentially harmful substances such as heavy metals, metalloids, synthetic organics, chlorinated organics, petroleum hydrocarbons, and polycyclic aromatic hydrocarbons (Dillon and Lynch, 1981; Rygg, 1986; O'Connor and Huggett,1988). This has to be properly acknowledged since large aquatic ecosystems are morphometrically, and hence, physically, chemically, and biologically predisposed to excessive susceptibility to toxic chemical insult. Their enormous surface area, hydraulic definition scales in the order of decades to centuries, and their relatively longer retention times of toxic substances particularly the heavy metals tend to increase the expose potential of biota in these systems. Further, these systems perhaps contain highly sensitive biota associated with relatively oligotrophic situations, in which one or more life stages may be particularly susceptible to the influence of toxic contaminants (Swain, 1988).

To manage aquatic resources and their habitats effectively one should relate results from laboratory research and field experiments with data from monitoring of contaminants and

effects on other resources. This is all the more essential for stopping or reversing aquatic habitat degradation in coastal waters (Pearce and Patanjo, 1988).

Apart from mechanical and amenities aspects, most pollution problems in final analysis are toxicological. The toxic effects of a pollutant may be acute, dramatic, short term, and readily discernible, but correction may be difficult, costly, and long term (Halstead, 1972; Hodson, 1988). Effects of pollutants on fishes and fisheries may be estimated from biological and biochemical responses to pollutant exposure and the incidence of pollution related diseases in aquatic organisms. Indirect and potential effects on ecosystems may be estimated from the accumulation of contaminants in biota. However, the plethora of contaminants discharged into the marine environment makes it impossible to determine which agent, or combination of agents have the greatest effects.

The challenge before an aquatic toxicologist is immense considering that virtually all man-made chemicals have a potential for entering aquatic environments indirectly or directly, and he is committed to determining how chemicals entering aquatic environments alter the well-being of organisms, and perturb community structures. He is faced with problems such as identification of these potentially toxic substances, and in relating their presence in environments and organisms to

alterations in life processes. The task becomes complicated by the presence of hundreds and perhaps thousands of xenobiotics that may influence observed biological changes (Malins and Jensen, 1981; O'Connor and Huggett, 1988). The general acceptance of the advantages inherent in the use of bioindicators to monitor aquatic pollution has given rise to the establishment of national and international programmes employing such species in many parts of the world over the last decade (Phillips, 1980; Phillips and Segar, 1986) since it is reasonably believed that bioindicators provide a direct picture of the pollutant abundance thus eliminating the need for exhaustive and complex studies on the chemical speciation and also the analysis of water samples for some trace contaminants, which is fraught with difficulty.

If the uptake of a given pollutant depends not only on its own ambient concentration but also on the presence, absence, or precise concentration of a second contaminant, the bioindicator concept finally breaks down. This question is increasingly addressed by many, and this thesis is also a small step in this respect, although other aspects as given elsewhere are also included.

1.2. Trace Metals in the Aquatic Environment

It has been known for several decades that trace quantities

of certain elements exert positive or negative influences on plants, animals, and human life. The specific role of each element has also been reasonably established. Generally, the term trace element is rather loosely used in current literature to designate the elements which occur in small concentrations in natural biological systems. The growing public concern over the deteriorating quality of the environment has led to generalised usage when referring to trace elements. For practical purposes, other terms such as heavy metals, trace inorganics, and microelements can be treated as synonyms of the term trace elements (Forstner and Wittmann, 1979). Heavy metals are considered to be an important class and cause of environmental pollution (Forstner and Wittman, 1979; Phillips, 1989). They are ubiquitous, readily dissolved in and transported by water, bioavailable, and strongly bound by sulphhydryl groups of proteins. Numerous studies conducted on the detrimental effect of metallic pollutants have documented that when the metal concentration surpasses a characteristic value for each species, the animal enters into a toxic situation that produces a wide range of effects and responses at all levels (Torres, et al., 1987). Almost all industrial process and urban activities involve release of at least trace quantities of half a dozen of metals in different forms, and increasing number of industrial plants and enhanced urban activities contribute to further increase of metallic ions in lakes and streams day by day. The estuaries are particularly exposed to metallic pollution more

than any other body of water, since the banks of the estuaries are often the hub of increased industrial activities. Another concern about metallic pollution in estuarine environment is the high retention time of metallic elements in such water bodies.

For assessing metal toxicity on aquatic organisms, biological availability, detoxication, accumulation, and active or passive release from the body play a basic role. Our present knowledge of the toxicity of heavy metals to aquatic organisms is almost entirely based on laboratory experiments conducted under high test concentrations, and a lack of understanding of the mechanisms underlying metal availability and accumulation makes it difficult to establish precise relationship between the concentrations of the metals in the aquatic environment and the toxicity of heavy metals to organisms (Kinne, 1984; Jenkins and Mason, 1988).

1.3. Significance of Toxicity Tests and Sublethal Stress Responses

Assessment of metal toxicity to aquatic organism is done mainly by conventional static tests for short periods. This is definitely the first step towards understanding and comparing the detrimental effects of the metals, and it is the basis for many of the management guidelines and environmental laws we have today which regulate the metallic contamination in the aquatic

environment. Moreover, water quality standards for protecting aquatic organisms require data which directly relate sublethal effects of contamination to the survival of natural populations (Little et al., 1985). (Quality criteria for water are intended not only to protect essential and significant life in water, but also to protect life that is dependent on life in water for their existence, or that may consume intentionally or unintentionally any edible portion of such life (Train, 1979)). Such water quality criteria are to be derived from scientific facts obtained from experimental or insitu observations that depict response of organisms to a defined stimulus or material under identifiable or regulated environmental conditions for a specific time-period.

Quantification of the functional status of an ecosystem as influenced or threatened by a potential toxicant is an important aspect and function of predictive ecotoxicology (Rand and Petrocelli, 1985). Many biological monitoring exercises are based on the structure of ecosystem rather than on functioning. In biological monitoring it is essential to develop functional parameters realistic to the ecosystem in question. For example, a continuous or repeated measurement of specific physiological or behavioral responses of suitable organisms exposed to the water/waste water stream in the field or under simulated field conditions may help to detect and prevent short term effects.

So, multifactoral tests in which sublethal physiological responses are taken into account are a pre requisite to obtain a meaningful assessment of the potential impact of metallic pollutants on aquatic organisms (Depledge, 1987). Aquatic organisms are known to respond physiologically and behaviorally on exposure to sublethal concentrations of toxic chemicals. Some of these functional responses are the result of the mode of action of the toxicant, while others are adaptive responses by the organism to the toxicant. There is a broad range of sublethal environmental combinations that influence the organism. Within the zone of compatibility, the organism may be subjected to many different environmentally significant organismic aspects. One significant aspect is that the environmental fluctuations may severely stress the organism and sustained environmental stress of any nature will reduce their survival potential and would give rise to loss of important species from an area under threat.

Another aspect which is of no less significance is the survival of the organisms by overcoming the temporary transient stresses. The morphological feature of the organism that increases its fitness or to the process of becoming fitted under such conditions needs further study. These tolerant organisms, though always a minor part of the population, deserve closer investigation not only because of the increasing frequency of low level transient stress experienced by them due to the

introduction of metallic pollutants into the aquatic media without any concern, but also due to the human health factor involved. Most organisms have evolved adaptive defensive responses, such as shell valve closure in mollusks and burrowing of crabs, that enable them to tolerate, survive, and overcome temporary transient stresses such as introduction of a chemical in their environment. However, at some level of stress or concentration of chemical, these responses may be inadequate and ability of the organism to respond normally may be impaired. It is evident in measuring any behavior, just as with other end points (or effect criteria) that a threshold exists such that exposure to one concentration of a chemical elicits an adaptive defense response, and exposure to another slightly higher chemical concentration may obliterate it and produce an aberrant behavior response (Rand, 1985).

As mentioned earlier our present knowledge of heavy metal toxicity to aquatic organism is mainly based on laboratory experiments conducted at *in situ* irrelevant test concentration. We are also handicapped with the following problems, (1) lack of metal specificity of analytical methods, (2) analytical difficulties in situation of multi factor stress exposure, and (3) insufficient awareness of the often efficient organismic compensatory mechanisms. In spite of deficiencies, techniques with the potential for general application to determining short and long term trends in the biota with the required degree of

sensitivity and specificity are currently available. Considerable fundamental research has been and continues to be undertaken into the toxicology of marine organisms at the cellular, tissue and whole organism levels and also into the response of populations and communities to anthropogenic disturbances (Dawson et al., 1988).

1.4. The Backwaters and Clam Resources of Kerala

The backwaters in Kerala have been one of the well studied environments in India, and considerable amount of information is available about the backwater system in general, and the Vembanad lake in particular. Rapid urbanisation and industrialisation, especially around Cochin have changed the ecology of the backwaters (Qasim and Madhupratap, 1979). The lengthy estuaries, backwaters and coastal areas of Kerala have abundant clam resources. It has been traditionally exploited by the coastal people, even though they are experiencing many ecological problems recently (press report). Clams form the basis of subsistence of the coastal poor, providing them with the much needed nutrition. Clams form a good source of food, and their shells are being used in industries. The wide range of industrial use of clam shells and dredged sub soil deposits include the manufacture of paper, rayon, leather, carbide, cement, and fertilizer. It is also used as shell grit for poultry. The frozen clam meat is also a foreign exchange earner

for India. As per the CMFRI report, the export which started with a meagre 16 tonnes in 1981-82 reached 1033 tonnes in 1984-85, a 65 fold increase in a short span of 3 years, and this figure has touched new heights in later years. The Vembanad lake, with an area of 200 sq.km, has an annual production capacity of around 25,000 tonnes of *Villorita cyprinoides* (Narasimham et al.,1986). It indicates the high conversion rate of calcium carbonate from the lake by these animals. The exploitation of clams is round the year. The period from December to February (January being the peak season) is the ideal harvest period as far as domestic consumption is concerned, since this is the period when the clams have high protein and lipid contents, and comparatively lesser water content (Lakshmanan, 1982).

1.5. Scope and objectives of the present investigation

Even though much work has been carried out on the biology and biochemical characteristics of molluscs in Cochin backwaters, no detailed attempt has been made to study metal-metal interactions in this commercially important clam. This is all the more important since it is recently reported that metallic interaction will finally result even in the breakdown of the well accepted bioindicator concept, besides considerably modifying the toxicities of metals to aquatic organisms (Phillips, 1980; Rainbow et al., 1990).

The main objectives of the present investigation are briefly summarised :

a) to understand the passive and active metal accumulation strategies, distribution, and retention kinetics of Cu and Zn in the estuarine clam *Villorita cyprinoides* (Hanley),

b) to have more idea about the physiological and biochemical adaptive responses of the organism during sublethal metal stress,

c) to have an insight into the interactive effect of the metal ions Cu and Zn, and their role, if any, in modifying the toxicity of the other metal,

d) to provide preliminary data on the effect of sublethal pre-exposure (acclimation) to metal ions, and to find out the influence of it in modifying the toxicity, and

e) to find out the role of other similar influencing factor(s).

No attempt, however, has been made to study the biology of the organism and no distinction has been done between male and females, since these are irrelevant as far as economical exploitation of clams, and the main objectives of the present investigations are considered.

CHAPTER 2

MATERIALS AND METHODS

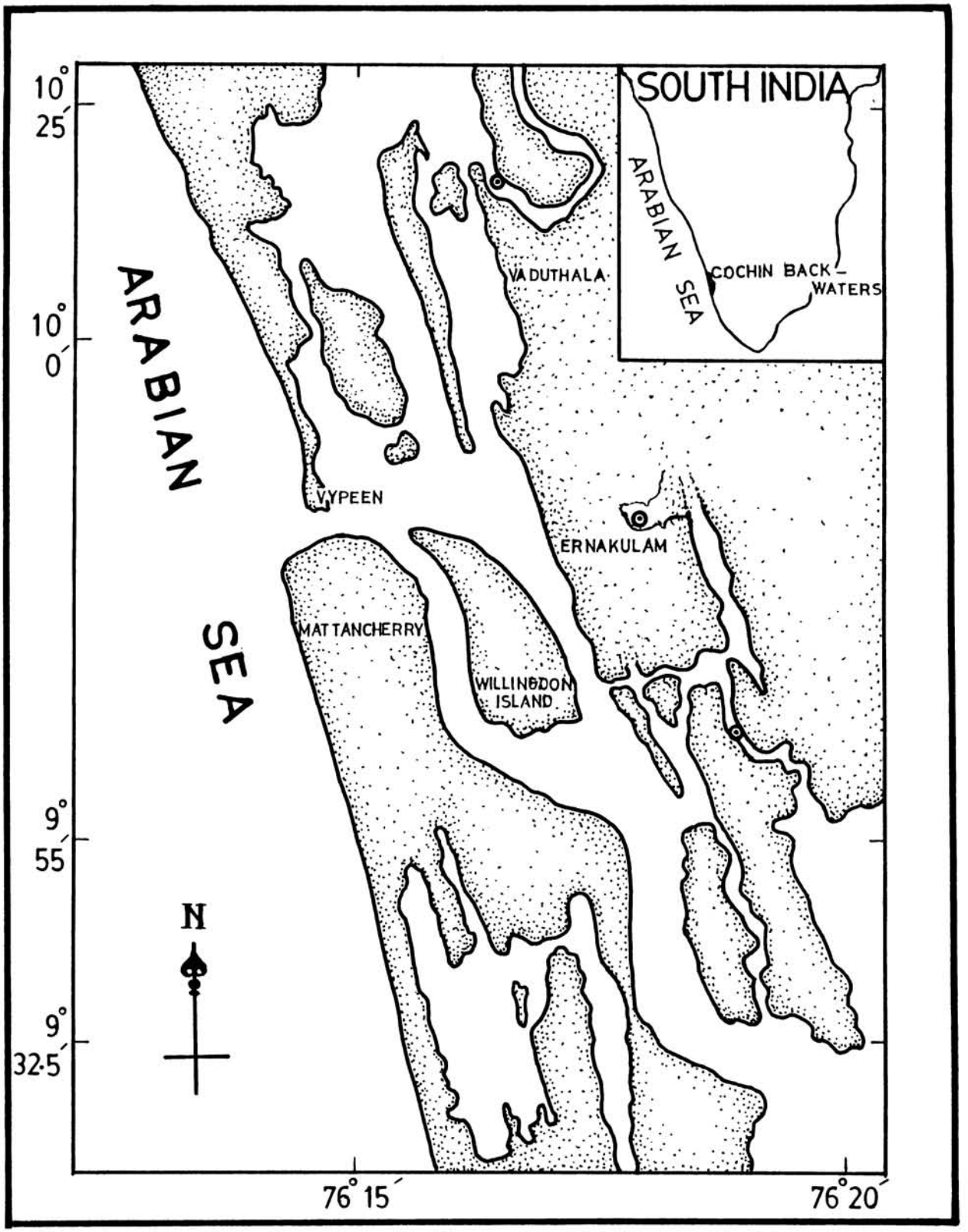
2.1. Materials

2.1.1. Collection of Organisms, and Water Samples

Specimens of the estuarine clam *Villorita cyprinoides* (Hanley) were collected from Cochin back waters ($9^{0}55'N$; $76^{0}17'E$). The locations of the collection centres are given in Fig 1. The size group of the organisms was 25-30 mm. Depth of the clam beds was two to three meters from the surface. Collection of the specimens was from a clean environment. Healthy, abundant specimens were available through out the year. The period of study was from January 1986 to July 1990. Collection of specimens and estuarine water samples was effected using the dinghy "Flying Fish", and of sea water using the research vessel " RV Nautilus ", both of the School of Marine sciences, Cochin University of Science and Technology. A stainless steel dredge, and a modified Van Don sampler, were used to collect organisms and deep water samples, respectively. Local fishermen helped in collecting the organisms. Most of the experimental work was carried out at the Chemical Oceanography laboratory, School of Marine Sciences, and the rest in the laboratory of School of Environmental Studies, Cochin University of Science and Technology.

2.2. Static Bioassay Tests

Fig.1



Map showing the site of collection of Villorita cyprinoides - ⊙.

2.2.1. Acclimation of Organisms

Following collection, the animals were carefully transported to the laboratory in healthy, live condition imparting as little shock as possible, and kept in large chemical resistant, non - dye polythene basins containing filtered- sea water of habitat salinity. The organisms were maintained for a day to remove the pseudofaecal materials and then transferred to the experimental salinity, and acclimated for 3-4 days. When a study in a different salinity regime was planned, the animals were progressively conditioned to that set of experimental parameters through a step-wise gradation process. For example, if the organisms were collected from a region where the salinity was $20x10^{-3}$, and the experimental salinity was $13x10^{-3}$ the animals were reared first in $20x10^{-3}$ salinity for 3-4 days, then in $17x10^{-3}$ for identical period, and finally in $13x10^{-3}$ salinity. As far as possible, however, the experiments at specific salinity were designed in such a way that the habitat salinity was the same or very near to it. For example, experiments meant at $<1x\ 10^{-3}$ salinity were carried out during monsoon season (June to August) when the habitat salinity was very near to zero. This type of investigation is time consuming, and this is one of the reasons for the extended study period (>5 years).

2.2.2. Long Term Sublethal Toxicity Studies

Long term/medium term (period extended up to about one to

two months) toxicity tests were conducted in 10-L white plastic basins previously tested and proved to be non toxic and chemical resistant (Sivadasan, Personal communication). Tests were carried out according to standard test protocols, or following the guidelines in Standard Methods, APHA (Clesceri etal; 1985, 1989). All troughs, basins, and glass wares were thoroughly washed, kept soaked in 6 N HNO_3 over night, and then washed with deionised water. Each basin was then filled with 5-L of sea water of desired salinity. The water was aerated slowly, but continuously ensuring oxygen saturation. The water sample was tested for different parameters like dissolved oxygen, temperature, pH, salinity, hardness, trace metals and nutrients. The above parameters were regularly monitored in a set of experiments, and water quality maintained steady through out the test period. Twenty five healthy acclimated animals of uniform size were carefully transferred to each basin. The media was spiked with different metal concentrations. Metal ions were added as their salts (Cu as $CuSO_4.5H_2O$; Zn as $ZnSO_4 7H_2O$). All chemicals were of ANALAR or equivalent grade. The medium was renewed every 24 hours. Mortal animals, if any, were immediately removed and mortality recorded at 24 hour intervals. Only those experimental concentrations in which percentage mortality during 96 hour period was less than 10 % were considered as sublethal. The animals were fed with the marine algae *Synechocystis salina* at the rate of 2.5 % of net body weight (Sprague, 1985), unless stated otherwise. Duplicates were run invariably through out.

2.2.3. Determination of 96 h LC_{50} Values

The concentration of the test chemical which kills 50 % of the test organisms in a fixed time is termed LC50 value. The log probit conversion and calculation were used for estimation of 96 h LC50 since they are valuable for description and statistical analysis, and since it is the most reproducible toxic response which can be estimated with the highest confidence (Rand and Petrocelli, 1985).

2.3. Determination of Other Chemical Parameters

2.3.1. Dissolved Oxygen

Dissolved oxygen in the water for all purposes was measured by the azide modified Winkler Method (Strickland and Parsons, 1972). This method is based on the oxidation of manganous hydroxide by dissolved oxygen. When a divalent manganese solution, followed by strong alkali is added to the water sample, first a white precipitate of manganous hydroxide is formed. The manganous hydroxide formed gets oxidised by the dissolved oxygen present in the sample giving an insoluble brown coloured tetravalent manganese compound, $MnO(OH)_2$. When the solution is acidified in the presence of iodine, the tetravalent Mn is reduced to divalent state again in amounts equivalent to the original dissolved oxygen content. The iodine liberated is titrated against standard sodium thiosulphate solution using starch indicator.

2.3.2. Determination of Salinity

Salinity of the water samples was determined by the conventional Mohrs-Knudsen Method (Andersen and Foyn, 1969; Strickland and Parsons, 1972). The principle of the method consists of taking equal volumes of International Sea water (Hydrographic lab -Denmark), and the sample, and determining the volume of silver nitrate required to precipitate completely the halogen using an ordinary burette and ordinary volumetric pipette. Potassium chromate was used as the external indicator. The volumes were corrected to correspond to the volumes in the Knudsen burette and pipette. If one Knudsen full pipette of standard sea water, requires about as many burette divisions (N) of standard silver nitrate as the chlorinity of standard seawater, the `α' value for the $AgNO_3$ is N-N, *i.e.*, zero. If the titre value A is not N, then N-A is called the `α' of silver nitrate used. The chlorinity of a sample of water, if a full pipette, which requires `a' burette divisions of $AgNO_3$ solution, will be (a+k) ppt where k is the correction factor read from the Knudsen's Table corresponding to a certain `α' value, and a certain titre value (a) in the Knudsen burette.

2.3.3. Determination of Trace Metals

The digestion of animal tissue, and preparation of samples for metal analysis were done as per the methods of Martincie et

al. (1984), and Denton and Jones (1986). The tissue samples of the live organisms were dissected using stainless steel scissors and scalpel, and dried at 105 $\pm$ 2^0 C to constant weight. Approximately 1 to 2 gms of the sample was taken in a Kjeldahl flask, covered with a funnel and 5:1 volume of Con.Nitric acid and Perchloric acid added. It was heated first gently and then strongly but very cautiously. The digestion was continued with additional quantity of acid mixtures, until all the organic matter was destroyed and the residue in the flask was colourless. The flask was cooled and the solution diluted to 25 ml in a standard flask using double distilled water. The sample was directly fed into the Atomic Absorption Spectrophotometer and absorbance taken (AAS model. Perkin - Elmer 2380 of Chemical Oceanography Division, School of Marine Sciences, and Hitachi 8000 of the Export Inspection Agency, Cochin).

The concentration range, wavelengths, slitwidth, lamp current, and fuel used in the estimation of the metals are given below:

Element	Wave length	Lamp current	Spectral band width	Conc.range.	Fuel
Copper	324.7nm	15 mA	0.7	2-8 ug/ml	Air/Acet
Zinc	213.9	15 mA	0.7	0.4-1.6	Air/Acet

2.3.4. Determination of Metabolic Rate

The metabolic rate of animals (oxygen uptake/hour/gm dry weight) was measured in Erlenmeyer flasks using classical Winkler Techniques (Strickland and Parsons, 1972). Liquid paraffin was used to seal the water-air interface. In wide mouth Erlenmeyer flasks, 500 ml of aerated water was taken. Two animals each from different experimental basins were transferred to each of the five different flasks (five sets were run simultaneously to ensure reliability). After 10 minutes (acclimation period), water was siphoned from the flask to standard oxygen bottles and initial oxygen fixed. Immediately liquid (non toxic, non absorbant) paraffin was added and water - air interface sealed. The set up was allowed to remain undisturbed for one hour in a perfectly calm and quiet environment. Final oxygen was fixed after this period. The difference between the initial and final dissolved oxygen content in the flask gave the amount of oxygen consumed by the organism. The animals were dissected, dry weight determined and the metabolic rate calculated and recorded as µg/h/gm dry wt. The metabolic rate of control animals was also simultaneously monitored following the same procedures.

2. 3.5. Determination of Glycogen

The modified Pfluger Method (Hassid and Abraham, 1957) was followed for the estimation of glycogen. The muscle tissues was separated from the animals and known quantity of tissue

(Approx. 2 grams) was taken in a 15 - ml graduated centrifuge tube containing 3 ml of 30 % potassium hydroxide solution. It was heated in a boiling water bath for 30 minutes. To digested tissue was added 0.5 ml of saturated Na_2SO_4 solution, and glycogen was precipitated by the addition of 1.1 to 1.2 ml of 95 % ethyl alcohol. The contents were stirred, heated to boiling, then cooled and centrifuged at 3000 rpm for about 20 minutes. The mother liquor was decanted off, the tube drained, the precipitate was dissolved in 2 ml of distilled water, and reprecipitated with 2.5 ml of 95 % alcohol, and the alcoholic supernatent liquid decanted. The purified glycogen was hydrolysed to glucose by refluxing with 6 ml of 0.6 N HCl in a test tube provided with an air condenser over a boiling water bath for about 3 hours. The solution was cooled, neutralised with 0.5 N NaOH and made up to 50 ml. A sample of 1-2 ml aliquots was withdrawn, and glucose estimated using $C_6H_{12}O_6$ as standard in spectrophotometer.

2.3.6. Determination of Lactic Acid

Lactic acid in the sample material was quantitatively converted into acetaldehyde by heating with concentrated H_2SO_4. P-hydroxy diphenyl reagent was added and colour measured in a spectrophotometer (Barker, 1957). The soft parts of the animals were dried by pressing within filter paper folds and wet weights taken. It was then homogenised in a glass mortor with the addition of 10% of cold trichloro acetic acid and purified sea sand. The liquid was then centrifuged at 10,000

rpm in a refrigerated centrifuge for 30 minutes. A sample of 1-2 ml of the protein free filtrate was treated with 1 ml of 20% $CuSO_4$ and diluted to 10 ml. Approximately 1 gm of powdered $Ca(OH)_2$ was added and shaken vigorously. It was then allowed to stand at room temperature for at least 30 minutes with occasional shaking, and was then centrifuged. Duplicate aliquots of 1 ml of the supernatent fluid was withdrawn into clean test tubes and one drop of 4% $CuSO_4$ added and test tubes were chilled in ice. Exactly 6 ml of cold con. H_2SO_4 was added slowly and the contents were mixed. The tubes were placed in a boiling waterbath for 5 minutes, removed and cooled to below 20°C. Two drops of 1.5% p-hydroxy diphynyl reagent were added,dispersed quickly and the tubes were placed in a waterbath at 30°C and allowed to stand for 30 minutes. Excess reagent, if any, was dissolved by heating the tubes for 90 seconds in a boiling waterbath. The absorbance was measured in a spectrophotometer at the wavelength 560 nm. Lithium lactate was used for preparing the standard curve.

2.4. Reagents, Solutions and Standards

All reagents were prepared in de-ionised, double distilled water. All chemicals were ANALAR grade BDH, India or equivalent.

2.4.1. Manganous salt solution (Winkler A)

240 gms of $MnSO_4.4H_2O$ dissolved in distilled water and made up to 500 ml.

2.4.2. Alkali-iodide -azide solution

10 gm NaN_3 dissolved in 500 ml distilled water, 480 gm of NaOH and 75 gm of KI added, stirred well, and dissolved in it.

2.4.3. Starch solution

2 gm of soluble starch mixed with 1 gm of salicylic acid and dissolved in 100 ml of water.

2.4.4. Standard sodium thiosulphate solution

Approximately 0.1 N thio was prepared by dissolving about 25 gm AR $Na_2S_2O_3\ 5H_2O$ in 1- L of distilled water. This was standardised against standard KIO_3, and thio diluted to the required strength.

2.4.5. Standard silver nitrate solution

37.11 gm of AR $AgNO_3$ dissolved in ion- free distilled water, and made up to 1 litre.

2.4.6 Potassium chromate indicator solution

8 gm of $K_2Cr\ O_4$ dissolved in 100 ml of distilled water.

2.4.7. Standard sea water

A sample of Eae de Mer Normale with a stated chlorinity (Cl%.) of 19.375.

2.4.8. Hydroxyl diphenyl reagent

1.5 gm of p-hydroxy diphenyl dissolved in 100 ml of 0.5% sodium hydroxide solution.

2.4.9. Standard $CuSO_4$ solution

3.9295 gm of AR $CuSO_4.5H_2O$ dissolved in 1 - L of double distilled water, and diluted to required strength using class A pipettes.

2.4.10. Standard $ZnSO_4$ solution

4.3982 gm of AR $ZnSO_4.7H_2O$ dissolved in 1 – L of double distilled water, and diluted to required strength using class A pipettes.

The Special methods, experimental protocols, and procedures used are described in appropriate chapters.

CHAPTER 3

INTERACTIVE EFFECT OF COPPER AND ZINC AND METAL BIO ACCUMULATION STRATEGIES BY THE CLAM VILLORITA CYPRINOIDES

3.1. INTRODUCTION

Marine and estuarine organisms are well known for their ability to take up and accumulate trace metals in their soft tissues in concentrations higher than the ambient environmental levels which form the basis for the well known bio indicator concept (Phillips, 1980).

Owing to the obvious advantages inherent in such pollution monitoring studies, the degree of metallic contamination in aquatic environment is now frequently assessed by comparing the contaminant concentration in associated biota.

But it goes often unnoticed that there are some other complicating factors which can influence the bioaccumulation other than the degree of contamination (Newman and Mitz, 1988). Some of these factors can also affect the toxicity of any metal to the particular organisms. Many of such influencing factors remain poorly defined in bioaccumulation studies. The physico -chemical parameters such as temperature, salinity, organic and inorganic ligands in the medium, season, feeding behavior,

microhabit utilization, and diet, which have direct influence on the contaminant body have been investigated by many (Unlu and Fowler, 1979; Bingham et al; 1984; Carrier and Beitinger, 1988; Krantzeberg and Stokes, 1989).

The presence or absence of other metals in the medium and their interactive effects have not been given adequate attention so far in such monitoring studies as well as in toxicological research. This is particularly relevant, and of concern in the estuarine and marine environment where several pollutants function in unison. To fulfill many of the aspirations implicit in biomonitoring programmes several such problems have to be identified and fears of physiologists have to be nullified; more clearly the problems caused by (1) metal-metal interactions, and (ii) metal regulation and the way by which they affect the environmental monitoring programmes (Simkiss and Mason, 1984).

The general accumulation strategies of aquatic invertebrates are reviewed, and the interactive effects of the two well known metal pollutants namely, copper and Zinc were investigated, and the related accumulation processes are discussed in this chapter.

Copper and Zinc, the two transition elements placed very close in the modern periodic table of elements (atomic number

29 and 30, respectively) attracted attention for study because of the fact that they exhibit extremely different toxic action to the organism, the former being the most, and the latter the least toxic metal (Lakshmanan, 1982).

3.2. Literature Survey, State of the Art

The voluminous research findings in toxicological study of metals are mainly addressed to accumulation or body burden of different species of organisms, from laboratory experiments or contaminated aquatic systems. Even though metal mixture toxicity studies, which have received focus of attention recently, have served in one way to reduce the drawbacks inherent in single metal exposure studies, studies on interactive effects of metals (metal- metal interactions) on aquatic organism have not acquired the deserving thrust so far, so also the different accumulation strategies adopted by organisms (Pascoe and Edwards, 1989).

The electrolytic chemistry of metals such as Cu and Zn is comparatively more complicated since they are able to form a wide range of co-valent compounds in physiological salines, and many of their complexes are relatively small, uncharged molecules, lipid soluble, and are potentially capable of crossing cell membranes. Obviously, a number of different uptake systems may be involved and there is reasonably good

evidence for endocytosis, membrane pumps, and organic lipophilic complexes affecting the metal uptake processes (Simkiss and Mason, 1984).

The mechanisms of interactions between constituents of pollutant mixtures and models for assessing multiple toxicity responses have been described by Anderson and D` Apollonia (1978). An account of the different types of pollutant interaction is given by Connel and Miller (1984). According to them the different types of pollutant interactions in kinetic and dynamic phases can be either chemical or physiological;which can be proceeded by altered mechanisms of toxicant uptake, distribution, deposition, metabolism, excretion, altered toxicant receptor binding affinity, and activity.

Sprague and Ramsay (1965) have reported even in early sixties that an additive effect was observed when Cu and Zn were supplied to the juveniles of Atlantic salmon. The pattern of metal uptake in the soft shell clam *Mya arenaria* was found to be markedly varying by the presence of low concentration of other metals (Eisler, 1977). Negilski et al.(1981) after assessing the toxicity of Zn, Cu and Cd to *Callianassa australiensis* (Dana) for a 14-day period concluded that all paired mixtures acted in an interactive manner. Ahsanullah et al. (1981) also reported some interactive effect in the shrimp

particularly Zn and Cd, since both metals enhanced the uptake of each other. The percentage survival of the organism *Clarias lazera* was considerably reduced when Cu and Zn were applied jointly, and it was concluded that Cu and Zn potentiate the toxic action of each other (Hilmy et al.a,b,c; 1987). An excellent example of pollutant interaction in bioindicators is afforded by the work of Luoma and Bryan (1978) on the interaction between lead and iron in the bivalve *Scrobicularia plana*, and the resultant effects on lead uptake. Later work by the same authors (Luoma and Bryan, 1982) also revealed an interaction between silver and copper in the same species.

Underwood (1979) has given a very good description of the interactive effect of trace metals in organism. According to him absorption, utilization, and excretion of many trace elements, and therefore their physiological, pharmacological, or toxicological actions within the cells and tissues of the animal body are greatly influenced by the extent to which other elements or compounds with which they interact are present or absent from the diet and from the body itself. Hence, there is a series of minimum needs or maximum tolerances depending upon the chemical form of the element, duration and continuity of intake, and the amount and proportions of other interacting elements and compounds.

From the available literature it can reasonably be concluded

that the toxicities of metallic pollutants can be altered by the co- presence of other contaminants. Although some mixtures of metal ions exhibit simple additivity of toxic action, many exhibit antagonism or less than additive effects, and some may interact in a synergistic or more than additive fashion (Phillips, 1980). While instances of non - additive interactions between toxicants may not always be due to alterations in the net uptake of one of the components by the other, some cases can certainly be ascribed to alterations in the net accumulation of toxicants. The fact whether such alterations are due to enhancement or depression of uptake or to similar changes in the excretion of pollutants, is uncertain in most cases, and differs in different species also (Phillips, 1980).

It appears that much of the literature reported for effects of mixtures of toxicants on both marine and freshwater biota have been based on the toxic-unit concept alone. This method does not seem to have a sound theoretical framework, even though there are empirical examples supporting the concept (Sprague, 1970; Negilski et al., 1981). It can be considered merely as a guide to levels of toxicants in mixtures that need investigation. This concept appears to be merely a redefinition of the simple similar action model, but unlike that model it has been used to characterize the toxicity of mixtures of pollutants without regard for the dose-mortality

curves of the individual constituents, in particular the shapes of the slopes. There appears to be a need for new models that take interaction into account when attempting to explain the results in such studies. This becomes all the more relevant since it was reported from different corners that when toxicities of mixtures of metals were assessed according to toxic-unit concept, the expected mortalities of organisms in paired and triad mixtures were overestimated. Even when concentrations of mixtures were as high as one third of the 14 d LC_{50} values, mortalities were negligible (Negilski et al., 1981). Possible explanation for some of these differences in results reported by various investigators are :

(1) different rates of absorption of the metals for different concentrations and environmental test conditions,

(2) differences in the physiological condition of test organism, and

(3) differences in models of hypothesized and interpreted results. It is obvious that no clear trends have emerged that would permit a simple generalisation.

Amongst molluscs, most information concerning pollutant interactions involve bivalve molluscs. Consistent dose dependent changes in the concentrations of both major ions (Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}), and trace metals (Fe and Zn) in tissues of the quahog clams, *Mercenaria mercenaria* were found to occur during pesticide exposure (Eisler and Weinstein, 1967). The effects were more evident for Zn in the mantle of the clam.

Animals exposed to pesticides exhibited consistent increase in concentration of Zn in the mantle. The authors failed to explain the mechanisms behind such effects and as in other cases, it remains speculative. But it is to be kept in mind that such interactive effects on pollutant kinetics are important in estuarine organisms particularly from the point of view of biomonitoring. A later report concerning the above species established marked interactions between Hg and Fe in the epithelial cells of the quahog mantle. Inorganic Hg taken from solution by the experimental animals was located (by electron microscopy) in cytosomes of epithelial cells of mantle tentacles. Energy dispersive X-ray micro analysis revealed high levels of both Fe and Hg in these cytosomes which are similar morphologically to metal rich lysosomes found in mammalian tissues after exposure to metals. Hg apparently caused decrease in Fe levels in mantle fringes of the clam at low dosage. The co presence of different metals in the same cytoplasmic organelle and granular sequestration of Zn and Cu in barnacles *Balanus balanoides* etc. reported by some authors are also of considerable interest but clearly such systems require further study, and use of ultra structural techniques of analysis should be increasingly encouraged since our current knowledge of subcellular metal storage is certainly inadequate (Phillips, 1980).

Some species of oyster were also reported to be exhibiting

metal -metal interactions (Brooks and Rumsby, 1967). When they exposed the oyster, *Ostrea sinuata* to very high concentrations of Cd in solution, significant loss of Mn, Pb and Ag from the visceral mass was observed. Uptake of the ^{65}Zn (radionucleide) by the whole soft parts of the oyster *Ostrea edulis* was found to be significantly depressed over the entire 40 day exposure by the addition of very low concentrations of ferric chloride or coboltous chloride, the latter depressing the uptake more than the former. By contrast, both elements increased the uptake of ^{65}Zn into the shell of this species, although the kinetics of ^{65}Zn uptake were different in the presence and absence of the interfering ions (Romeril, 1971). Coombs (1974) studied the biochemistry of Zn and Cu complexes in this oyster species and found that some metal was reversibly bound to amino acids and other small molecules, but about 60% of the total was present in at least two complexes which were associated with cell debris rather than any soluble components. In a back reference by Bryan(1976b) it was reported that Coombs succeeded in isolating specific metallothioneins from this oyster.

It is surprising to note the report that Cd uptake in a scallop was depressed by high ambient levels of DDT and its metabolites discharged from the Palos Verdes sewage outfalls (Schroeder et al., 1967). Phillips (1976) studied the uptake of Cu by *M.edulis* from a solution containing Cd, Pb, and Zn for a thirty five day period, and the results point to the

interactive effects of metals and consequent changes in the uptake processes . As pointed out earlier and as commended by Rainbow et al. (1990) much of the literature on trace metal concentrations in marine invertebrates contains list of data, with authors not in a position to comment on the significance of results. This view point makes a plea for lab experiments as a necessary compliment to field data to gain information on the metal accumulation strategies available to marine organisms. An understanding of the accumulation strategy adopted by a particular organism will then place the investigator in a position to conclude whether a measured trace metal concentration in an organism is high, low, or of little significance (Rainbow et al., 1990). As a general rule, organisms unable to match rates of excretion of trace metals to rates of uptake will necessarily be net accumulator of trace metal. This category includes organisms that do not regulate and those in which regulatory mechanisms are not functioning (break down of regulation).

The storage detoxification process operating in invertebrates has also been reasonably well studied. It is reported that barnacles are unable to excrete significant amount of accumulated Zn and yet have high Zn uptake rate (Rainbow and White, 1989). The possible explanation offered is that barnacle move large volumes of water across the permeable surfaces of their cirri (thoracic limbs) in order to feed, and

they feed on metal rich food sources and Zn is accumulated without significant excretion over its life time and hence the body contents reach high values according to local bioavailabilty. The body Zn content of *Balanus improvisus* from the Thames estuary registered a staggering high concentration of 150000 $\mu g\ l^{-1}$ and the excess Zn is expected to be stored in granules in the form of detoxified zinc pyrophosphate, which is apparently unavailable to play a deleterious metabolic role (Rainbow et al., 1990).

It can be reasonably concluded from the literatures surveyed that marine invertebrates show different accumulation strategies which vary intra- specifically and inter-specifically with different trace metals, and particularly pre-determined by the rate of uptake of the metal, which is again influenced by many factors like *in situ* interactions, and physiological history of the species. In terms of evolutionary generalisations, physiologically advanced members of any group appeared to be capable of partial or complete metal regulation. Less advanced members seem to adopt storage accumulation strategies. Metal accumulation is also affected by the ecological habit of the animal. Sedentary suspension feeding invertebrates with large permeable areas may be committed to high uptake rates that cannot be matched with excretion rates. Regulation of trace metal concentration may not be then a feasible strategy for adoption (or may be

energetically too expensive). Behavioural changes, feeding habit, eccophysiological adaptations to respiratory stress or dessication may also affect important processes of metal uptake and loss, and hence partly predetermine accumulation strategies (Rainbow et al., 1990).

3.3. MATERIALS AND METHODS

Protocols for collection of organism, acclimatisation etc.were the same as described in Chapter 2. Seawater was collected from unpolluted area in Arabian sea far away from the coast in big jerry cans of about 50-L capacity. It was kept in total darkness in the laboratory for 7 days for aging. Particulate fractions(living and nonliving) were allowed to settle. It was then filtered using fibre glass filter, glass wool, and activated charcoal. The seawater was diluted to appropriate salinities using aerated, aged, and pretreated domestic tapwater. Dechlorinated, nutrient compensated, and aged tap water was used for experiments at $< 1x10^{-3}$salinity. Two different test salinities ($13x10^{-3}$and $< 1x10^{-3}$) were selected for the present study due to the following reasons. Previous metal toxicity study on this organism (Lakshmanan, 1982) has revealed important aspects on metal toxicity , but the specific role of salinity,particularly during metal-metal interactions has not been taken into account. It is known that metal toxic responses are very much influenced by this

important factor. The salinity $13x10^{-3}$ was chosen since trial experiments have shown that it nearly represents the optimum salinity regime for this organism (only with respect to the investigating parameters). The metal input into the estuary is on a higher side during monsoon season (Balachand, personal communication) when the salinity is minimum and the habitat population will be exposed to metal ions to a greater extent during this period due to many factors. Hence, the study is extended to near freshwater condition (salinity $< 1x10^{-3}$) also in order to gain some information regarding the general toxic responses, and metal-metal interactions of the organisms.

3.4. Experimental Protocol

Chronic studies usually involve exposure to a toxicant over an extended period of time, typically 30 to 60 days (Rand and Petrocelli, 1985; La point et al., 1989). The effects measured may be lethal or sublethal. Chronic tests are regarded very useful in establishing threshold toxicity values and measuring time independent toxicity relations(La Point et al., 1989). Thirty two days were found to be a sufficient period to derive meaningful trends in such tests (Denton and Jones, 1981), and hence the experimental period in the present study was restricted to 32 days.

3.4.1. Determination of Percentage Survival of the Organisms

Specimens of *Villorita cyprinoides* were maintained in control and metal spiked media for 24 days in salinity <1x10 (freshwater). The metal concentrations in (µg/l) used to expose the organism at salinity $<1x10^{-3}$ was Cu 60, Cu 100, Zn 600, Zn 1000, Cu 60 + Zn 1000, and Cu 100 + Zn 1000. Twenty five animals of approximately the same size (25-30 mm) were exposed to each of the test concentrations for 24 days, and percentage survival recorded on 4^{th}, 8^{th}, 12^{th}, 16^{th},20^{th}, and 24^{th} day. Duplicates were run simultaneously. Mortal animals were removed immediately but mortality recorded at the end of the day. The experiments were repeated with fresh batch of specimens if more than 10 % mortality was observed in control animals during the whole experimental period.

At $13x10^{-3}$ salinity, the toxicant exposure period was extended upto 32 days, and higher test concentrations were used (due to increased survival rate observed). The metal concentrations (in µg/l) used were Cu 300, Cu 600, Zn 600,Zn 1000, Cu 300 + Zn 1000, and Cu 600 + Zn 1000. The percentage survival of the organisms exposed to the above metal concentrations was recorded at 4 days intervals upto 32 days.Twenty five animals of the same size group (25-30 mm) were exposed to each of the test concentrations and 25 specimens

reared in $13x10^{-3}$ salinity served as the control. Duplicates were run simultaneously.

3.4.2. Metal Accumulation Studies

Separate experiments were conducted to assess metal accumulation by the organism. For this, 100 specimens were exposed to each of the following concentrations of the metal ions (Cu and Zn) individually and in combination in salinity $1x10^{-3}$: (freshwater): Cu 60, Cu 100, Cu 60 + Zn 1000, Cu 100 + Zn 1000, and Zn 1000(Tables 3, and 4).The body burden of the metals Cu and Zn, in specimens exposed to the metals individually and in combination was estimated on 4^{th}, 8^{th}, 12^{th},and 16^{th} day. For determining the tissue metal content, two live animals were sacrificed at the above time periods , their flesh dissected, dried, and the metal content estimated as per the procedure described in Chapter 2. Tissue samples of more specimens (pooled) from each time-period were used when dried sample quantity was not sufficient for metal estimation. Duplicates were run through out.Identical experiments were conducted in salinity $13x10^{-3}$, and the tissue metal content of the organisms was estimated at 4^{th}, 8^{th}, 16^{th}, and 24^{th} day post-exposure of organisms to the following metal concentrations, Cu 100, Cu 300, Cu 600, Zn 1000, Cu 300 + Zn 1000, and Cu 600 + Zn 1000 (Table 5, 6).

(NB:The unit for Salinity has been invariably printed as "ppt" in all the the Tables and Figures)

3.4.3. Depuration Study

Depuration study was also conducted as separate experiments. For this the exposure periods, salinity, and concentration were selected based on the metal accumulation data of the organism. Moreover, to compare the depuration trend of different metals, and between individual metals and binary combination of metals, initial metal content in tissues was also taken into consideration. Taking into consideration of these factors, the following pre-fixed concentrations and exposure periods were chosen for depuration study of Cu and Zn.

Salinity < $1x10^{-3}$

1) Exposure period - 8 days

Metal concentrations in the medium prior to depuration - Cu 60 and Cu 100 µg/l

2) Exposure period - 12 days

Metal concentrations in the medium prior to depuration - Cu 60 + Zn 1000, and Cu 100 + Zn 1000 µg/l

Salinity $13x10^{-3}$

1) Exposure period - 16 days

Metal concentrations in the medium prior to depuration - Cu 300 and Cu 300 + Zn 1000 µg/l

Salinity < $1x10^{-3}$

1) Exposure period - 16 days

Metal concentration in the medium prior to depuration - Zn 1000 µg/l

2) Exposure period - 12 days

Metal concentrations in the medium prior to depuration - Cu 60 + Zn 1000, Cu 100 + Zn 1000 μg/l

Salinity $13x10^{-3}$

1) Exposure period - 24 days

Metal concentrations in the medium prior to depuration - Zn 1000, Cu 300 + Zn 1000, Cu 600 + Zn 1000 μg/l

For each metal concentration, single or binary, at salinities < 1 x 10^{-3} and 13 $x10^{-3}$, One hundred specimens were used at the beginning of the depuration study. After the exposure periods of 8, 12, 16, and 24 days as mentioned earlier, the specimens were transferred to metal- free media and maintained there upto 32 days. Tissue metal content was studied at 4, 8, 16, 24, and 32 day intervals. The method followed to study tissue metal(s)content was the same as described in detail in Chapter 2. In binary combination of metals simultaneous analysis of tissue Cu and Zn content was carried out to evaluate the influence of one metal on the other.

The control values of each metal at < 1 x 10^{-3}, and 13 x 10^{-3} salinities were found out at day 4, 8, 16, 24, and 32 from tissues of specimens reared in the respective salinities (without metal load), and the control value given and used in calculating the percentage loss of metal after 32 days of

depuration is the average of the values obtained at these time- periods. At each time-period, in each salinity, pooled tissue samples from a minimum of 2 specimens were used for metal assay. The control group consisted of 100 specimens in each salinity.

3.4.4. Statistical Analysis

The results were analysed statistically according to Snedecor and Cochran,(1967) and Caulcutt and Boddy,(1983). The significant difference between experimental groups and control groups were determined using Students *t*-test (extended co-variance). To compare the effect of different treatments and exposure time data were subjected to statistical analysis using the Anova techniques. The mathematical model used for the purpose was

$$X_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

where X_{ij} = effect of i^{th} treatment on j^{th} exposure days

μ = environmental effect

α_i = i^{th} treatment effect

β_j = j^{th} exposure day effect

ε_{ij} = random error

3.5. RESULTS

3.5.1. Toxicity Evaluation

The percentage survival of the organism exposed to various concentrations of copper and zinc individually and in binary combinations at salinities < 1 x $10^{-3}$and 13 x 10^{-3} is presented in Tables 1 and 2. The dose mortality/survival curves are presented in Fig.2 and 3. Statistical analysis of the data in Table.1. revealed that survival rate was higher in Zn 600, followed by Zn 1000, Cu 60 + Zn 1000, Cu 100 + Zn 1000, Cu 60, and Cu 100 µg/l (salinity <1x10^{-3}). At 13 x 10^{-3} salinity (Table 2) survival rate was higher in Zn 600, followed by Zn 1000, Cu 300 + Zn 1000, Cu600 + Zn 1000, Cu 300, and Cu 600 . There is significant difference ($P<0.01$) in the percentage survival of the organisms due to the toxic action of the metals Cu and Zn when applied singly and in combination. Comparison of the dose mortality/survival curves also showed that there is significant difference in the toxic action of the metals Cu and Zn, when applied singly and in binary combination.

3.5.1.1. Toxicity of copper

The impact of chronic copper stress on survival of the organisms is more visible at lower salinities, particularly in

Table 1

Percentage Survival of Villorita cyprinoides: Salinity <1 ppt

Exposure time/ days	Concentrations of metal ions in µg/l					
	Cu 60	Cu 100	Cu 60+ Zn 1000	Cu 100+ Zn 1000	Zn 600	Zn 1000
0	100	100	100	100	100	100
4	96	88	100	92	100	100
8	72	68	80	76	100	96
12	52	40	68	48	96	92
16	40	28	48	44	96	88
20	32	24	36	32	92	88
24	28	16	36	28	92	84

Significance:
P<0.01(n=25 for each concentration)

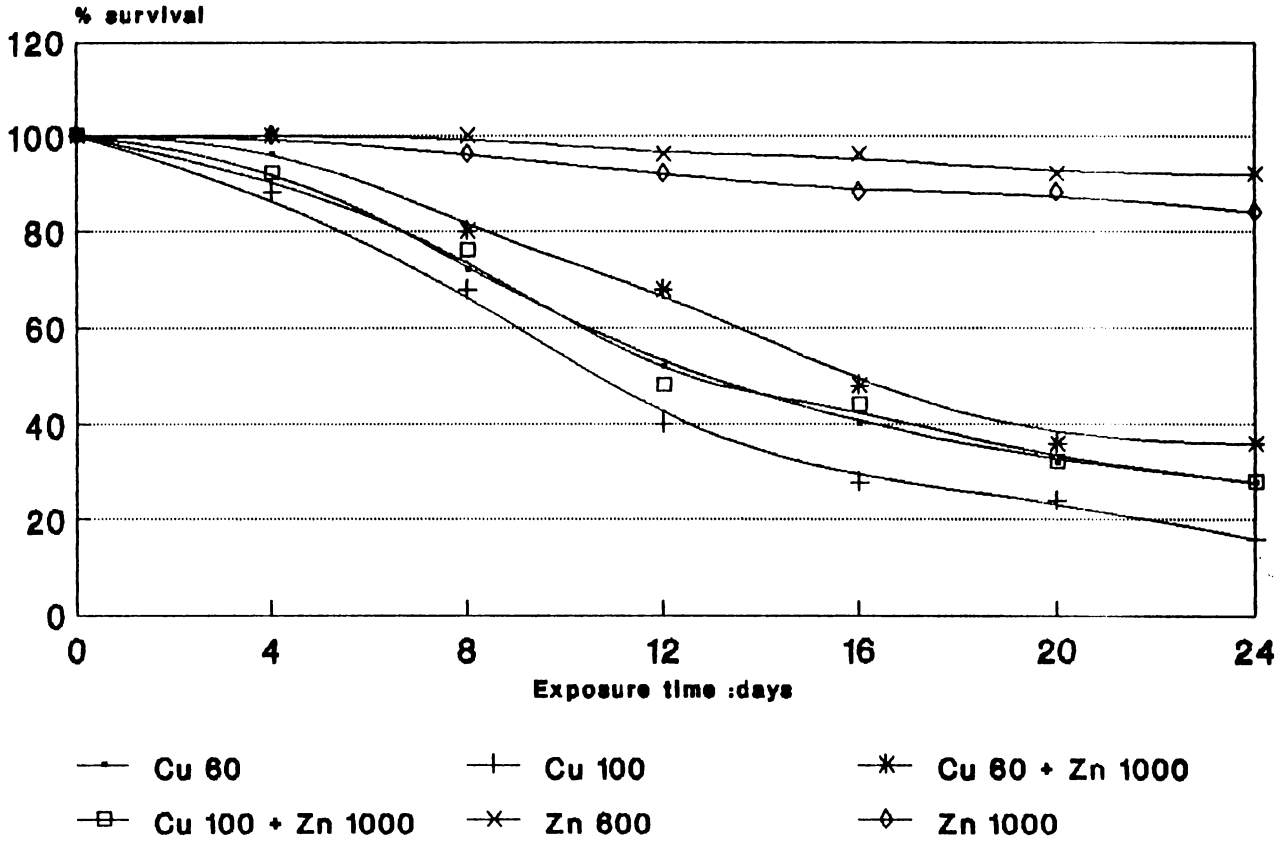
Table 2

Percentage Survival of *Villorita cyprinoides*: Salinity 13ppt

Exposure time Days	Concentrations of metal ions in µg/l					
	Cu 300	Cu 600	Cu 300 + Zn 1000	Cu 600 + Zn 1000	Zn 600	Zn 1000
0	100	100	100	100	100	100
4	96	92	100	100	100	100
8	88	76	96	88	100	100
12	80	72	96	84	100	96
16	56	44	84	72	96	88
20	52	36	76	48	92	88
24	44	28	68	48	88	84
28	40	28	62	40	84	80
32	36	20	48	32	80	76

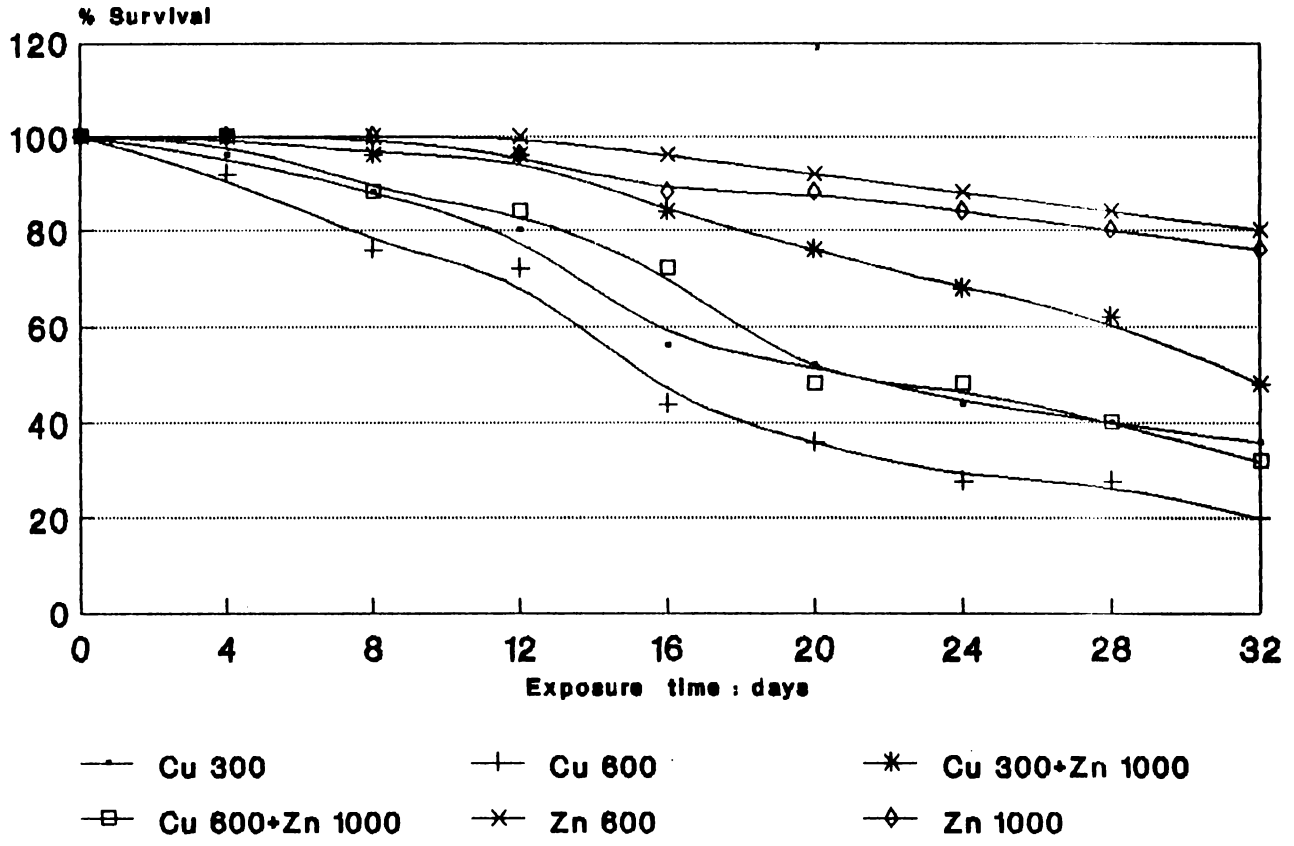
Significance:
$P < 0.01$ (n= 25 for each concentration)

Fig 2 : Dose-Survival Curves
Salinity <1 ppt



(Conc. in µg/l)

Fig 3: Dose-Survival Curves
Salinity 13 ppt



(Conc. in µg/l)

fresh water (salinity < $1x10^{-3}$). A few symptoms of slow Cu poisoning such as secretion of mucus sheet near the opening of shell valves, swelling of the foot, and loss of control in closing the shell valves were noticed in the clam exposed to higher concentration of the the metal ions in both the salinities. The onset of mortality started from 4^{th}day onwards, even at the concentration of 60 µg/ml in fresh water. The survival rate depressed faster after a week, and only 28% of the organisms survived after 16 days in higher concentration of the metal (Fig 2). The 96h LC_{50} in freshwater is only 1/3 of the LC_{50} at $13x10^{-3}$salinity. At higher salinity not only was the onset of mortality delayed, but also the animals survived longer periods even at higher concentration of the metal ions. The slopes of dose mortality curves for all time intervals were not similar, which suggest that the toxic action of Cu did change with time and concentration. This again confirms that short period (96h) toxicity studies will not provide meaningful organismic toxic responses or trends as suggested by Ahsanullah et al. (1981).

3.5.1.2. Toxicity of Zinc

Zinc was found to be the least toxic metal to the clam (toxicities of other metals Hg, Pb, and Cd were also tested but data not presented). The 96h LC_{50} value could not be

determined because of the very low mortality rate even at higher concentrations (>2000 µg/l). The mortality of animals started very late after a lag period of about a week in test concentration of 1000 µg/l (Table 1). The lower toxic responses of the organism to the metal is reflected in the corresponding Dose-Survival curves also (Fig. 2 , 3).

3.5.1.3. Impact of Zinc on Copper Toxicity

Significant variations (P< 0.01) in survival rate of the organism was observed in paired combination exposure of the metals when compared to individual metal exposures. The onset of mortality was delayed in Cu exposed animals when the medium contained Zn also. The percentage mortality was found to be reduced at all days of exposure in all the tested concentrations in both the salinities (Table 1 , 2; Fig. 2, 3). For example, the percentage survival on 4^{th} and 12^{th} days in 60 µg/l exposed organisms in < $1x10^{-3}$salinity increased to 100 and 68 % from 96 and 52 % , respectively. Similarly, in Cu 100 µg/l plus Zn 1000 µg/l exposed organisms the % survival on 16^{th} and 24^{th} days were 44 and 28 % compared to 28 and 16 % survival in Cu 100 µg/l exposed ones (Table 1). At the higher salinity regime also the impact of Zn on Cu toxicity was visible. For example, 96 and 68 % organisms survived in Cu 300+ Zn 1000 µg/l on $12^{th}$and 24^{th}days compared to 80 and 44 % in Cu 300 µg/l exposure (Table 2). The Dose-Survival curves (Fig. 2 , 3)

clearly depict the influence of one metal on the other.

3.5.2. Bioaccumulation of Metals

The efficiency of metal accumulation in the organism is best reflected in Bio Concentration Factor(BCF). In simple terms it is the quotient of the concentration of the substance in the organism and ambient medium (Ernest, 1985). The bioaccumulation potential of a pollutant can be assessed solely in terms of the water vector if the concentration of pollutant in the food vector is not exceedingly high(it is so here). The expression describing the accumulation of a chemical in such a case may be the following. Differential equation of Roberts and Mc.Garrity (1985).

The expression showing the bioaccumulation potential of a pollutant (Roberts and Mc.Garrity,1985) is as follows:

$$\frac{dC_{an}}{dt} = \frac{k_w C_w}{W} - k_{cl} C_{an}$$

where

C_{an} = Concentration of the chemical in the animal

K_w = Rate constant associated with the uptake of the pollutant via water

C_w = Concentration of chemical in water

K_{cl} = the first order rate constant describing the clearance of

pollutant from the organism

W = weight in grams

Until steady state is reached, the measured ratio C_{an}/C_w is a function of time elapsed from the beginning of the exposure. If C_w is relatively constant (concentration of the chemical in the medium can be kept constant by changing the medium daily) C_{an}/C_w at time t is described by the following equation

$$C_{an}/C_w = K_w/K_{cl}\ w\ (1-e^{-kclt})$$

At steady state C_{an}/C_w is constant and equals the ratio $K_w/K_{cl}W$. This is usually referred to as the Bioaccumulation / Bioconcentration Factor (BCF), and is the accepted indicator of the tendency for a chemical to bioaccumulate (Roberts and Mc Garrity, 1985)

3.5.2.1. Accumulation of Copper

The tissue Cu content of the organism after exposure to various concentrations of Cu individually and in combination at different intervals of time in freshwater and at salinity $13x10^{-3}$ is given in Table 3, 5, and Fig. 4, and 5. Statistical analysis (ANOVA techniques) of the data revealed that there is significant difference between treatments ($P<0.05$), and between days($P<0.01$). The least significant difference (LSD) at 5 %

Table 3

Accumulation of Cu in Villorita cyprinoides
Salinity < 1 ppt

Exposure time/ days	Conc. of metal ion in the medium:µg/l			
	Cu 60	Cu 100	Cu 60 + Zn 1000	Cu 100 + Zn 1000
	Tissue Cu content $\bar{x}$ ± SD µg/g (n=2)			
0/ control	19.82 1.36	19.07 1.03	19.22 1.54	19.67 1.87
4	38.76 2.91	54.59 4.88	26.43 2.16	38.67 2.64
8	85.90 6.68	96.73 7.26	44.37 4.92	75.19 6.27
12	109.61 8.92	148.28 11.78	78.36 5.19	109.49 8.94
16	135.48 10.99	165.49 14.73	119.48 9.43	128.73 11.39
BCF	2258.00	1654.90	1991.30	1287.30

SD = Standard Deviation (n=2)
Significance: $P<0.01$

Table 4

Accumulation of Zn in Villorita cyprinoides
Salinity <1 ppt

Exposure time/ days	Conc.of metal in the med. µg/l		
	Zn 1000	Cu 60 + Zn 1000	Cu 100 + Zn 1000
	Tissue Zn content $\bar{x} \pm$ SD		
0/ control	78.73 3.34	76.11 3.27	77.64 3.49
4	92.84 5.04	102.84 5.95	109.52 6.1
8	122.41 9.51	132.46 10.83	146.77 11.25
12	158.54 10.59	198.35 13.74	210.66 13.72
16	169.5 11.79	230.96 15.71	263.49 16.92
B C F	169.5	230.96	263.49

SD = Standard Deviation (n=2)
Significance:
Concentrations: $P < 0.05$
Periods: $P < 0.01$ (except 0, and 4 days)

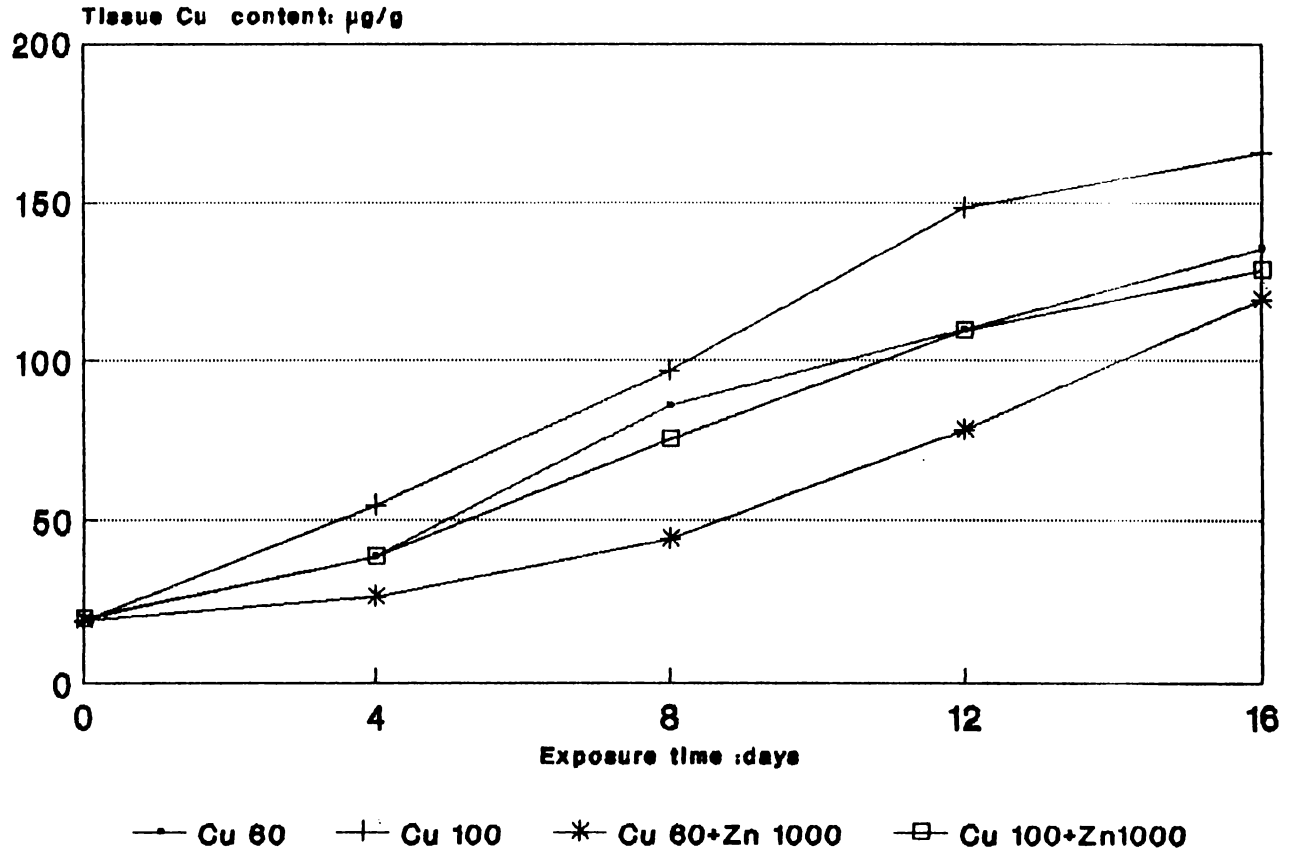
Table 5

Accumulation of Cu in Villorita cyprinoides:Salinity 13 ppt

Exposure time/ days	Conc. of metal ion in the medium : µg/l				
	Cu 100	Cu 300	Cu 600	Cu 300 + Zn 1000	Cu 600 + Zn 1000
	Tissue Cu content $\bar{x}$ ± SD: µg/g (n=2)				
0/ control	19.55 1.23	19.81 1.34	18.94 1.29	19.63 1.93	18.57 1.73
4	21.29 1.27	22.92 1.71	25.78 2.06	20.57 1.39	23.63 1.99
8	45.32 2.79	67.65 5.05	97.62 8.29	54.73 3.11	66.54 5.79
16	62.75 4.98	116.34 7.59	124.59 10.07	98.89 6.25	84.38 7.55
24	79.43 5.31	130.07 11.24	152.67 14.53	114.66 8.57	125.79 10.68
BCF	794.3	433.6	254.5	382.2	209.7

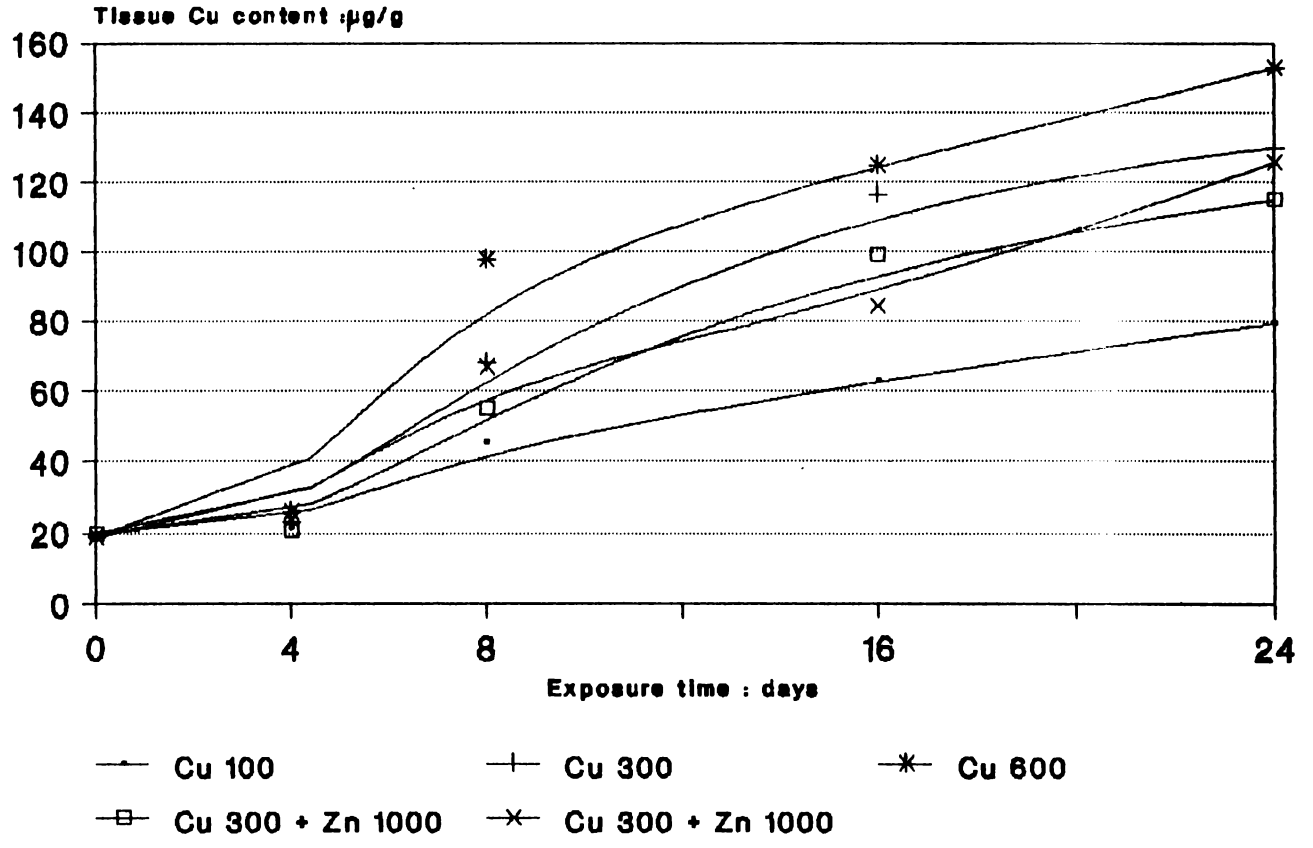
SD = Standard Deviation
Significance :
Concentrations : $P < 0.05$
Periods : $P < 0.01$ (except 0,and 4 days)

Fig 4 : Accumulation of Cu
Salinity < 1 ppt



(Conc. in µg/l)

Fig 5 : Accumulation of Cu
Salinity 13 ppt



(Conc. in µg/l)

level was calculated and means of concentrations and days were separated according to their significance. Table 3 shows that accumulation of Cu was more in Cu 100, followed by Cu 60, Cu 100 + Zn 1000, and Cu 60 + Zn 1000 μg/l. The minimum was observed in Cu 60 + Zn 1000. Among exposure days there was no significant difference between 0, and 4 days. Among 8, 12, and 16 days accumulation was significantly higher in 16 days followed by 12 and 8 days. As shown in Table 5, there was no significant difference between the means of days 0, and 4, but others were significantly different. Regarding concentration of exposure, Cu 100 was having minimum, and Cu 600 μg/l was having the maximum. There was no significant difference between the combinations Cu 300 + Zn 1000 and Cu 600 + Zn 1000 (Saliniity $13x10^{-3}$). The BCF was higher at lower concentrations and lower salinity. The BCF decreased when concentration and salinity of the medium increased. The BCF was 2258.3 when concentration was 60 μg/l in freshwater, while it was reduced to 254.5 when the concentration was increased to 600 μg/l, and the salinity to $13x10^{-}.^{3}$ The initial rate of uptake of the metal by the organism was faster in freshwater and a plateau seems to have reached during progress of time (Fig. 5). The tissue metal content rose to 85.90, and 96.73 μg/g after 8 days of exposure at concentrations of 60 and 100 μg/l, respectively, of the metal ion Cu in freshwater (Table 3), while exposure to the same period at concentrations of 100,300, and 600 μg/l at salinity $13x10^{-3}$,

registered a tissue content of 45.32, 67.65, and 97.62 μg/g only (Table 5). At the end of the observation period (24 days), the corresponding values at salinity 13x10 were 79.43, 130.07, and 152.67, respectively, with 794.3, 433.6 and 254.5 as BCF values.

3.5.2.2. Accumulation of Zinc

Zn accumulation in the organism followed a pattern different from Cu. The body Zn burden increased slowly compared to Cu (Table 4, 6 ; Fig. 6, 7). Hence, the BCF value was also low. The tissue Zn content increased slightly to 169.5 μg/g from the average control value of 78.73 μg/g during the 16 day exposure in 1000 μg/l metal concentration at $1x10^{-3}$salinity (Table 4). The corresponding bio concentration factor was only 169.5. Similar trend was observed in higher salinity regime also (Table 6).

3.5.2.3. Influence of Zinc on the Accumulation Pattern of Copper

Significant changes ($P<0.05$ between concentrations, and $P<0.01$ between days) in the accumulation pattern of Cu in the organism was noticed when the organisms were subjected to combined exposure (Cu and Zn in different permutations and combinations of concentrations in the medium). The body Cu

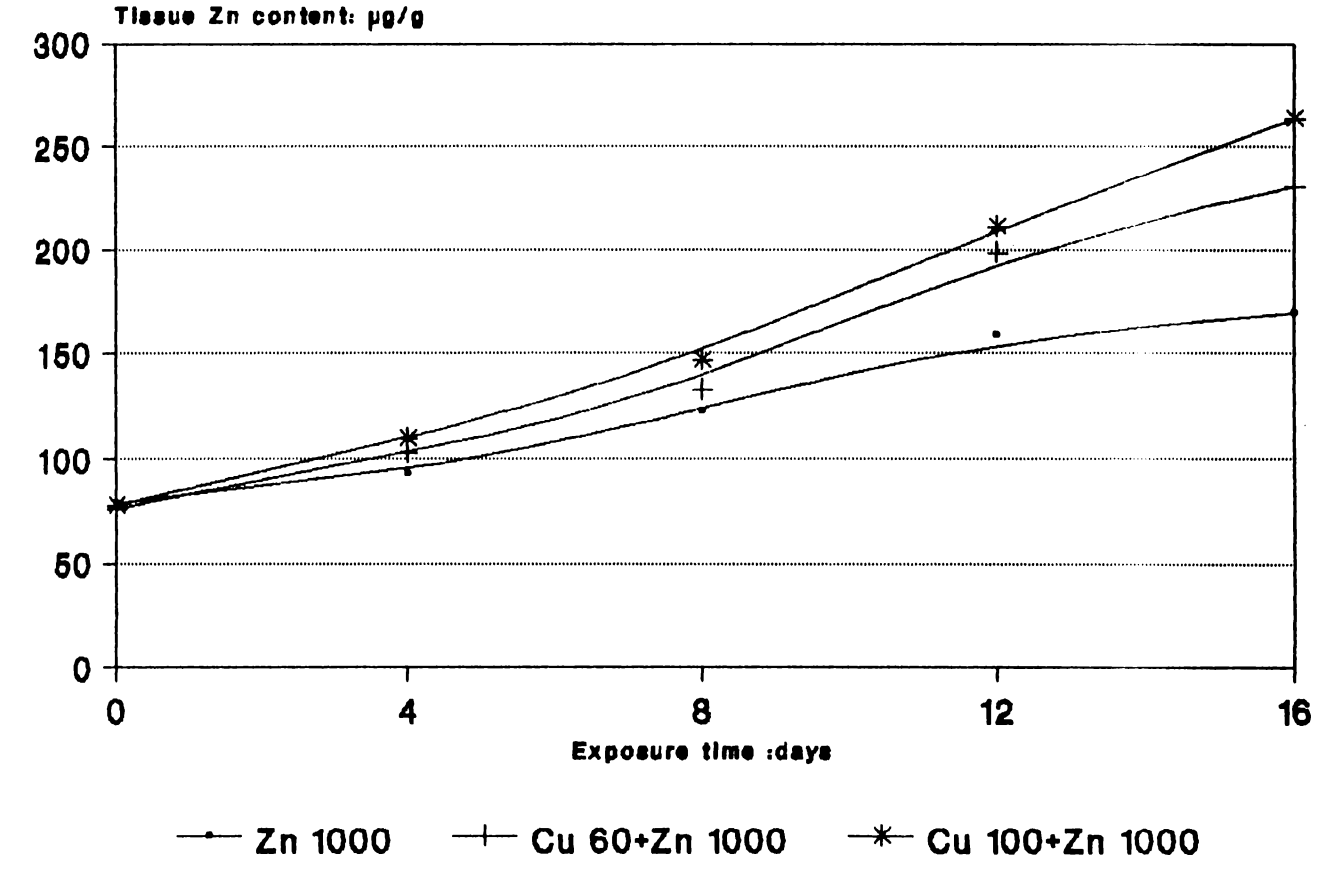
Table 6

Accumulation of Zn in Villorita cyprinoides
Salinity 13 ppt

Exposure time/ days	Conc.of metal in the med.µg/l		
	Zn 1000	Cu 300 + Zn 1000	Cu 600 + Zn 1000
	Tissue Zn content $\bar{x}$ ± SD µg/g		
0/ control	79.45 4.67	78.57 4.39	79.1 4.07
4	88.74 6.53	92.72 7.94	97.65 8.2
8	120.53 9.69	147.62 10.59	158.24 11.21
16	150.52 11.76	176.77 12.62	189.55 13.79
24	190.37 13.79	221.34 15.2	245.74 17.32
BCF	190.37	221.34	245.74

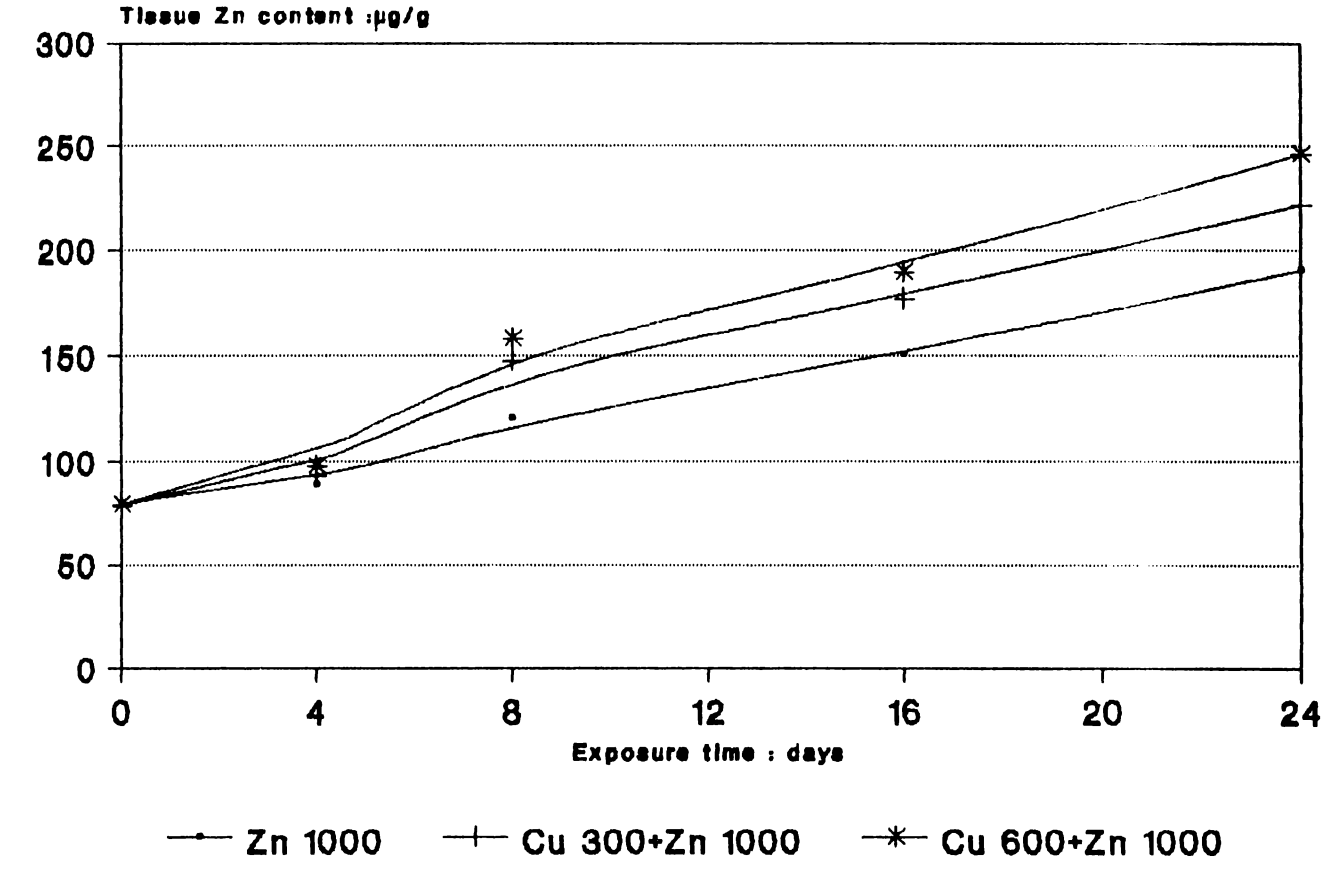
SD = Standard Deviation (n=2)
Significance:
Concentrations: $P<0.05$
Periods: $P<0.01$

Fig 6 : Accumulation of Zn
Salinity <1 ppt



(Conc. in µg/l)

Fig 7: Accumulation of Zn
Salinity 13 ppt



(Conc. in µg/l)

burden was found to be less on all days of exposure when compared to that of the organisms exposed to Cu alone(Table 3, 5, Fig. 4, 5). For example, the body Cu content was only 119.48 in the medium containing Cu 60 + Zn 1000 against 135.48 µg/g in the medium containing Cu 60 alone after 16 day exposure period at salinity < 1 x 10^{-3} The BCF also got reduced to 1991.3 from 2258.0. In the case of Cu 100 + Zn 1000 µg/l exposed organisms, the BCF was reduced to 1287.3 from 1654.9(Table 3). At higher salinity regime also the effect of Zn on the accumulation pattern of Cu was visible (Table 5 , Fig. 5). The metals Cu and Zn in the organism was found to be significantly correlated statistically
(r = 0.977)(r = 0.9611 from computer analysis) (Table 12)

3.5.2.4. Influence of Copper on the accumulation of Zinc

Zn uptake by the animals seems rather enhanced when the medium contained Cu even in lower amounts. A breakdown in the quantitative relationship between Zn in the ambient medium and the body was evident in such an environment. When the organisms were maintained in a medium containing a mixture of Cu and Zn (Cu = 60 and Zn = 1000 µg/l) the tissue Zn content was 230.96 µg/g after 16 days against 169.5 in the medium that contained Zn alone (in salinity <$1x10^{-3}$)(Table 4). In binary combination Zn content in the organism showed higher values on all days of exposure in both salinities (Table 4, 6) .

Statistically there was no significant difference in the values of 0 and 4 days but in other days the values were significant (Table 4). The minimum was observed in 0 day and the maximum in 16 days. The accumulation of Zn was minimum in Zn 1000 but maximum in the combination Cu 100 + Zn 1000. A comparison of accumulation trends (Fig. 6,7) in single metal exposures, and paired metal exposures in both salinities clearly shows the influence of Cu on Zn.

3. 5.3. Efficiency of Depuration

The amount of Cu and Zn retained in the body after different periods of depuration is presented in Table 7, 8, 9, and 10. The depuration trends are presented in Fig. 8, 9, 10, and 11. The data of binary combinations were compared with the data on depuration trend of the organisms exposed to single metals, Cu and Zn, and were found to be statistically significant ($P < 0.01$). Eventhough minor discrepancies were visible, the depuration trend was comparable and predictive.

3.5.3.1. Depuration of Cu

The rate of loss of Cu from the body was comparatively slow. *i.e;* the body retained higher amount of Cu. Even after 32 days of maintenance of the organism in metal- free freshwater, the loss in accumulated Cu content was only 71.04 % in Cu 100 (

Table 7

Cu depuration in Villorita cyprinoides : Salinity < 1 ppt

Exposure-Metal con. prior to depuration - µg/l	Tissue Cu content-Initial- µg/g	Depuration interval:days					%loss at 32 days
		4	8	16	24	32	
		Tissue Cu content $\bar{x} \pm SD$ µg/g (n=2)					
Cu 60	79.07	74.22	61.32	40.55	39.69	37.45	69.77
*	4.77	4.07	3.75	2.16	1.98	2.62	
Cu 100	95.96	87.52	79.83	53.33	46.25	41.58	71.04
*	8.67	7.24	4.07	4.82	3.63	5.04	
Cu 60 + Zn 1000	83.12	59.34	50.56	39.5	34.11	29.07	84.26
**	9.18	3.12	6.97	2.77	3.83	1.97	
Cu 100 + Zn 1000	108.65	86.14	56.73	47.39	38.98	34.54	78.15
	10.71	7.17	3.83	3.18	2.02	2.74	

* 8 days exposure
** 12 days exposure
Control = 19.42 µg/g
Significance: $P < 0.01$

Table 8

Cu depuration in Villorita cyprinoides : Salinity 13 ppt

Exposure-Metal con. prior to depuration - µg/l *	Tissue Cu content initial µg/g	Depuration interval:days					%loss at 32 days
		4	8	16	24	32	
		Tissue Cu content $\bar{x} \pm$ SD (n=2)					
Cu 300	122.53 7.14	120.16 6.97	116.11 8.75	88.19 7.52	69.73 7.22	56.95 8.68	63.91
Cu 300 + Zn 1000	113.28 8.95	107.76 7.84	95.43 8.45	61.76 6.64	42.77 5.46	39.78 4.21	78.32

* 16 days exposure
Control (Average) = 19.5 µg/g
Significance: $P < 0.01$

Table 9

Zn depuration in Villorita cyprinoides : Salinity < 1 ppt

Exposure-Metal con. prior to depuration - ug/l	Tissue Zn content-Initial- ug/g	Depuration interval : days					% loss at 32 days
		4	8	16	24	32	
		Tissue Zn content x + SD : ug/g (n=2)					
Zn 1000	178.84 * 17.96	168.73 10.34	154.02 11.51	135.7 7.11	120.29 10.14	103.23 8.17	79.19
Cu 60 + Zn 1000	185.22 ** 16.14	180.74 15.09	170.42 13.96	165.22 12.93	155.18 12.31	128.66 9.75	52.14
Cu 100 + Zn 1000	201.04 ** 17.22	191.55 16.77	186.25 13.12	173.13 14.54	166.88 13.01	148.74 15.91	42.45

* 16 days exposure
** 12 days exposure
Control (Average) = 77.81 ug/g
Significance: P < 0.01

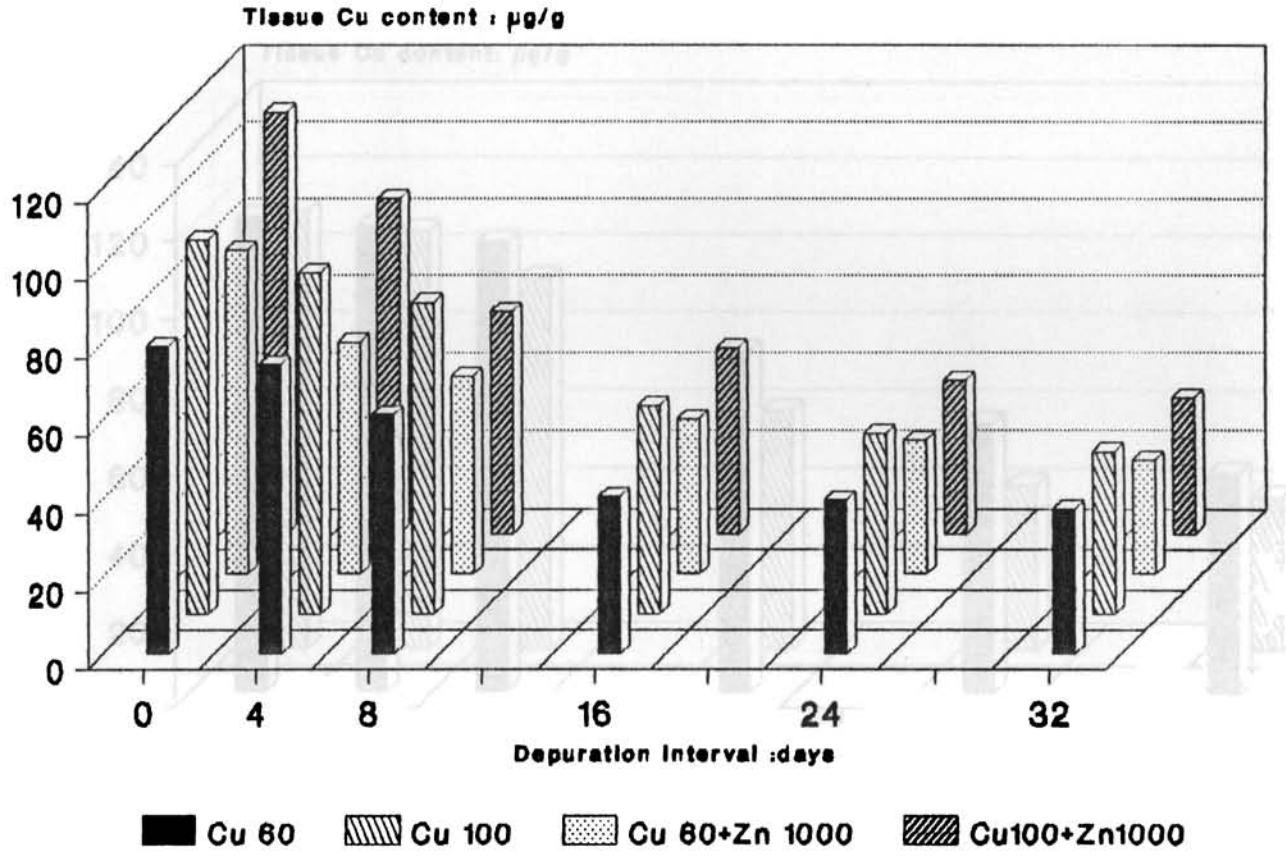
Table 10

Zn depuration in Villorita cyprinoides : Salinity 13 ppt

Exposure-Metal con. prior to depuration - µg/l *	Tissue Zn content-Initial- µg/g	Exposure time : days					% loss at 32 days
		4	8	16	24	32	
		Tissue Zn content $\bar{x} \pm$ SD : µg/g (n=2)					
Zn 1000 ug/l	196.59 15.28	173.54 14.7	158.51 14.1	123.16 15.79	104.1 9.34	94.37 8.35	86.8
Cu 300 + Zn 1000	219.28 15.73	186.39 15.28	179.38 14.99	168.52 14.86	156.12 13.88	139.66 12.54	56.8
Cu 600 + Zn 1000	238.51 17.84	209.96 15.73	196.14 13.7	179.59 12.98	165.95 11.22	153.98 10.57	52.9

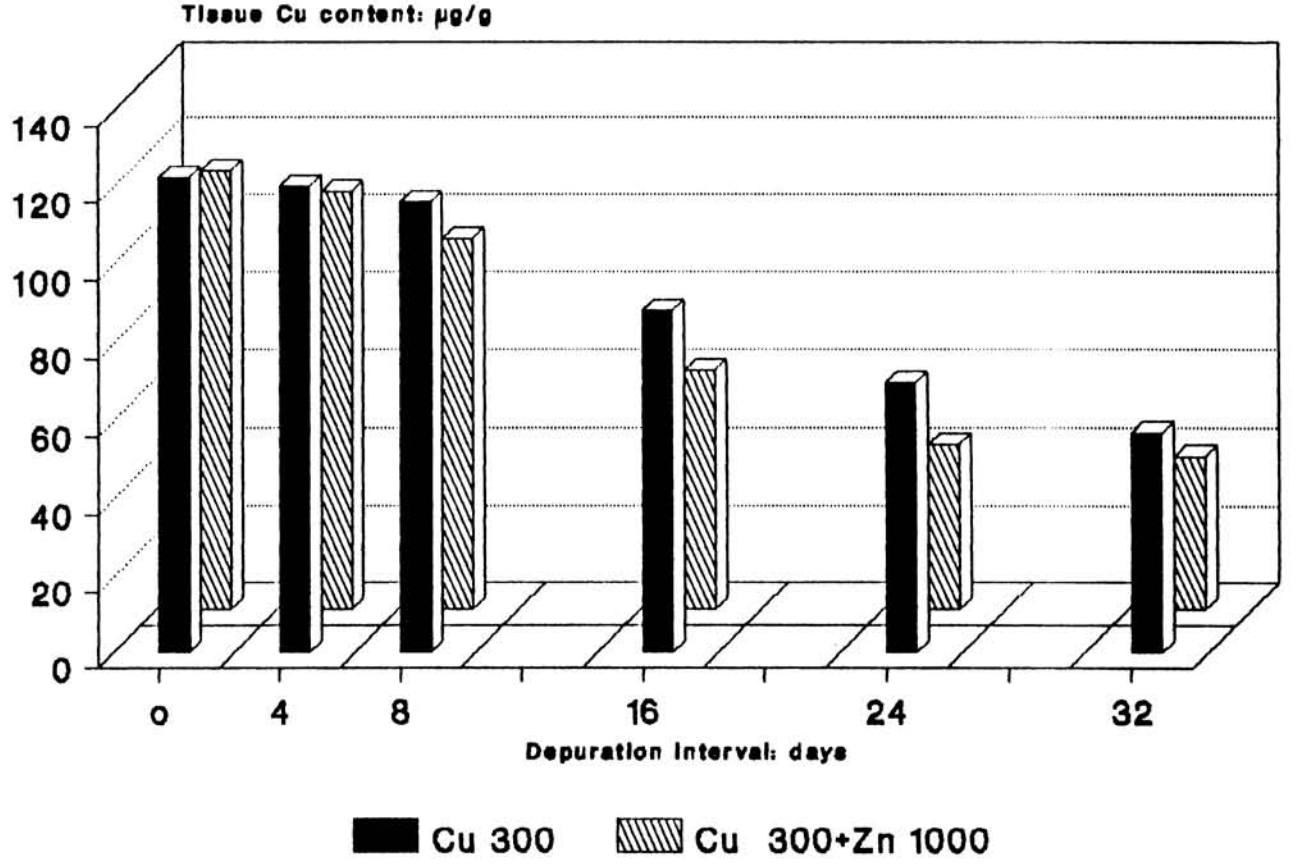
* 24 days exposure
Control (Average) = 78.96 µg/g
Significance: $P < 0.01$

Fig 8: Depuration of Cu
Salinity <1 ppt



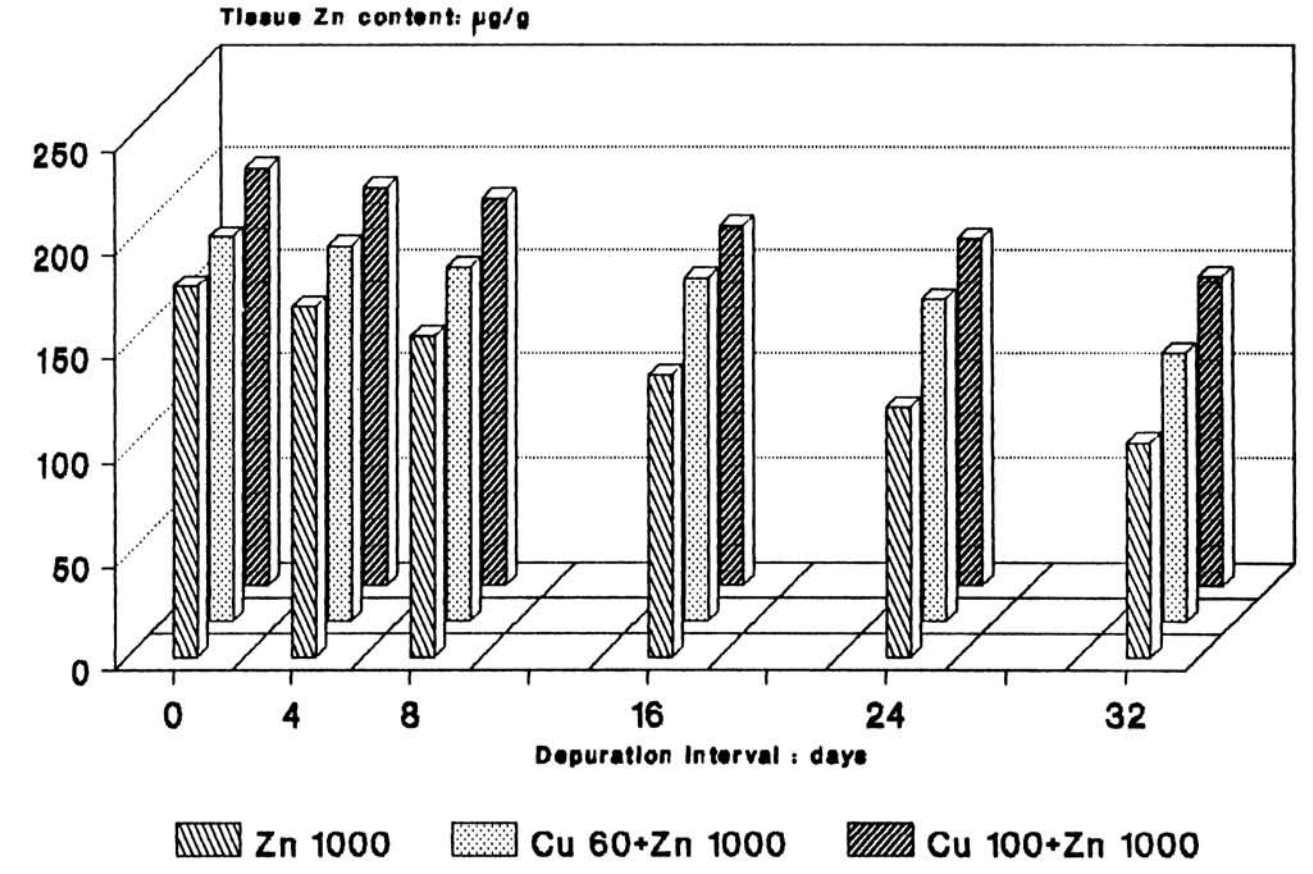
(Conc.in µg/l)

Fig 9 : Depuration of Cu
Salinity 13 ppt



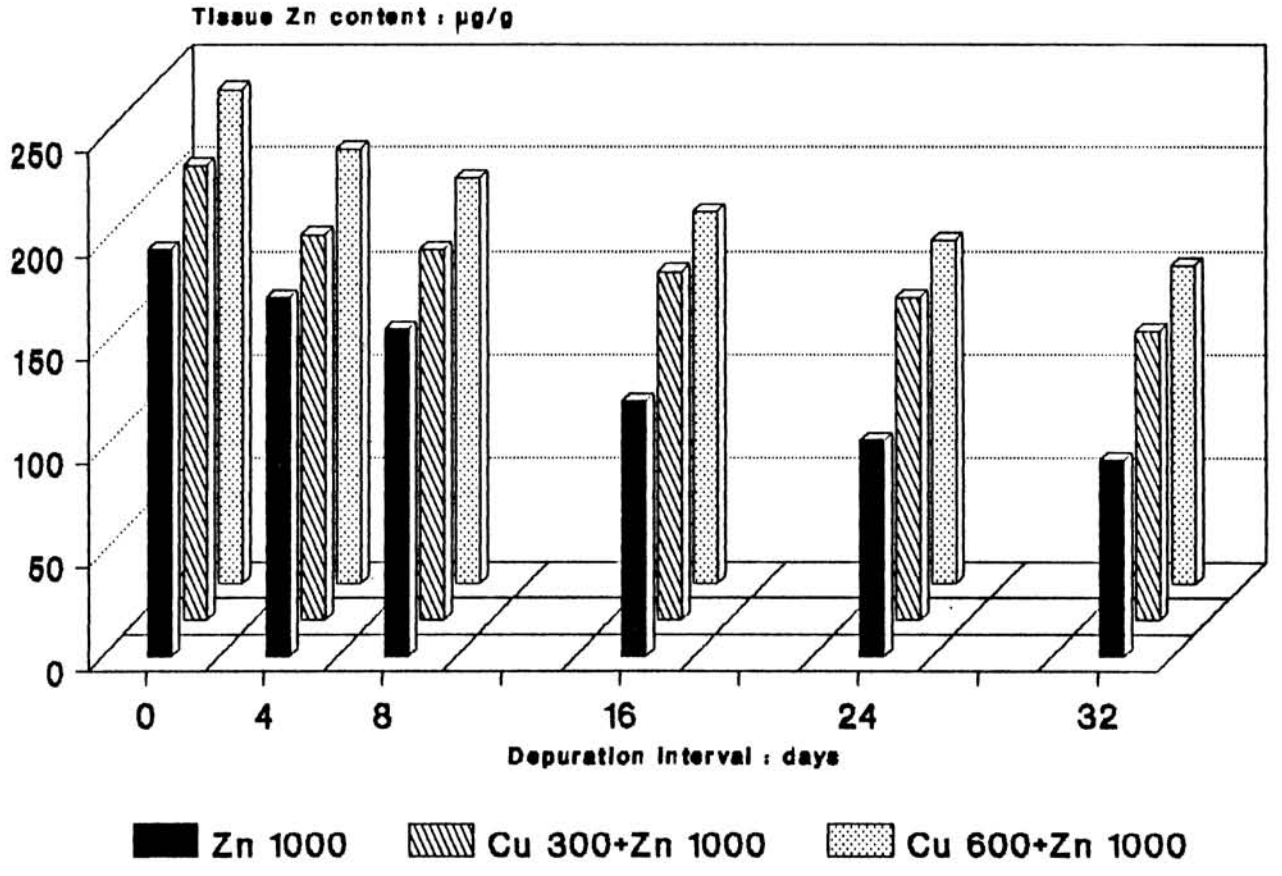
(Conc.in µg/l)

Fig 10 : Depuration of Zn
Salinity <1 ppt



(Conc.in µg/l)

Fig 11 : Depuration of Zn
Salinity 13 ppt



(Conc.in µg/l)

from 95.96 to 41.58 µg/g), and 69.77 % in Cu 60 µg/l (from 79.07 to 37.45 µg/g) (Table 7). The depuration rate was even less in the higher saline medium. .The percentage loss of accumulated Cu was only about 63.9 % in Cu 300 µg/l for the same depuration interval (Table 8). Tissue Cu content failed to reach control values even after prolonged depuration period.

3.5.3.2. Depuration of Zn

The rate of loss of Zn was rather faster than that of Cu.More than 79 % of the accumulated Zn in Zn 1000 was lost in freshwater during 32 days depuration period (Table 9),*i.e*; the tissue Zn content decreased to 103.23 from 178.84 µg/g , while in $13x10^{-3}$ salinity Zn content in Zn 1000 dropped to 94.37 from the initial value of 196.59 µg/g, registering a loss of more than 86 % of the accumulated Zn from tissues (Table 10). After 32 days of depuration the Zn body burden seems to reach more or less control values, in salinities $1x10^{-3}$, and $13x10^{-3}$.

3.5.3.3. Influence of Zn and Cu on the Depuration of each other

The kinetics of metal loss of the organism was found to be affected by the presence of the other metal. Copper was found to be lost at a faster rate when the tissue contained higher amounts of Zn. On the other hand the body tried to retain

higher amounts of Zn, when the Cu residue in the tissues was in excess. In freshwater depuration the tissue lost about 84% of the accumulated Cu in Cu 60 + Zn 1000 when compared to about 69 % in Cu 60, and 78 % in Cu 100 + Zn 1000 as against 71 % in Cu 100 (Table 7). The higher depuration rate of Cu is attributable to the presence of Zn. Similarly in $13x10^{-3}$ tissue Cu content dropped to 39.78 in Cu 300 + Zn 1000 when compared to 56.95 µg/g in Cu 300 µg/l (Table 8). The depuration trend of Zn was found to be slower in the presence of Cu. The tissue lost only 52.14 % in Cu 60 + Zn 1000, and 42.45 % in Cu 100 + Zn 1000 of the accumulated Zn within 32 days in freshwater compared to 79.19 % when Zn alone was depurating (Table 9). In salinity $13x10^{-3}$ tissue Zn content after depuration was 139.66 in Cu 300 + Zn 1000, and 153.98 µg/g in Cu 600 + Zn 1000 when compared to 94.37 µg/g in Zn 1000 (Table 10). There exists strong correlation between the metals Cu and Zn in the tissues of the organisms. The correlation coefficient *r* between the metals was 0.9611 (Table 12).

3.6. DISCUSSION AND CONCLUSION

The results indicate that Cu is highly toxic to the organism particularly at very low salinity. This metal gets accumulated at a faster rate, and is retained for a considerable period of time in tissues; while Zn is less

Table 11

Physico Chemical Properties of Cu and Zn

Property	Cu	Zn	Reference
Electro negativity	2.0	1.6	Pauling (1975) and Meites (1963)
Ionic radius M ++ ion	0.72 A	0.83 A	
Pks values metal sulphides	35.2	23.8	

Table 12

Regression Output:

Constant	83.19325
Std Err of Y Est	8.471596
R Squared	0.923731
No. of Observations	6
Regression Output:	4
Constant	2
X Coefficient(s) 0.646959	0
Std Err of Coef. 0.092949	1
Correlation co efficient r	0.9611

toxic irrespective of very high ambient concentrations and tissue load. The presence of one metal in the medium is affecting the uptake and retention of the other. The toxicity of Cu is reduced significantly in presence of Zn. The depuration rates were also affected. The interactive effects of Cu and Zn is evident in the organism.

The toxic effects of heavy metals in the organism are due to a great extent to the damage caused to the the enzyme systems. Inhibition of enzymatic activity is suggested to be preceeded by incorporation of the metal in the enzyme molecule through selective chemical modification, affinity labelling, metal chelation, non- covalent binding of inhibitors, and analogues (Lavie and Nevo, 1986). Once the metal ions are influxed into the body co- ordinated complexes are formed with ligands like -SH, amino, hydroxyl, phosphate, carbohydrate, imdazol etc., which result in structural changes and enzymatic activities of proteins culminating in toxic effects evident at the whole organism level (Oeheme, 1976; Hodson, 1988).

Since the metals Cu and Zn were found to be behaving very differently in the organism, the former imparting high toxic responses and the latter remaining less harmful, it is assumed that the degree of toxic action of the metals is different. Even though each metal ion is having its own function as far as the enzymatic activity is considered, metal ions exert their

toxic influence by covalent binding at cell surfaces, and that only a small difference in ionic size is sufficient to cause one metal to be biologically active and another not to be. The general concept of inorganic chemistry that the higher the electronegativity and PK_s values of the metal sulphides and lower the ionic radius of the metal ion the more toxic the metal would be (Heslop and Jones, 1976; Lakshmanan, 1982) seems valid in this context . The Physico- chemical properties of Cu and Zn presented in Table.11 will be helpful in comparing the toxic action of the metals. The difference in the steriochemistry of Zn and Cu co-ordination complexes, and the interactions between the ligands and charge field created by ligands themselves may possibly disrupt the biological system favourably or unfavourably resulting in reduction or increase in toxicity of the metal ions (Lange, 1969; Heslop and Jones, 1976; Cotton and Wilkinson, 1979). Moreover, the electrolytic and co-ordination chemistry of the metals, Cu and Zn, are very different, particularly with respect to their oxidation state and considerably more complicated since they are able to form a wide range of covalent and co-ordination compounds in physiological salines. As pointed out earlier, many of the complexes are capable of crossing cell membranes due to their relative smaller size and neutral charge. Obviously, a number of different uptake systems may be involved, depending upon the metal ions (Simkiss and Mason, 1984), and causing different toxic actions.

The results have indicated that the organism exhibits higher tolerance to Cu in presence of Zn which implies the interactive effect of the metals (tolerance in this context is used to express the ability of the organism to show less response to a specific dose of a chemical than it showed on a prior occasion from the same dose). The reduced toxicity of Cu to the organism may be due to the reduced uptake of Cu into the tissues in presence of Zn (Table 3 , 4). Zn , in this context, may be acting as an ameliorating agent as far as Cu toxicity to the organism is considered. Hence, the onset of mortality was delayed and the percentage survival of organisms was higher (Figs.2 ,3), when the medium contained excess amounts of Zn. Zn may be expected to give some kind of protection to the clam from the toxic action of Cu. The enhanced accumulation of Zn from the medium and higher retention time of it support this argument. This is followed by reduction in assimilation of Cu also. There may exist some threshold ratio of Cu to Zn which is most effective in this regard.

The interactions between constituents of pollutants can be, according to Connell and Miller (1984a), either chemical or physiological within the kinetic phase, by altering mechanisms of toxicant uptake, distribution, deposition, metabolism and excretion, and in the dynamic phase by altering toxicant-recepter binding affinity and activity . Based on the

theory of Rainbow et al.,(1990) reduction in toxicity of a metal consequent to depression in assimilation rate can also happen if the passive diffusion along the concentration gradient involving the binding of free Cu $^{2+}$ions to some carrier is hindered or blocked. The degree of involvement of the different mechanisms is a matter of speculation. The reduction in assimilation of Cu observed here in presence of Zn is almost similar to that reported by Turner and Swick (1983), and Boisson et al. (1989) in the uptake of Hg in presence of selenium. The protection offered by Se to organism from the toxic effect of heavy metals is widely discussed (Pymbroeck et al., 1982; Boisson et al., 1989).

Hilmy et al. (1987c) have also observed, in a similar context, that the uptake of one metal (Cu or Zn) was decreased in presence of the other, even though they were also not able to derive the underlying physiological mechanisms. Lavie and Nevo (1986) have commented that the specific results obtained as a result of interactive effect of the metals may be as well as a consequence of the formation of an amalgam of Cu and Zn which behaves in a specific way different from Cu.

In the present study, the obvious role of Zn in extricating/ extunating the toxicity of Cu will be definitely related to the threshold ratio of the metals in tissues. Even though post-mortom analysis were carried out to have some insight into this

and also to derive the ratio, statistically significant data were not available, mainly due to the severe experimental constraints in this regard. As per the general observation it can reasonably be concluded that the effective ratio of Cu to Zn in tissues which can offer reasonable protection to the organism from Cu toxicity is 1:7.

According to Lobel (1987), the ratio of Zn to other metals is an important factor in determining the extent of damage to the cells of organism. Bryan (1984), has also, after observing very high concentrations of Zn in contaminated oysters (toxicity was not visible), suggested that toxicity is not simply a function of dietary concentrations but rather imbalances between concentrations of different metals, and added that the tolerance level of a toxic metal depends not only on its chemical form but also on its ratio to metals with which it interacts.

Absorption from solution by the organisms involves passive diffusion of the metal probably as a soluble complex, down gradients created by adsorption at the surface, and binding by constituents of the surface cells, body fluids and internal organs. The permeability of various ions is of considerable importance in determining the tolerance to metals. Even though there is no evidence that any animal can prevent the entry of metals by changing the permeability rapidly, in this organism

the possibility cannot be ruled out in presence of Zn . A change in metal oxidation state can alter the permeability of cells to metal ions (Davis, 1976). Whether such a possibility exists with respect to Cu in presence of excess Zn in physiological salines, resulting in reduction of overall toxicity remains a matter of speculation. Sutherland and Majors (1981) have reported an important finding in this respect, *i.e.*, a 300 % increase of Zn in the gills during elevated external Cu levels in *Mytilus edulis*.

The detoxification mechanisms operating in the organism during single metal and combined metal exposures may involve storage of metals at inactive sites on a temporary basis by binding to proteins, polysaccharides, and amino acids in soft tissues or body fluids (Connell and Miller, 1984). Accumulation usually involves detoxification of the metal to render it unavailable metabolically and restrict potential toxic effects. Detoxification strategies appear to be of two extreme types, viz., detoxification by temporary storage, and excretion. Accumulated metals may be processed into detoxified metal-rich granules which may have the potential to be excreted, eg., bivalves detoxify accumulated trace metals into granules in the kidney (thus promoting a high body metal concentration), and such granules are excreted (George and Pirie, 1980). Ultrastructural and biochemical analyses of granules isolated from isopod hepatopancreas (the primary soft

tissue site of heavy metal storage in isopods is the hepatopancreas, which stores more heavy metal than any other soft tissue of any other animal) revealed that the metals are localized in both membrane bound granules and smaller non-membrane bound aggregations. In some cases the hepatopancreas becomes so saturated with the metals that the excess is found in the hemolymph (Tomita et al., 1991).

The physiological and detoxifying roles of the metalloproteins, metallothioneins, are described in a separate chapter. It may be noted that since Zn is the primary constituent of metallothioneins, and if the concentration of the metals taken up into the cell is high and the metals saturate the physiological pool of (Zn-Cu) thioneins, then the excess cations can stimulate the synthesis of new thioneins (Viarengo, 1985). The high rate of uptake of Zn (in presence of copper), even when the Concentration of Zn in the medium was very less points to this aspect also.

Munzinger and Guarducci (1988) have described the process of excess accumulation of Zn under heavy metal stress to act on diverse enzymatic reactions, even under lowered metallothionein production. The lysosomes, the subcellular organelle, which are one of the targets of heavy metals (hence accepted indicator of cellular stress) and the most important sites of compartmentation in the cell may also be playing its role since

elimination of metallothioneis is also a known function of lysosomes (Moore et al., 1982; George, 1983). The physiological / biochemical bases for the effect of Cu on the distribution of other metals particularly Zn are not yet fully understood. The mechanisms by which metals are mobilized, transported, and sequestered by critical tissues in organisms are also not fully known and further study is necessary to elucidate their role in heavy metal detoxification (Tomita et al., 1991).

The following may be mentioned about the *in situ* environmental implication of the present observations:

i) It is to be believed that in any aquatic bivalve population there may exist a smaller percentage (~ < 30 %) of members which are relatively very tolerant and resistant to toxicants. Their survival probability during exposure to toxicants may even become brighter if the medium contains ameliorating agents like Zn or similar type of constituents . But it will be intriguing to note that these tolerant organisms carry higher concentrations of toxicants particularly heavy metals in their soft tissues and this can pose severe health hazards to predators including man,

(ii) The quantitative relationship of metal burden particularly Zn in the body of the organism and the ambient medium is no longer valid unless the qualitative and quantitative aspects of other metals in the medium are also taken into account and

necessary corrections and/or allowances are incorporated, iii) The accumulation strategies of this organism and similar type of organisms require further studies, particularly with respect to the metal-metal interactions and especially from the angle of biomonitoring. This becomes all the more relavant, since it is now increasingly being reported in sentinel organisms from different parts of the world about the unusual existence of metabolic process which protects the organisms from the potential toxic effects of the metals by its lack of uptake, or its active excretion, (Phillips, 1989).

The body metal residue in any organism results from the net balance between the processes of metal uptake and loss. The relationship between the metal uptake and loss brings about a particular metal accumulation strategy in any organism. At this point the interactive effect of metals *in vivo* and *in vitro* plays a crucial role. The conclusion that can be drawn from this study and similar type of studies is that the uptake mechanisms undoubtedly influence the results that are obtained in bioaccumulation studies. More attention should be paid on this aspect and especially the way through which inter metal influences affect the processes involved. As suggested by Rainbow et al.(1990) knowledge of the accumulation strategy of a particular trace metal is a pre-requisite for understanding the significance of an observed metal concentration in an organism and this knowledge can only be gained from such continued laboratory experiments.

CHAPTER 4

SUBLETHAL METAL STRESS AND PHYSIOLOGICAL ADAPTIVE RESPONSES OF VILLORITA CYPRINOIDES

4.1. INTRODUCTION

When changes occur in the environment, individual organisms either resist the change (resistance adaptation), or adapt to it in a compensatory manner(capacity adaptation) at a rate and to a degree that is within their particular scope of achievement.When this ability is overly taxed in either duration or magnitude, the organism is said to be stressed and less competitive (Dillon and Lynch 1981; Hoar, 1987). The effect of the stress may be lethal to the organism, or simply alter a physiological process, or cause increased susceptibility to other sources of mortality. Most man-induced stresses are similar to those which occur naturally, but they may differ in intensity and quality. Stress exerts an energy cost and interferes with the normal function of a system.

The observed toxic effect of organism depends upon the overall balance between pollutant uptake, metabolism, bioaccumulation, clearance etc., and consequently factors which influence physiology may alter the rate at which the toxokinetic and toxodynamic processes occur. To study the intrinsic factors which affect the physiolgical status of the organisms, careful monitoring of the physiological responses is

essential to asses the impact of low level stressors (Pascoe and Edwards, 1989).

We are less advanced in our understanding of the sequelae of ecosystem distress (Nriangu, 1989). The mammalian response to stress is characterised by three stages, (1) the alarm reaction, (2) the stage of resistance where adjustment and accommodations can be made, and (3) the stage of exhaustion leading ultimately to death. Since the main objective of ecotoxicology is to detect pollution related problems during the reversible stages or before exhaustion occurs, measurement of reliable physiological and behavioural responses may serve as an important component of any toxicological studies. This is mainly due to the fact that the earliest symptomology of ecosystem distress may be subtle, and transient physiological behavioural changes in the sensitive organisms or groups may go undetected.

Many of the reasonable and prudent approaches which try to evaluate the potential stressful effects of low level contaminants in estuarine environment are based on the premise that any environmental perturbation will result in the response of some measurable biological parameter (Dillon and Lynch 1981). As in clinical chemistry where biochemical changes are widely used in disease diagnosis, chematological indicators are coming increasingly into play in the assessment of the

ecosystem health. This area of biochemical indicators of ecosystem dysfunction, though remains a fertile field of study, was not investigated in the present study due to severe experimental constraints.

A particularly significant attribute of sublethal physiological responses is that it is amenable to both laboratory and field measurements unlike traditional toxicant testing based on LC 50 etc.(Widdows, 1985). Physiological responses are also capable of quantifying an organism's condition and predicting the impact of pollutants at population and community levels of biological organisation even though there may be serious conceptual constraints to such a thesis (Payne et al., 1987).

4.2. Literature Survey

4.2.1. Ecotoxicological Approach to Stress

Ecotoxicological approach to the contamination of an organism and consequent stress is based on two sets of mechanisms that are closely dependent on each other. First, the bioaccumulation processes (as briefly described in the previous chapter) are the result of transfer of contaminants between the surrounding medium and the organism. In the ecosystem they are particularly charecterised by the

significance of amounts of toxicants absorbed and absorbed, by the evolution in their distribution at tissue and cell levels,and by the effectiveness of excretion mechanisms. Second, the toxicological effects on biological structures and their functioning are closely associated with the characteristic of bioaccumulation (cellular and molecular levels) from which the set of toxicological effects ensues (Boudou and Ribeyre, 1989).

Literature is full of reference to `stressed organisms'or `responses to stress'. *Eventhough* the mammalian stress is well defined in terms of Selye's general adaptation syndrome and the accompanying changes in blood chemistry, stress in estuarine organisms has not been clearly delineated (Dillon and Lynch, 1981) . The large volume of departmentalised, medium by medium, and pollutant by pollutant studies have only yielded some clues on the principal distress syndromes of severely distressed environment.

As with the results of any toxicity tests it is essential that the sublethal responses be related to those of control animals before any conclusion about the toxicity of any chemical to any species is drawn. So, it is clear that studies carried over a wide range of concentrations and leading to acute and chronic, sublethal as well as lethal responses are necessary if a clear appreciation of ecotoxicological effects

is to be obtained (Pascoe and Edwards, 1989). The advantages of utilising physiological responses as an index of stress lie in the fact that early detection of potential biological harm in an impacted area may be possible. This level of examination allows one to investigate the initial interaction between an organism and a potential stress before population and community structures have been disrupted (Pascoe and Edwards, 1989). According to Franz (1981) there are two approaches in studying stress. The first considers stress as cause which acts as an independent variable, external to the organism, being a stimulus or input which causes strain and which is potentially unfavorable. The latter approach considers stress as a dependent variable, internal to the organism, being a response or output which is caused by some factor that is usually identified as the stressor. An appropriate definition of stress which may suit the present context was given by Bayne (1975), and Ivanovici and Weibe (1981). It describes stress as a measurable alteration of a physiological (or behavioral, biochemical or cytological) steady state which is induced by an environmental change, and which renders the individual (or the population or the community) more vulnerable to further environmental change.

Along with the enormous range of pollutant sources and models of contamination, the effects of toxicants on aquatic organisms are also extremely varied depending on the level

selected for analysis (population, individual, organ, cell, molecule) or the criteriaconsidered (lethality, growth, reproduction, and behaviour). In the literature different types of effects are discerned, depending on the degree of toxicity, length of exposure (from acute effects, that occur rapidly as a result of short term exposure, to chronic effects that occur in long term), or reversibility of the consequences. From the methodological point of view two types of approaches can be discerned in the numerous studies based on the effects of stressors. First, those that use the effect as criteria to reveal or, in certain cases, to quantify the risks associated with the presence of toxicants in the medium. These applied methodologies do not intend to understand ecotoxicological mechanisms, but to disclose structural or functional perturbations revealing more or less specific toxic effects. Second, those that use the effects as a means of studying contamination processes, impairment of the biological structure or of a biochemical or physiological function indicating intoxication mechanisms (Boudou and Ribeyre, 1989).

4.2.2. Change in Metabolic Rate as an Indicator of Stress

The measurement of metabolism may be the most sensitive parameter of stress in individual organisms since it integrates many elements like enzyme activity, modulator substrate pools, and physiological responses (Bayne, 1976; Anderson, 1977;

Dillon and Lynch, 1981). Respiratory metabolism was found to be appropriate for quantifying the energy expenditure under different multifactoral stress conditions since it denotes the general well-being of the organism(Bryan, 1984). Measurement of oxygen consumption provides an overall indication of the respiratory and cardiovascular function, and respiratory metabolism, as determined by total and per unit weight oxygen consumption, is known to be susceptible to changes depending on several ambient and endogenous factors . Marked changes in the total and per unit weight oxygen consumption consequent to exposure to aquatic pollutants especially heavy metals have been reported by many researchers (Baby, 1987 ; Varghese et al., 1992). A brief survey of the metabolic responses of different aquatic species to various pollutants was offered by Varghese et al. (1992).

4.2.3. Other Functional Indices of Metal Stress.

Survival is considered the best index of metal stress particularly copper since it is found to be the least variable. When estuarine organisms are exposed to heavy metals dissolved in water, the gills function as the major route for uptake of these compounds. The rapid accumulation through gill tissues is usually accompanied by deleterious effects to gill structure and may therefore interfere with its respiratory and osmoregulatory functions. Many workers have attributed acute

heavy metal toxicity in many organisms to impairment of one of these main functions of the gills (Putte et al., 1981; Mangum et al., 1987). Manley and Davenport (1979) observed the behavioral responses of some bivalves to increasing concentration of Cu, and concluded that it is a three stage process of shell valve movement-shell valve adduction is followed by a period of testing, and then complete closure. Redpath and Davenport (1988) noticed an increase in shell gap area of the inhalent aperture of the mussel *Mytilus edulis* in the presence of many metal ions including Cu, and this was not found to occur in presence of Zn. A primary inhibition of oxygen uptake rate followed by a temporary activation of oxygen consuming capacity was also reported in these mussels when external stress was experienced by the organisms. Certain marine invertebrates were reported to metabolically regulate trace metals particularly Zn to an approximately constant level over a wide range of ambient dissolved metal availabilities (Rainbow et al., 1990). Chan(1988), and Phillips(1989) also have mentioned about some undefined exposure concentrations above which there exists some metabolic process which protects the organism from the toxic effects of the metals.

Heavy metals have been found to exert inhibitory effects on several other physiological processes, and the stress effects of metals particularly of Cu on glycogen stores, and lactate levels etc. of aquatic organisms have also been studied by many

(Shaffi, 1978; Lakshmanan, 1982; Abdullah and Ireland, 1986; Suresh and Mohandas, 1987 ;Nath and Kumar, 1988).Different species of organisms may be responding differently to its metabolic functions and the biochemical components such as glycogen, glucose,and blood sugar levels may have different biochemical links with the total metabolism. For example, in the crab *Carcinus maenas* blood holds almost four times the amount of polysaccharides than the hepatopancreas, and glucose is actively incorporated into glycogen in the haemolymph which is an active seat of carbohydrate metabolism (Williams and Lutz, 1975). Many decapod crustaceans respond to physiological stress by elevating their blood sugar levels. Studies on fish have also revealed that blood sugar level changes with the effect of exogenous foreign substances. Toxic effects of some selected polutants including Hg, Cu, and Zn on the cardiophysiology , respiration and enzyme profile of a few freshwater animals, and respiratory responses, and variations in the blood sugar level of *Barytelphusa cunicularis* (Westwood) have been reported by Varghese et al. (1992) separately.

4.2.4. The Physiological Role of Carotenoids

Carotenoids are yellow or red pigments which are widely distributed in plants and animals, and play their indispensable role in the orienting and directing mechanisms that co-ordinate

feeding behavior and social organisation (Hoar, 1987). Carotenoids are one of a group of fat soluble substances (polyenes with approximate molecular formula as $C_{40}H_{56}$) characterized by a long skeletal chain of carbon and hydrogen atoms with alternate double bonds. According to Britton (1985) they are sensitive to oxygen, light, heat, acids and alkali, and especially a combination of these factors. Even though much is known about the carotene mechanisms in higher animals, little is known in lower animals. The physiological role of carotenoids in bivalve molluscs was first reported by Karnaukhov (1971). It is postulated that (Karnaukhov et al.,1977) carotenoids participate in the oxidative metabolism of molluscs by stocking a part of the oxygen by the use of carotenoid unsaturated double bonds and releasing the same, when the gas diffusion into the tissues is insufficient. Unfortunately, this aspect or possible role of carotenoids in the tolerance of organisms are not widely discussed, or seems not properly directed. Krishnakumar et al. (1987) have observed that the tissue carotenoid content in the mussel *Perna viridis* increased proportional to the ambient metal content and reported an inverse relationship with the oxygen uptake of the animal. Sathyanathan et al. (1988) also observed a similar trend in the organism under study, *Villorita cyprinoides*, exposed to sublethal concentration of Cu and Hg. Carotenoid pigments are reported to take part in the defense mechanisms implicated in the prevention of lipid peroxidation in aquatic

organisms (Viarengo, 1985). Information on the metabolic adaptations, physiological adaptive responses to sublethal metal stress etc. of this organism is scanty. The physiological role of tissue carotenoids in the tolerance of the organism to metals is also not investigated. Hence, an initial attempt was made (estimation of the quantitative changes in tissue carotenoids of Cu exposed organisms) and the results are reported in this chapter.The investigations will be complete only if the study is extended to qualitative analysis of tissue carotenoids.

4.3. MATERIALS AND METHODS

The general methods were the same as described in Chapter 2, the only change being that the animals used for carotenoid determinations were not fed during the experimental period. For monitoring the metabolic rate of the metal exposed organisms the following procedure was adopted. Two hundred specimens (in 8 troughs containing 25 animals each) were exposed to Cu 60, Cu 100, Cu 60+Zn 1000, and Cu 100+Zn 1000 µg/l of Cu in fresh water separately. Two animals from each of the metal dosed media were transferred to Erlenmeyer flasks at day 1,2,3,4,6,and 8 post-exposure, and the oxygen consumption of the animals determined as per the procedure described in Chapter 2. The animals were dissected, dry weight recorded, and metabolic rate calculated. The metabolic rate

of control animals was also determined at the above intervals following the same procedure. In the case of $13x10^{-3}$ salinity the concentrations of the metal in the medium chosen was higher. The experimental period was also extended to 10 days. The selected Cu concentrations were Cu 300, Cu 600, Cu 300+Zn 1000, and Cu 600+Zn 1000 µg/l. The metabolic rate of control animals was also determined simultaneously. For each concentration and control five replicates were used, and Winkler's modified Iodometric method was used for measuring dissolved oxygen.

Carotenoid content(total and unsaponifiable) of the clams (of control and Cu exposed) was determined at intervals 24,48,72 and 96 hours (in salinity $13x10^{-3}$) by designing a separate experiment following the procedures of Karnaukhov et al.(1977). The animals were not fed during this experimental period. The experiment was not extended for longer periods . The procedure used for the estimation of carotenoids was as follows. The whole body tissue was removed from the shells, blotted on filter paper, and weighed wet. Carotenoids were extracted from the tissues by grinding under acetone in a mortar or glass homogeniser. The acetone extract was filtered under reduced pressure in a scintered glass funnel and the solid residue was returned to the mortar for further extraction, which was repeated until the acetone extract was colourless. Absorbtion spectra of the acetone extracts were recorded in a

spectrophotometer (Hitachi Model 2000) at 450 nm for calculation of the total carotenoid concentration in the animals.

The acetone extracts were subsequently saponified for 12-14 hours at room temperature by addition of 1ml of 60 % KOH solution to each 10 ml extract. Unsaponifiable carotenoids were extracted from this mixture by light petroleum(b.p 40-60). For calculation of the unsaponifiable carotenoid concentration in the organism the visible absorption spectra of the petroleum extracts were recorded. The carotenoid concentration in mg/100 g of the tissue (wet wt) was calculated from the equation

mg/100 g = 0.4 DV/P

where

D - is the absorbance of the extract in 1 cm (thickness) cuvette measured at the wavelength of carotenoid absorbtion maxima, 450-460 nm;

V - total volume of carotenoid extract in ml;

P - total wt(wet)of specimen tissue (g) from which the carotenoids were extracted.

When the total carotenoids concentration in acetone extract was determined, the absorbance of noncarotenoid compounds at the wavelength, 450 nm was subtracted from the total absorbance of the acetone extract measured spectrophotometrically at the wavelength 450 nm.

The Cu exposed (300 and 600 ug/l) organisms at salinity $13x10^{-3}$ were also subjected to study muscle glycogen level for upto 72 h at intervals 12,24,36,48,60,and 72 h. The lactic acid content of the animals was estimated at 6,9,12 and 24 hours. For estimation of the above two parameters two animals each were sacrificed at the corresponding intervals,wet tissue dissected, and the respective procedures followed as described in Chapter 2. Estimation of glycogen, lactic acid, and tissue carotenoid contents was not carried out in organisms maintained at salinity $<1 \times 10^{-3}$, and in combined exposure due to some technical problem.

4.4. RESULTS

The results were analysed statistically. The Dose-Survival curves reflecting the impact of chronic copper stress are presented in Chapter 3. The changes in metabolic rate, variations in glycogen levels, lactate content, and tissue carotenoid (total and unsaponifiable) concentrations are presented in Tables and Figures (Tables 13 to 16, Figures 12 to 16).

Statistical analysis (ANOVA) revealed significant difference ($P<0.01$) in the metabolic rate of the organisms exposed to the metal concentrations Cu 60, Cu 100, Cu 60 + Zn 1000, Cu 100 + Zn 1000, and the controls (Table 13). Regarding exposure time

Table 13

Changes in Metabolic Rate of the Organism
Salinity < 1 ppt

Exposure time/ days	Metabolic rate: $\bar{x}$ ± SD µg/h/g dry wt. Conc. of metal ion in the medium: µg/l			
	Cu 60	Cu 100	Cu 60 + Zn 1000	Cu 100 + Zn 1000
0/ control	1865 127	1834 119	1874 131	1855 112
1	1677 134	1571 146	1748 119	1666 128
2	1441 129	1348 121	1627 114	1527 123
3	1716 135	1467 132	1779 125	1698 96
4	1739 118	1468 129	1792 130	1624 113
6	1698 120	1358 127	1764 106	1575 125
8	1449 124	1210 112	1699 114	1428 142

SD = Standard Deviation (n=5)
Significance: $\underline{P} < 0.01$

Table 14

Changes in Metabolic Rate of the Organism
Salinity 13 ppt

Exposure time/ days	Metabolic Rate: $\bar{x} \pm$ SD µg/h/gm dry wt.			
	Conc. of metal ions in the medium: µg/l			
	Cu 300	Cu 600	Cu 300 + Zn 1000	Cu 600 + Zn 1000
o/ control	2402 182	2414 208	2437 196	2463 218
1	2022 149	1786 167	2376 171	2291 155
2	1829 219	1680 145	2248 163	2043 149
3	2156 194	1957 151	2317 160	2213 175
4	2142 188	1854 165	2307 144	2237 163
6	2065 171	1742 149	2285 215	2263 128
8	1946 166	1650 169	2160 197	2076 179
10	1770 158	1570 172	2065 158	1849 137

SD = Standard Deviation (n=5)
Significance: $P<0.01$

Table 15

Changes in Tissue Lactic Acid Content of the Clam
Salinity 13 ppt

Conc.of Metal ion µg/l	Lactic acid content $\bar{x}$ ± SD µg/g wet wt.			
	6 h	9 h	12 h	24 h
Cu 300	45.3 4.4	44.6 3.5	56.5 5.2	94.4 9.6
Cu 600	72.5 7.9	78.5 8.3	95.3 5.2	136.1 11.4
control	21.01 1.1	18.3 2.7	22.3 1.8	19.5 2.2

SD = Standard Deviation (n=2)
Significance: $\underline{P}<0.05$

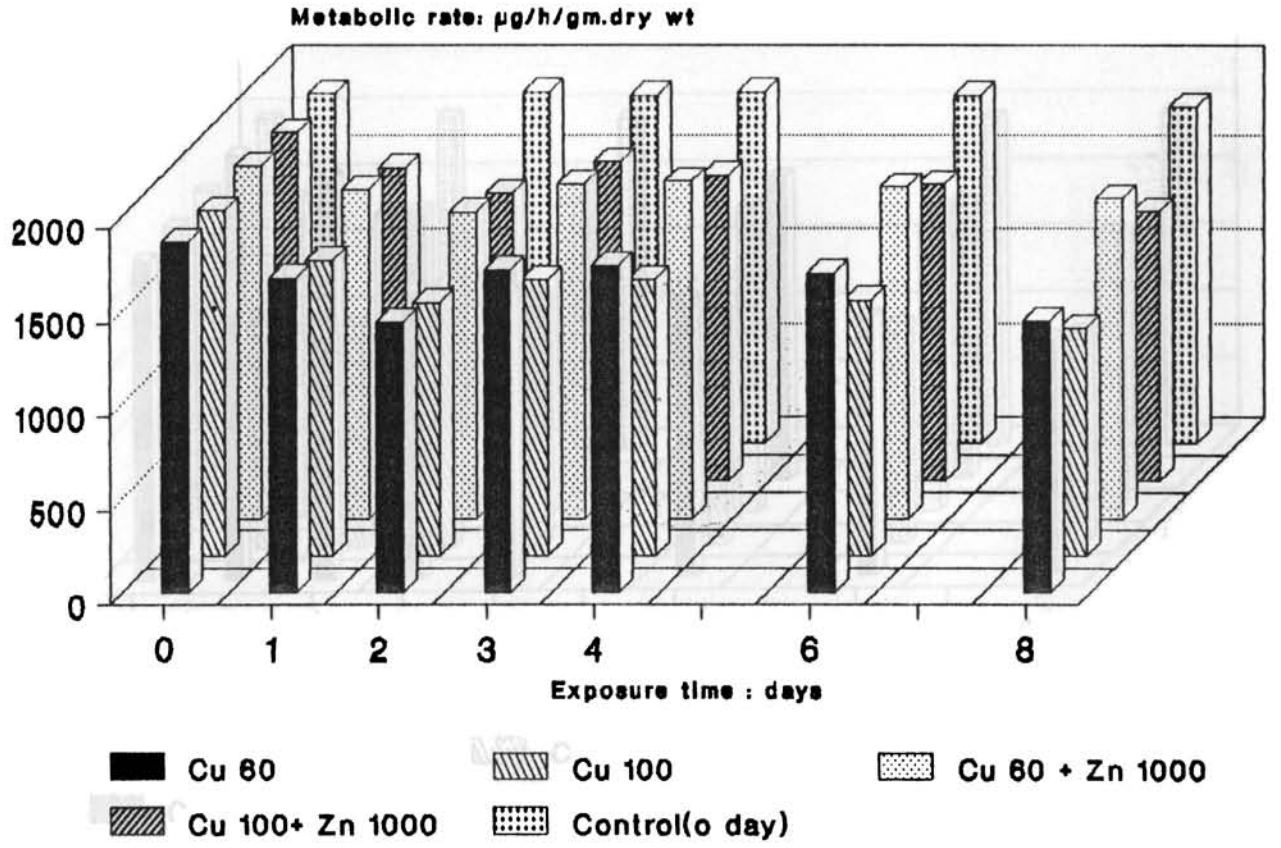
Table 16

Changes in Muscle Glycogen Content
Salinity 13 ppt

Conc. of Metal ion µg/l	Glycogen content $\bar{x}$ ± SD µg/g wet wt.					
	12 h	24 h	36 h	48 h	60 h	72 h
Cu 300	1793 29.2	1485 32.2	1480 26.5	1409 43.2	1396 49	1285 32.7
Cu 600	1545 29.2	1211 44.7	1061 42.5	980 30.8	968 20.8	910 42.9
Control	1944 23.7	1950 30.5	1959 24.5	1940 31.9	1963 46.9	1954 29.2

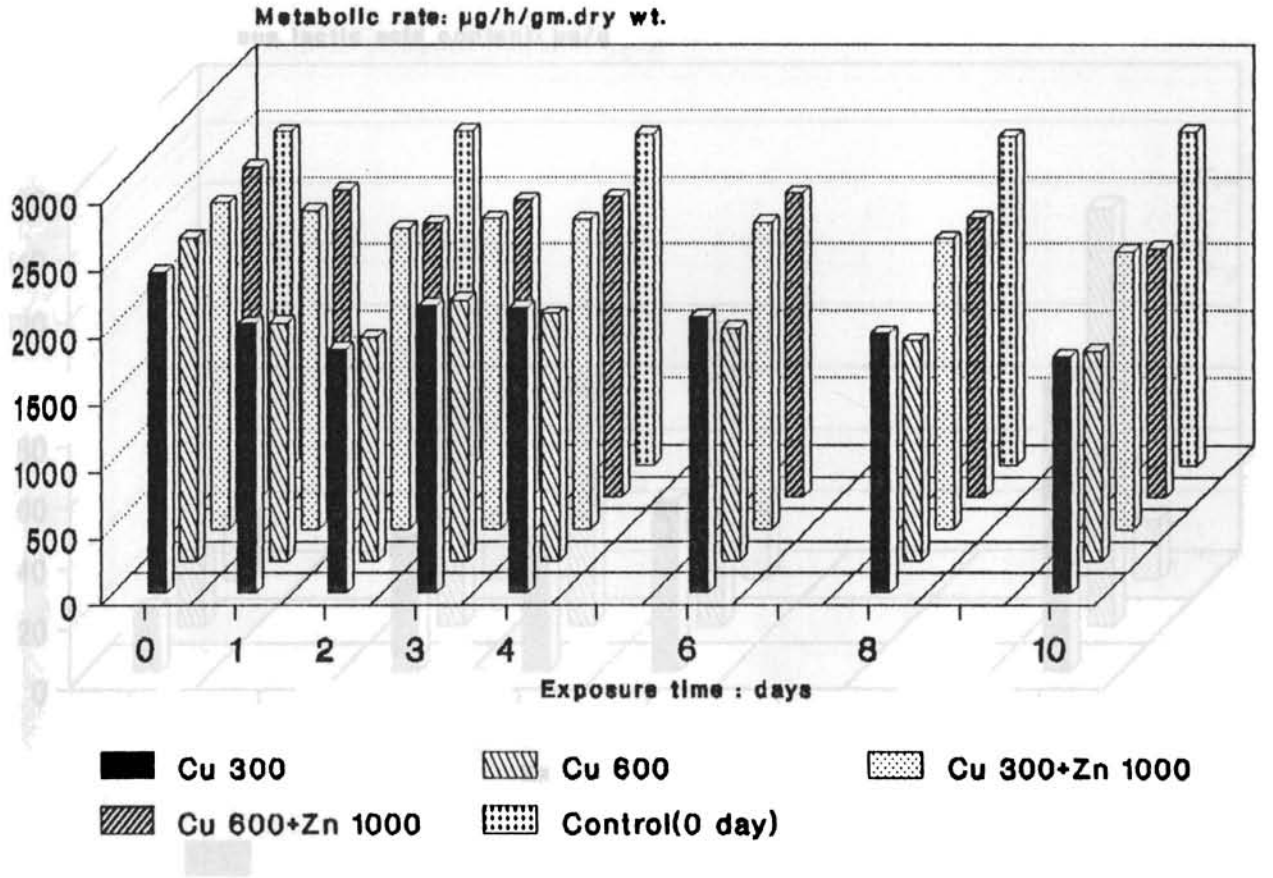
SD = Standard Deviation (n=2)
Significance: $\underline{P} < 0.05$

Fig 12: Changes in Metabolic Rate
Salinity < 1 ppt



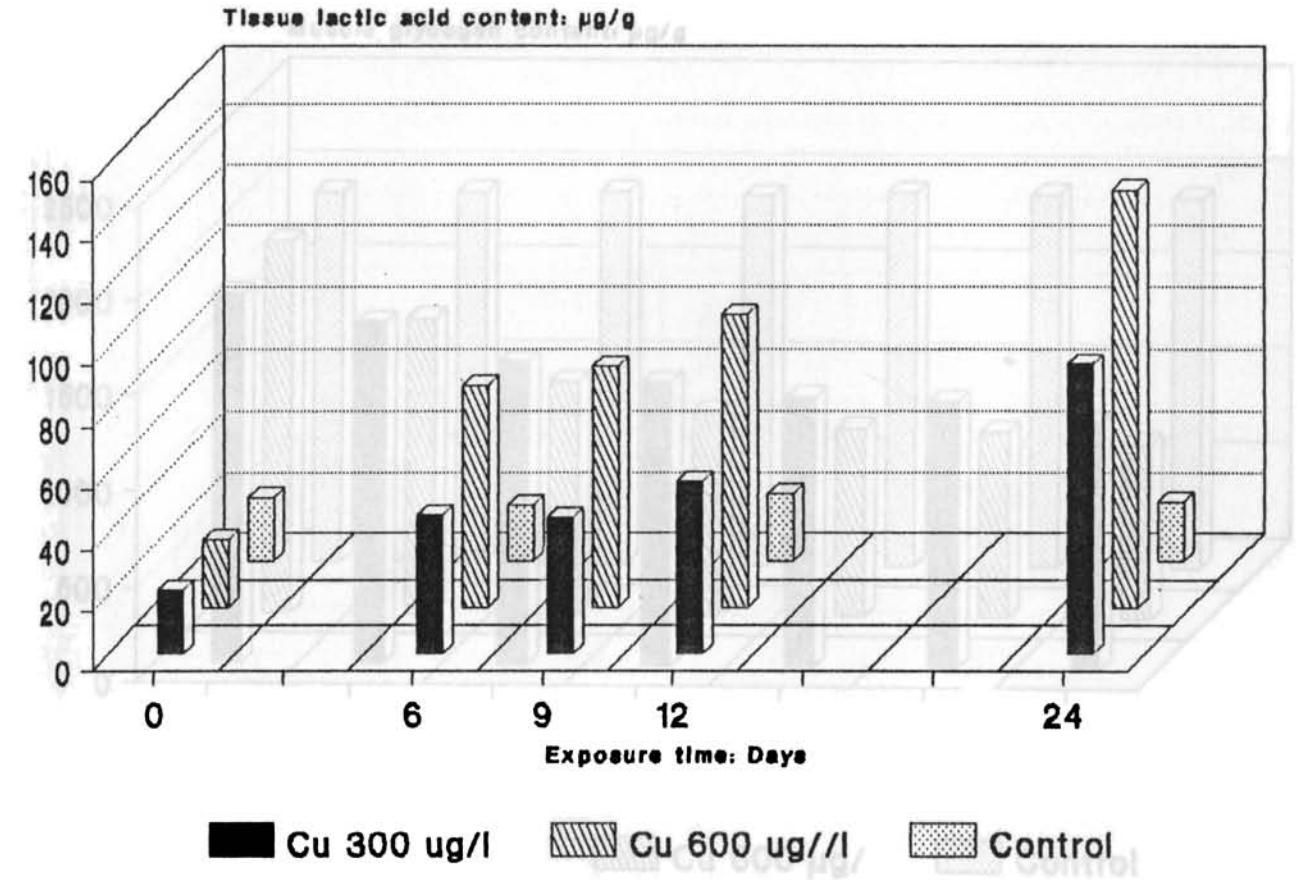
(Conc. in µg/l)

Fig 13 : Changes in Metabolic Rate
Salinity 13 ppt



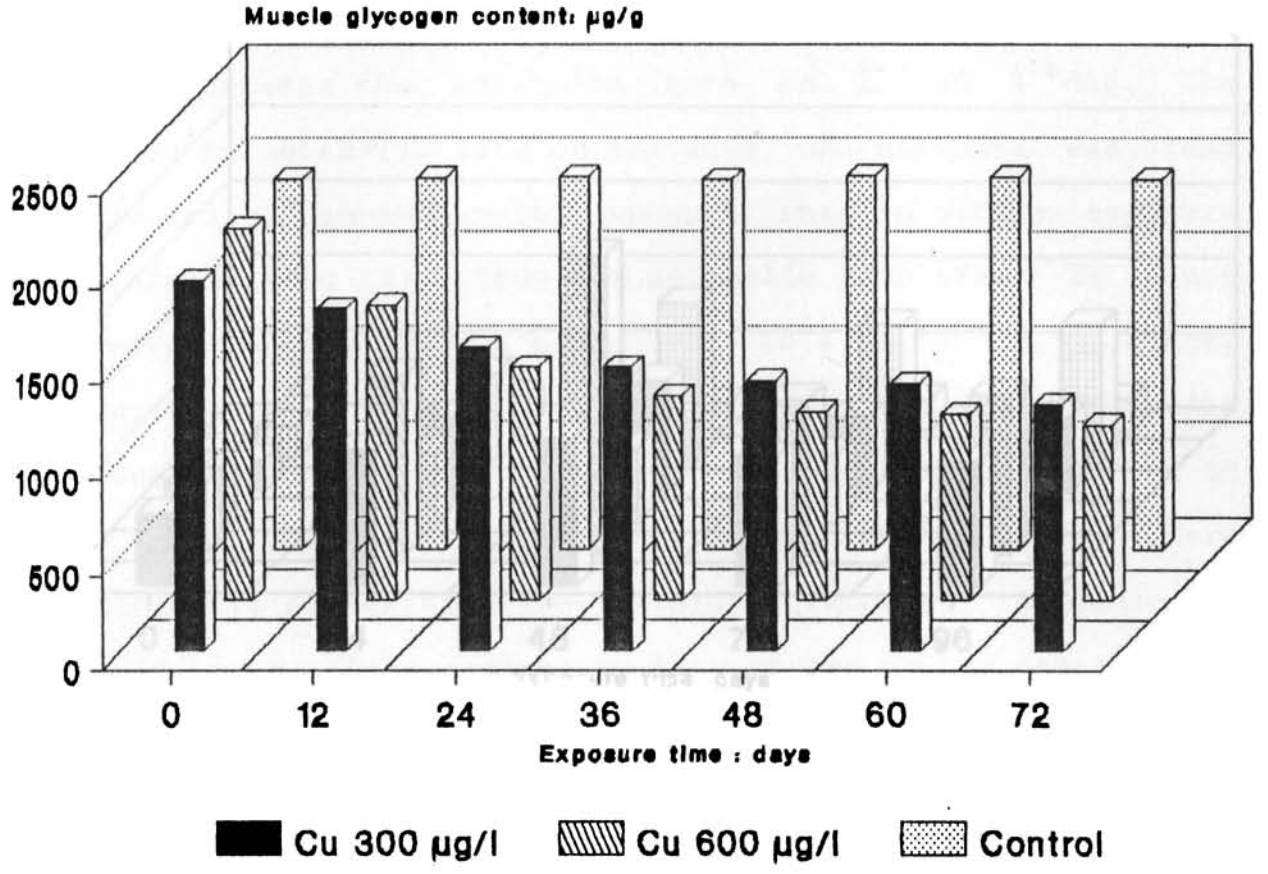
(Conc.in µg/l)

Fig 14:Changes in tissue lactic acid
Salinity 13 ppt



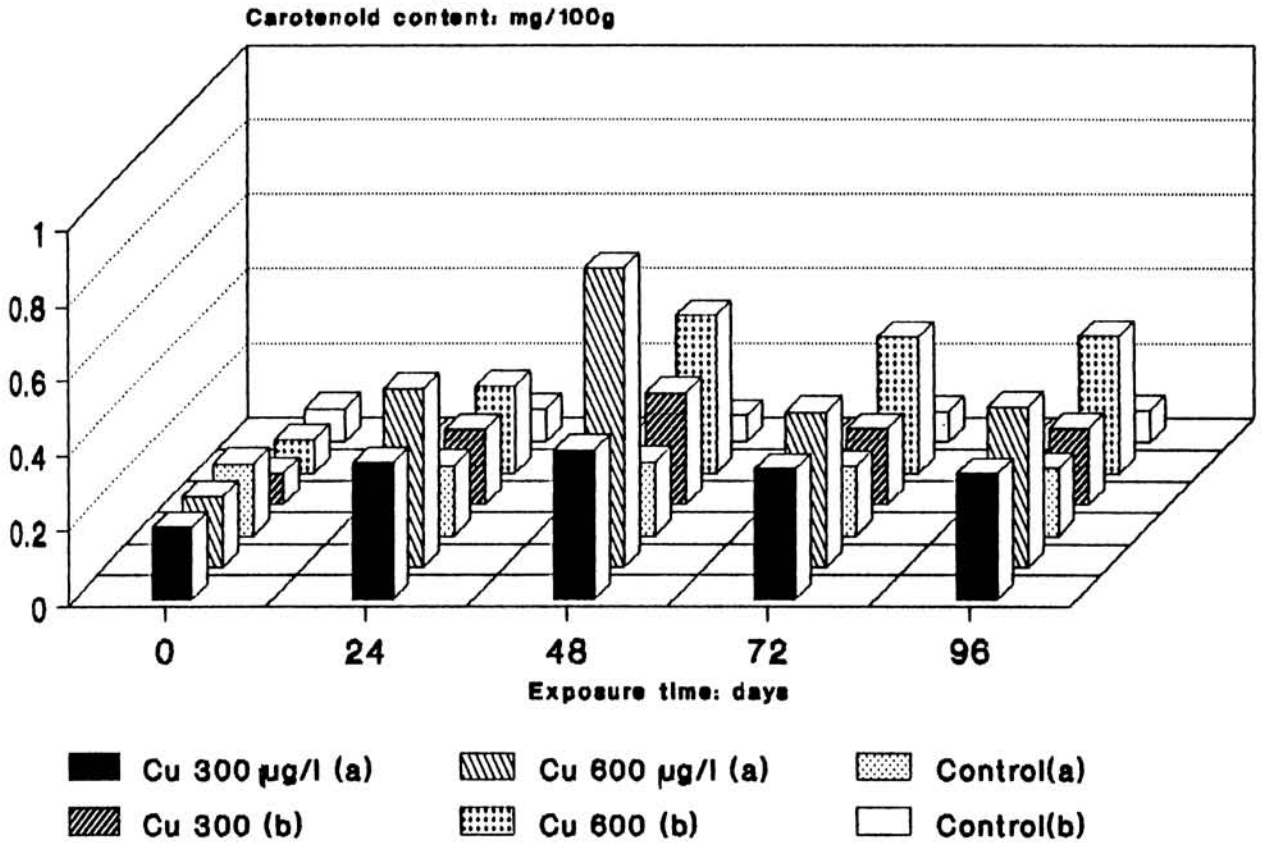
(Conc. in µg/l)

Fig 15 : Changes in Glycogen Content
Salinity 13 ppt



(Conc. in µg/l)

Fig 16: Change in Carotenoid content
Salinity 13 ppt



a-total; b-unsaponifiable

(in days) also there was significant change in the metabolic rate on all days of exposure when compared with the control (0 day). The metabolic rate of the organisms exposed to 60 and 100 µg/l of Cu got reduced to 1677 and 1571 µg/g (from the control values of 1865, and 1834, respectively) within 24 hours, and to 1441 and 1348 within 48 hours, respectively. The metabolic rate on 1^{st}, 3^{rd}, and 4^{th} day showed no significant difference between them. So also, no significant difference was recorded between the metabolic rate on $2^{nd}$and 8^{th}day. The decrease in metabolic rate on all days of exposure was less pronounced in combined metal exposure than in single exposure (Cu alone) (Fig 12). Thus the metabolic rate after 24 hours depressed to only 1748 µg/g (control 1874 µg/g) in binary exposure of Cu 60 + Zn 1000. After 48 hours the values recorded in combination exposure of Cu 60 + Zn 1000, and Cu 100 + Zn 1000 were 1627 and 1527 µg/g, respectively which were significaty higher than the values recorded in organisms exposed to Cu alone;indicating less strain on the organism.

There was significant difference ($P<0.01$) in metabolic rate of the organisms exposed to different metal concentrations on different exposure days at 13 x 10^{-3} salinity (Table 14). All the values were significantly different from the control (0 day). The metabolic rate of the animals depressed to 2022, and 1786 µg/g (control values - 2402 and 2414 µg/g, respectively) within 24 hours at 300 and 600 µg/l Cu exposure. The metabolic

rate further decreased to 1829, and 1680 µg/g, respetively after 48 hours (Table 14). The decrease in metabolic rate consequent to metal exposure was less pronounced in combined metal exposure(Fig. 13). The values were 2248 and 2043 µg/g after 48 hours (control 2437 and 2463 µg/g) in combination exposure of Cu 300 + Zn 1000, and Cu 600 + Zn 1000 µg/l, respectively.

The metabolic rate of the organism (including control) in freshwater was found to be considerably less when compared to the rates in organisms inhabiting at $13x10^{-3}$ salinity.The metabolic rates of control animals in freshwater were 1865, 1834, 1874,and 1855 µg/g (Table 13) while those of control animals at $13x10^{-3}$ were 2402, 2414, 2437,and 2463 µg/g (Table 14).

An abrupt depression in metabolic rate was noticed within 48 hours in metal exposed organisms at both the salinities. The depression was more sharp at higher salinity (Fig.13). This sharp depression in metabolic rate observed within 48 hours of exposure to metal ions was not so conspicuous in combined exposure. The physiological dysfunction also varied depending upon the concentration of the metal ion in the medium, and the salinity. A decrease of about 26 % and 32 % in metabolic rates from control values was noticed after 48 hours of exposure of organisms to 300 and 600 µg/l of Cu, respectively at $13x10^{-3}$

salinity . But after 72 hours a temporary recovery in oxygen uptake was noticed in organisms maintained in both salinities. The metabolic rates at 60 and 100 µg/l of Cu exposure (after 72 hours) in freshwater were 1716, and 1467, respectively (Table 13). The metabolic rate improved significantly at $13x10^{-3}$salinity, and it was more pronounced in binary combination of the metal ions. The improved metabolic rates were very near to the control values. But this trend was not maintained and the animals fell into a situation from which it never recovered, particularly at higher test concentrations (Sathyanathan and Nambisan, 1990).

In animals exposed to 300 and 600 µg/l of Cu, the total carotenoid content increased sharply with time and reached the maximum value by about 48 hours(Fig. 16). The total carotenoid content increased two times in the former and about four times in the latter, the increase being proportional to the concentration of metal ions in solutions (Fig. 16). After 48 h a decrease was noticed. The control values remained remarkably steady (variations $\pm$ 5 %) through out the experimental period..

The change in metabolic rate was found to be inversely proportional to the carotenoid content . Within 48 h, the metabolic rate depressed to about 32 % and 26 % in 600 and 300 µg/l Cu exposed organism while the total carotenoid content

increased to approximately four and two times. A reverse trend was shown after 72 h.

No significant change in metabolic rate, compared to control organisms, was noticed in Zn exposed organisms even upto 1000 µg/l in both the salinities.

Significant variations ($P<0.05$) in glycogen, and lactic acid contents of the organisms were noticed during metal exposure(Table 15, 16). The muscle glycogen stores generally depleted fast during Cu exposure(Fig. 15). Glycogen content in 300 and 600 µg/l exposed organisms decreased to 1285, and 910 µg/g, respectively within 72 h (control 1954 µg/g) (Table 16). Lactic acid content, however, increased during exposure period. The tissue lactic acid content increased to 94.4, and 136.1 µg/g during the test period, in the two concentrations of exposure (Table 15, Fig. 14).

DISCUSSION AND CONCLUSION

The results indicate that Cu affects the normal physiological and biochemical functions, including metabolic activity and energy budget. The animals can be assumed to have passed through two classical stages of stress response; the alarm reaction, and the stage of resistance. The organisms were able to adjust the initial stress or may have released

partly that stress. This may be the reason for the transient state of recovery in metabolic rate after initial stress. The recovery in metabolic rate was not found to be significant in lower salinity regimes. This may be due to the higher energy cost including that for osmoregulation. In higher salinity regime also after showing symptoms of resilience the animals went into a no return state, and finally died. In bivalves, the action of gill cilia facilitates respiratory gaseous exchange. Hence, any alteration in gill epithelium would cause respiratory changes. The steep suppression in the rate of oxygen consumption observed within 48 h of exposure to the toxicants may be probably due to the reduced efficiency of gills, besides the irreversible interaction of metal ions with cellular metabolic enzymes (Singh and Singh, 1979). A gradual recovery in metabolic rate on further exposure indicates the effort taken by the animals for their survival in a toxic environment. Fortification of gill epithelium, high metal-storing capacity, and synthesis of metal binding proteins may also be considered as some of the possible reasons for the metabolic recovery (Engle and Fowler, 1979).It is also to be assumed that the animal may have withdrawn into their shells when exposed to alien environment. But they were likely removing oxygen from its environment despite withdrawing. Animals may be capable of pumping water through their respiratory chambers even when they have retreated into their protective shells. Most animals which have retreated and ceased

ventillation must rely upon oxygen dissolved in the water within the confines of the shells, or must switch to anaerobic processess(Davenport et al., 1980). The effectiveness of the withdrawal mechanisms obviously relies upon the animal reducing contact with the exterior to as small an area as possible while trapping the polluted water within the shell.Consequently, once widrawn into the shell the animal's sense organs will no longer be in direct contact with the environment. It is to be presumed that the diffusional processes will be adjusted and the internal organs better equipped to cope up with the external stressful environment.But it is clear that the internal efforts of the organisms were not sufficient to keep the organism alive for longer periods in the alien environment. The above adaptive processes (fortification of gill epithelium, detoxication mechanisms, etc) may be more actively operating in the organisms, during combined exposure of Cu and Zn. It is already known that Zn acts as an ameleorating agent as far as Cu toxicity is concerned, and the uptake of the latter is significatly reduced in presence of the former.

Metal induced stress, particularly Cu in aquatic organisms has been demonstrated by many (Scott and Major, 1972; Lakshmanan, 1982; Meador, 1991). The gills function as the major route of uptake of Cu compounds. The gill tissues rapidly accumulate most heavy metals, in such a manner that its content, at least initially, far exceeds that in other tissues,

which obviously results in physiological dysfunction. This organism is also not an exception (Lakshmanan ,1982). The rapid accumulation of metal ions in gill tissues is usually accompanied by deleterious effects to gill structure and may therefore interfere with its respiratory and osmoregulatory functions. Many workers have attributed acute heavy metal toxicity in many organisms to impairment of one of these main functions of gill tissues,and passive binding of metallic ions with organic ligands (Putte et al., 1981).

The higher toxic response of the organisms at the lower salinity regime can be easily explained. Free or ionic metal of Cu is very toxic. Ionised hydroxides of Cu may also be toxic. These species predominate in lower salinities while at higher salinities unionised carbonates of Cu which are less toxic exceed the other species there by contributing less to the overall toxicity to the organisms. Further more, in the case of Cu, toxicity changes considerably when alkalinity is varied. Metals are subjected to large change in toxicity not only through ionization but through precipitation, sorption, and binding to suspended and dissolved matter (Rand and Petrocelli, 1985). Ionoregulatory dysfunction and respiratory impairement due to increased fusions of filaments and lamellae, reduced filament and lamellar growth, poor filamental vascularization and hyperplasia of the respiratory epithelium resulting in reduction of the surface area of the gill

available for gas excahange were also reported to be associated with fish inhabiting in soft waters with little acidic charecter (Conklin et al.,1992)

It may be noticed that the metabolic rate of the organisms in the lower salinity regime is considerably less than in the higher salinity. For example, the metabolic rate of control animals at salinity $<1x10^{-3}$was only 1865, 1834, 1874 and 1855 µg/h/gm compared to 2402, 2414, 2437, and 2463 µg/h/gm at $13x10^{-3}$ salinity (Tables 13, 14). This is explainable in terms of the energy regulatory process occurring within the organisms for ion regulation. It is hypothesized that the energy requirement for ion regulation would be minimal at an isotonic water salinity, where the ionic gradients between blood and water are minimal (Morgan and Iwama, 1991). They observed that the average metabolic rates of different species of salmonids increased with salinity, being significantly higher in hypertonic treatments than in freshwater. The magnitude of these changes in metabolic rate from freshwater to 16 to 20 x 10^{-3} salinity ranged from 25 to 30 % . They have also offered a regression model to explain the relationship between metabolic rate and salinity. Life habits will, to a certain extent, determine the type of metabolic response to change in salinity. For example, euryhaline fish which move freely through freshwater do not exhibit wide metabolic change consequent to salinity variations (Morgan and Iwama, 1991).

Euryhaline forms which are found in freshwater as adults or in estuaries as juveniles tend to show a metabolic rate minimum in isotonic salinity and higher at lower and higher salinities (Febry and Lutz, 1987), whereas fish which are found as juveniles in freshwater or as adults in estuaries show low metabolic rate in freshwater and it increases with salinity (Morgan and Iwama, 1991). The latter pattern in salinity related metabolic rates seems to be followed by the organisms under study. The lowest metabolic rates in response to salinity are associated with the environments in which the species are most commonly found, and presumably most physiologically adapted for a particular life stage. There are conflicting reports in literature regarding the effects of isotonic salinity. Considering that the mechanisms of ion-osmotic regulations are similar among species, habits, and life stages the variety of metabolic responses to different salinities suggests that these responses are being influenced by physiological processes in addition to the energy required for ion-osmotic regulation (Febry and Lutz, 1987).

During initial metal stress and subsequent intoxication, there appears to be alterations in cellular energy metabolism. This is simultaneously followed by utilization of stored energy compounds or normal energy budget of the organism. So, the glycogen stores were abruptly depleted consequent to Cu stress (Table 16). Preliminary hypoxia induces mucus secretion.

Appreciable mucus secretion was observed in organisms exposed to even low concentrations of Cu. Mucus may protect the organism from immediate contact with the toxic ions to some extent. But normal metabolic functions may be also affected. Lactic acid is the end product of glycogenolysis and hence lactate accumulation is seen consequent to glycogen depletion in tissues affected by metal stress. (two lactate forming pathways, semi-independent, with discrete starting subsrates, and non-mixing pools of chemically identical intermediates, one showing Pasteur Effect, and the other reversed Pasteur Effect were suggested by Hochachka (1985). Moreover, in anaerobic metabolism (due to reduced oxygen intake caused by pollution stress) animals are able to utilize the free energy from chemical processes, that do not require oxygen such as the formation of lactic acid from glucose. The complete oxidation of one mole of glucose yields 686 k.cal. The splitting of glucose into lactic acid yields 56 k.cal per mole glucose, or about 8 % of the total available energy. Shaffi(1978), Abdullah and Ireland(1986), and Nath and Kumar(1988) also observed glycogen depletion and lactate accumulation in bivalves during heavy metal stress. Katticaran and Salih (1992) observed a significant drop in oxygen consumption simultaneously followed by an increase in lactic acid levels in the adductor muscle, and digestive gland in the clam *Sunetta scripta* exposed to certain sublethal concentrations of copper ions. Describing briefly the end products of the lactate

pathways and its implications they have commented that capacity for mobilisation of carbon via anaerobic pathways depending upon the tissue oxygen supply may represent an evolutionary process of adaptation, enabling the animal to occupy unfavourable environments with variable oxygen content.

The biochemical, especially the enzymatic, adaptive responses of the organism could be elucidated only by detailed enzyme analysis. Eventhough such an attempt was not made in the present study, available literature and latest research work on the aspect elsewhere gives enough insight into the functional strategies adopted by organisms in metal stressed situations. James et al. (1992) offer such a treatment in which *Oreochromis mossambicus* (Peters) was found to adopt a strategic step for its survival in less toxic environment (sublethal concentrations of metal ions individually and in paired and triad combinations), a kind of adaptation observed in energy metabolism under chronic period of metal stress. They have observed significant gradual decrease in the respiratory enzyme succinate dehydrogenase (SDH) with concomitant increase in glyceraldehyde dehydrogenase (GDH) in liver, brain, muscle, and gill of the organism exposed to the metals Cu, Cd, and Zn individually, and in paired and triad combinations of the metals. They suggested a matabolic shift from aerobiosis to anaerobiosis due to metal action (supression of SDH activity and elevation of GDH activity are indications of anoxic or

hypoxic conditions in fishes). It was also interesting to notice that exposed individuals when transfered to metal impoverished water(depuration study!) showed an improvement in SDH activity, and decline in GDH activity suggesting slow reversal to aerobic metabolism.

It has been commonly assumed that organisms harness three general mechanisms for solving their adaptational problems. These include:

1) adjustments in the kind or amount of macromolecular (especially enzymatic) components of cell metabolism,
2) adjustments in the intracellular microenvironment within which macromolecules function, and
3) adjustments in the outputs of metabolism on a moment-by-moment basis.

What roles are played and what general, or specific functions do such adjustments serve which enable the organisms to survive in alien environments particularly during oxygen lack is under intensive study and many theory have been formulated (Hochachka, 1985). The general theorem states that environmentally sensitive cells, tissues, organs, or organisms display a cascading series of mutually reinforcing responses when their environment changes: first the metabolic rate processes change strictly according to thermodynamic effects,

i.e., metabolic regulation is disrupted and rate of ATP synthesis drops, and second, membrane, structural, and functional adjustments are not realisable. In cells or organisms tolerant to environmental change selective pressure is clearly to reverse the downhill cascading cycle; breakdown in metabolism, and metabolic regulation (Hochachka, 1985).

As pointed out elsewhere it is only in the last ten years that the importance of metal ions in biochemistry has become fully apparent (Suckling and Suckling, 1980). This seems very true as far as Cu and Zn are considered. Now it is well known that without metal ion no cell could retain its integrity or carry out metabolism. Besides the known toxic actions of Cu, its intake may also be causing immediate deficiency of desirable elements such as Zn to the organisms, thereby causing serious functional disorders. Higher Zn content in the medium may be protecting the organismic cells from the above casuality and this may be the reason for the better improvement in metabolic rate of the organisms during combined exposure of Cu and Zn.

The physiological functions and the role of carotenoids in tolerance of molluscs to metallic contaminants deserve further study. The investigations by Karnaukhov (1971) showed that carotenoids took part in oxidative metabolism of the cells. It was suggested that carotenoids common with haemoproteins can

orm the system of oxygen reserve (accumulator) in special intracellular organoid which contains some respiratory enzymes as well. It allows them to provide energy requirements of the cell under conditions of low rate oxygen penetration into the tissues. Therefore, the increase in carotenoid concentration in the animals can be observed under their adaptations to hypoxic conditions or to the mitochondrial inhibitor actions (Karnaukhov et al., 1977). What all processes are taking place internally in organisms to compensate or adjust to the external stress is a matter of speculation (Magnum et al., 1987).

The behavioural responses of bivalves to increasing concentrations of Cu has been reported to be a three stage process of shell valve movement-shell valve adduction is followed by a period of testing and then complete closure (Manley and Davenport, 1979). As pointed out earlier, there may be some physiological mechanisms functioning within the organism to mitigate the toxic effects of heavy metals. Considerable energy is required to establish the adaptive mechanism but less energy to maintain it (Dixon and Sprague, 1981 b). A sharp decrease in glycogen followed by a temporary steady state observed in the present study may be of relevance in this context(Table 16). Since metals such as Cu and Hg are more toxic at low salinities, the pressure to adapt is probably greatest at these conditions. Observations indicate a switch towards glycolysis by the organisms in order to overcome the

anaerobic stress caused by the toxic metal. As pointed out earlier when the animals absorb little oxygen from the environment, the respiratory metabolism is depressed, consequently stored intracellular glycogen is increasigly utilized. Eventhough the organisms have a definite need for heavy metals, their presence in excess will produce various molecular abnormalities. The synthesis of metal binding proteins like metallothionein, and the unknown functions of Zn containing enzymes like carbonic anhydrase etc. in this animal has to be investigated further inorder to gain informations in this regard.

The difficulty in distinguishing the deleterious effect of sublethal exposure of the metal with normal physiological mechanisms of the organism is a major handicap in determining the extent and efficiency of resilience of the organisms. In any case a long period of increased oxygen consumption is necessary to restore the normal metabolic process damaged by metals. James et al. (1992), after studying some enzymatic behavior of metal exposed fish, have also concluded that the organisms need more time (time not specified) for complete recovery from the metabolic processes damaged by metals.The mechanisms possessed by organisms for handling natural fluctuations in the availability of heavy metals under contaminated conditions have been well described by Bryan (1976 a,b). The organism under study was found to be able to adjust

the initial metal stress imposed at lower concentration within 72 h. But this observation seems valid only at the optimum salinity range and the continuity of the process cannot be assured for prolonged periods (Sathyanathan and Nambisan, 1990). In close contrast, the sockeye salmon was found to adjust the stress imposed by low concentration of Cu within 4 h (Donaldson and Dye, 1975). Varghese et al. (1992) have also observed some depression in metabolic activity in the crab *Barytelphusa cunicularis* followed by transitory excitation in the metabolic rate consequent to exposure to heavy metal ions, Cu Hg, and Zn. According to their observations, crabs exposed to copper showed decrease in oxygen consumption right from 15 minutes while animals exposed to Zn (10mg/l) showed an increase in oxygen uptake. They have speculated the involvement of some neurohormonal factors also in alien environment.

Boesch and Rosenberg (1981) have indicated that simple estuarine benthic communities were more resistant and more resilient to man induced and unusual natural stress than similar, more oceanic salinity conditions. Moreover, the estuarine communities seemed to return more quickly to their typical state after an acute stress or once a chronic stress was released. This is particularly true with the organisms inhabited in mildly polluted environments.

Since this organism is a typical euryhaline species, the higher tolerance and symptoms of resilience at the optimum

salinity regime may be related to its osmotic properties. All organisms in freshwater are hyperosmotic with respect to their environment and are hypoosmotic in saline water. Different strategies are required to maintain osmotic balance, but in either case there is an imposed physiological load that could be considered a form of stress. If the organism is euryhaline, it would be in overall osmotic balance at an intermediate salinity, and it would be hypothesized that at this balance point the organism would be physiologically more effective (Sprague, 1985). For example, *Cyprinodon macularius* shows better food conversion efficiency in half strength sea water than in either freshwater or full strength sea water(cited from Sprague,1985). As mentioned earlier, euryhaline species may be more effective in dealing with pollutants at their isosmotic point. There may be a decreased osmotic stress as salinity was increased toward the isosmotic point, with a decreased inward flow of water, which would presumably be accompanied by a reduced intake of toxic ions. For *Villorita cyprinoides*, which is a typical euryhaline species, is regularly encountering a variety of rates and amplitudes of salinity changes; a flexible response to salinity change may be advantageous to overcome even the extra contaminant load in addition to fluctuating salinity regimes. But to what extent this will be effective is to be observed in the real environment itself.

So there can exist two situations in the habitat environment. The more tolerant organisms subjected to exposure to low levels of metallic pollutants, like Cu, may be able to accomodate and adjust the initial stress partially or fully. Moreover, the presence of ameleorating agents like Zn may be giving more relief to the stressed organisms. From the public health point of view, the protective mechanisms determine the degree of contamination of edible fish and shell fish, even if the organisms themselves are unaffected. From the point of view of the organism and polluters the ability to adapt to metals seems to be a favourable aspect to the animal, however, it should be remembered that many of these tolerant organisms contain two to three orders of magnitude higher concentrations of metals than normal, and the obvious threat to predators is already mentioned elsewhere. The second facet of the picture is that heavy metals like Cu are stable compounds that are not readily removed by oxidation, precipitation, or other processes and the gill lamellae of the bivalves are intimately associated with the toxicants in case of polluted environment. The contaminated water current continuosly flowing around the gills reduces the efficiency of the gills and will eventually create physiological imbalance in the organisms, finally resulting in death of the individual organisms and loss of habitat population. Both are of severe environmental implications. Moreover, since physiological responses caused by pollutants are considered to be useful in conjugation with others as

sensitive bio-indicators for detecting water pollution, more attention has to be focussed on the interactive effects of metals on the biochemical responses of the monitoring organisms.

CHAPTER 5

PRE-EXPOSURE TO COPPER AND ZINC, AND INDUCTION OF ENHANCED TOLERANCE PHENOMENON

5.1. INTRODUCTION

Eventhough it is known that physiological history of aquatic organisms can influence their ability to withstand environmental changes, physiological compensation by organisms exposed to toxicants has received little attention recently (Bradley et al., 1985).

Although most toxicity tests involve continuous exposure of test animals to a constant concentration of the test substance, this does not necessarily simulate a natural pollution situation. Many pollutants occur in fluctuating concentrations, and others as single episodic events in which the pattern of exposure to toxic substances and its consequences may be different from those provided by conventional tests (Pascoe and Edwards, 1989). For example, animals briefly exposed to a pollutant and then transferred to non-contaminated water may have an opportunity (not available in a normal test) to eliminate the toxicant and recover (Green et al., 1988). Moreover, the competitive interactions among heavy metals during absorption are complex and have little been studied (Misra and Mani, 1992).

While the term 'compensatory change' could be interpreted as meaning adaptive change, Fry's definition suggests that an observed change within an animal need not be adaptive, since it is often difficult to relate an observed change to an animal's fitness (Fry, 1971).

An animal has not only the capacity for tolerance and resistance (as described in Chapters 3 and 4) but also for acclimation and acclimatization. This means that its previous history with respect to any factor may modify its subsequent tolerance and resistance to changing conditions of this factor (Hoar, 1987). Although the terms acclimation and acclimatization have essentially the same meaning in the English language and are frequently used interchangeably in biological literature, there is a tendency on the part of environmental physiologists to restrict their usage to somewhat different compensatory changes (Hoar, 1987). Acclimation may be the descriptive term applied to compensatory changes which occur in the laboratory where animals are maintained under controlled conditions of one particular environmental variable, or acclimation may be well defined as the compensatory change in an organism under maintained deviation of a single environmental factor or the process of bringing an animal to a given steady state by maintaining it under constant conditions of one or more factors. Even though these process are attributed usually in the laboratory it is a frequently

occurring natural phenomena also (Bryan and Hummerstone, 1973).

With animals pre-exposed to low concentrations of essential metals like Cu and Zn and then maintaining them in higher test concentrations of the toxic metal ions for an extended period of time, important informations should be obtained including the prediction of survival probability of a population .

5.2. Literature Survey

In an early investigation, Mc Leese (1956) studied the interaction of temperature, salinity, and oxygen in the survival of the American lobster. He found that an acclimation to any two of these factors produced a marked alterations in the incipient lethal levels of the third and that acclimation could be readily demonstrated for each factor singly. An improved capacity to tolerate hypoxic environments subsequent to acclimatization has been reported for several invertebrates. Compensation, through acclimation may alter an animals critical oxygen demands; and the changes in oxygen utility, metabolic and respiratory responses of such organisms have been well demonstrated (Steen 1971; Newell, 1971; Hoar 1987).

Luoma (1977), while discussing the potential use of toxicant resistance as a tool in assessing contaminant effects

in natural ecosystem, states that the level of resistance of the organisms reflects the degree of contamination of the environment; the statement directly implies the effect of acclimation of organisms to sustained low level pollutants in the natural environment.

Apostolopoulou et al. (1986)reported that higher tolerance (acclimation phenomena, adaptation) to oil and oil dispersant can be induced in *Artenia salina* after pre-exposure to these toxicants. The higher tolerance demonstrated includes acute toxicity (LC_{50}), and sublethal physiological dysfunction (respiration). According to their observation high pre-exposure concentrations lead to rapid induction of acclimation phenomena, but the higher resistance is partly lost after exposure to clean water. It was also observed that exposure to low concentrations of toxicants induces a slow appearance of adaptation phenomena, but higher tolerance does not disappear after exposure to clean sea water and is even strengthened after detoxification period.

Duncan and Klaverkamp (1983), and Thomas et al. (1985) have also shown that tolerance to one metal can be induced by exposure to another metal (eg. Zn), usually with a corresponding increase in hepatic metallothionein.

In controlled laboratory experiments, pre-exposure to rainbow trout to Cu and Zn enhanced tolerance by 2 to 2.5 times relative to non-preexposed control fish. Bradley et al. (1985) demonstrated that this tolerance was accompanied by increased levels of hepatic proteins corresponding to metallothionein. When the trout were transferred back to uncontaminated water, both tolerance and levels of metallothionein like proteins declined to control levels.

5.3. MATERIALS AND METHODS

Methods of collection of organisms,and water samples were the same as described in Chapter 2.

The experiment protocols were as described by Dixon and Sprague (1981 a, b), and Bradley et al. (1985). Two sets of separate tests with the metal ions Cu and Zn were run in the tolerance experiments in two salinities (< 1 x $10^{-3}$and 13 x 10^{-3}). In the first set of tests a group of organisms (200 specimens) was subjected to 14 day pre-exposure of 10 µg/l of Cu. The exposure period was then extended to 21 days after allowing the organisms to remain in metal impoverished water for 7 days.. In the second set of experiments another group of (200 specimens) organisms were subjected to continuous pre-exposure of 21 days in 60 µg/l of Zn, and conventional toxicity tests were carried out with higher test concentrations

of Cu. The pre-exposure period and pre-exposure concentrations were selected based on the mean incipient lethal levels and LC_{50} of the metals to the organisms.Trial experiments were also conducted to determine the optimum effective pre-exposure concentrations. The observed mortality was less than 10 % during 14 day period and less than 20 % during 21 day Cu pre-exposure period. Nil mortality was observed in 60 µg/l Zn pre-exposure period (upto 21 days). On termination of pre-exposure, organisms were removed and used in toxicity tests to determine their tolerance to increased ambient levels of the metal.Percentage survival of Cu and Zn acclimated organisms was observed on 4, 8, 16, 24, and 32 days after maintaining them in 300 and 600 µg/l of Cu at salinity $13x10^{-3}$(Table 17 and 18). The percentage survival of Zn acclimated organisms was monitored in freshwater also at days 4,8,12,16, and 24(Table 19). The concentration of Cu used for dosing the post Zn acclimated organisms in freshwater was 60 and 100 µg/l. The same data presented in Table 1 and 2 (Chapter 3) were used to compare and assess the higher tolerance,if any, inducted due to acclimation of the metal ions.. Organisms were also subjected to tissue metal residue analysis (Cu) at various intervals (at 4,8,16,and 24 days) in the case of 10 µg/l Cu , and 60 µg/l Zn pre-exposed organisms at $13x10^{-3}$ salinity (Table 20, and 21), and at 4,8,12, and 16 days of 60 µg/l Zn pre-exposed organisms in freshwater(Table 22). For this, separate tests were conducted after acclimating another group

of organisms (200 specimens), each to 10 μg/l of Cu and 60 μg/l Zn for the specified period (21 days) and then post-exposing them to Cu ions for different concentrations. The post-exposure concentrations were as described above *i.e.*, 300 and 600 μg/l Cu at salinity $13x10^{-3}$, and 60 and 100 μg/l in freshwater.The Cu accumulation data from Tables 3 and 5 (Chapter 3) were used to compare the effect of metal pre-exposure on the organisms.

The metabolic rates of Cu and Zn acclimated organisms post-exposed to higher concentrations of Cu in both the salinities were also carried out but only important general observations were mentioned in the text. The acclimated organisms were allowed to remain in metal-free medium for additional 7 days (recovery) before final toxicity test was run to assess retention of the enhanced tolerance, if any. Groups of control organisms were maintained in metal free media, and were simultaneously subjected to the same procedures.

5.4. RESULTS

The results were statistically analysed using ANOVA techniques. The results are presented in Tables 17 to 22, and Fig. 17 to 22.

Table 17

Percentage Survival of Villorita cyprinoides after Acclimation to Cu Salinity 13 ppt

Conc. of metal ion µg/l	Exposure time : days					
	0	4	8	16	24	32
Cu 300	100	100	100	92	80	68
Cu 600	100	100	92	84	64	56
Non-accli Cu 300 *	100	96	88	56	44	36
Non-accli Cu 600 *	100	92	76	44	28	20

* From Table 2
Significance: $P < 0.01$

Table 18

Percentage Survival of Villorita cyprinoides after Acclimation to Zn Salinity 13 ppt

Conc.of Metal ion µg/l	Exposure time:days					
	0	4	8	16	24	32
Cu 300	100	100	100	96	84	72
Cu 600	100	100	100	92	76	60

* Data for non-acclimated organisms as above
Significance: $P < 0.01$

Table 19

Percentage Survival of Villorita cyprinoides after Acclimation to Zn Salinity < 1 ppt

Conc.of metal Ion µg/l	Exposure time:days					
	0	4	8	12	16	24
Cu 60	100	100	92	88	80	64
Cu 100	100	100	88	76	68	48
Non-accli Cu 60 *	100	96	72	52	40	28
Non-accli Cu 100 *	100	88	68	40	28	16

* Data of non-acclimated organisms from Table 1
Significance: $P < 0.01$

Table 20

Accumulation of Cu in Villorita cyprinoides after Acclimation to Cu
Salinity 13 ppt

Conc. of Metal ion in the-medium- µg/l	Exposure time : days					BCF
	0	4	8	16	24	
	Tissue Cu content $\bar{x}$ ± SD µg/g *					
Cu 300	33.86 2.14	34.23 2.57	45.96 2.37	96.39 4.24	157.91 6.78	526.36
Cu 600	32.95 2.42	35.57 2.69	59.39 2.98	102.93 6.42	163.54 7.11	272.56

* Metal accumulation data from Table 5,Chapter 1 used for comparison of changes in accumulation pattern of Cu from non-acclimated animals
Significance:$\underline{P}<0.05$,$\underline{P}<0.01$

Table 21

Accumulation of Cu in Villorita cyprinoides after Acclimation to Zn
Salinity 13 ppt

Conc. of Metal ion in the-medium- µg/l	Exposure time : days					BCF
	0	4	8	16	24	
	Tissue Cu content $\bar{x}$ ± SD (n=2)					
Cu 300	14.54 1.83	16.38 1.93	30.14 2.18	48.43 3.63	92.47 8.16	308.23
Cu 600	14.69 1.78	17.84 1.97	46.89 2.54	66.79 4.61	121.59 10.72	202.65

[To compare with Table 5]
Significance: $P<0.05$, $P<0.01$

Table 22

Accumulation of Cu in Villorita cyprinoides after Acclimation to Zn
Salinity < 1 ppt

Conc. of Metal ion in the-medium- µg/l	Exposure time : days					BCF
	0	4	8	12	16	
	Tissue Cu content $\bar{x}$ ± SD (n=2)					
Cu 60	13.12 1.18	15.73 1.63	19.36 2.03	38.77 2.84	65.19 4.93	1086.5
Cu 100	13.86 1.34	17.56 2.01	25.32 2.63	49.79 3.81	73.84 5.83	738.4

[To compare with Table 3]
Significance: $P<0.05$, $P<0.01$

Fig 17:Dose-Survival Curves of the clam after acclimation to Cu:Salinity 13 ppt

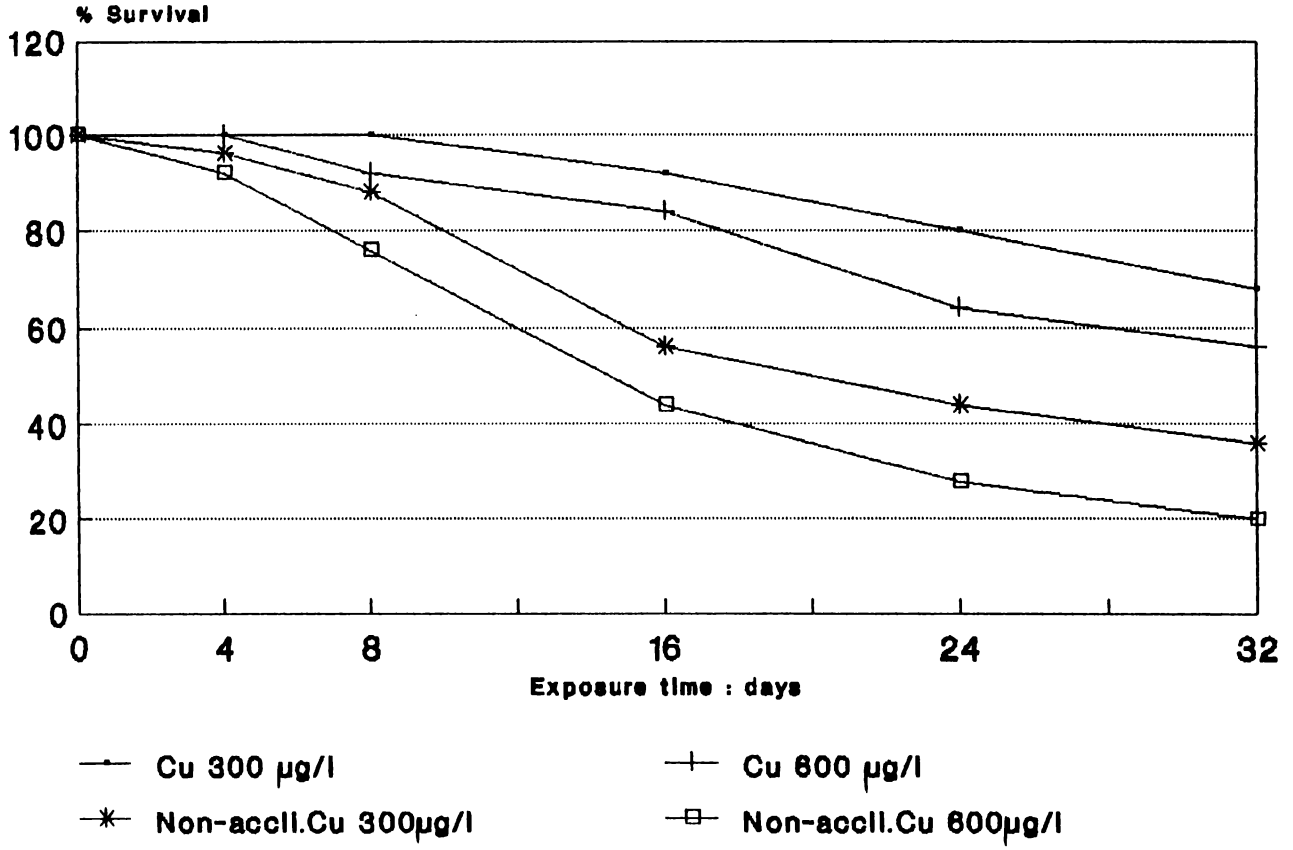


Fig 18: Dose-Survival Curves of the clam after acclimation to Zn:Salinity 13 ppt

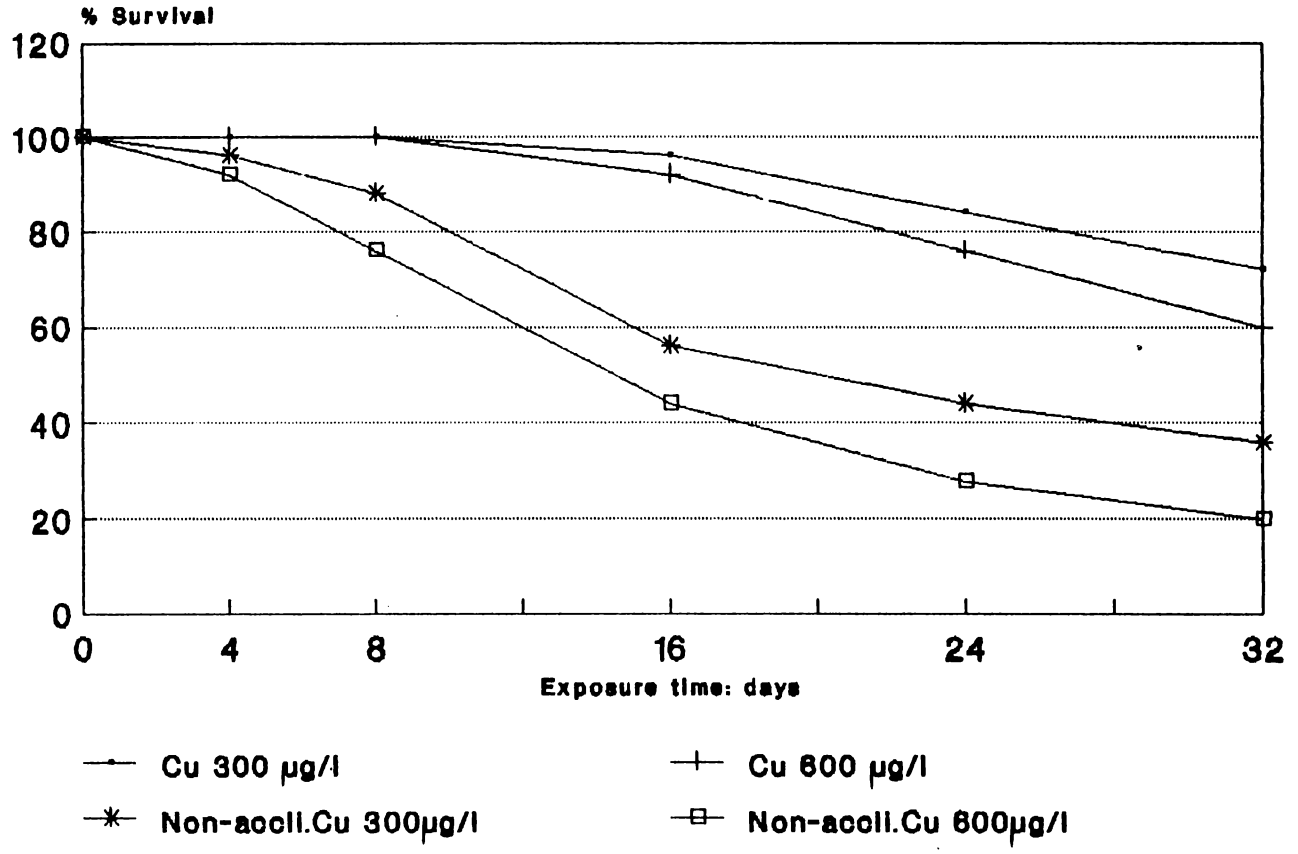


Fig 19:Dose-Survival Curves of the clam after acclimation to Zn:Salinity <1 ppt

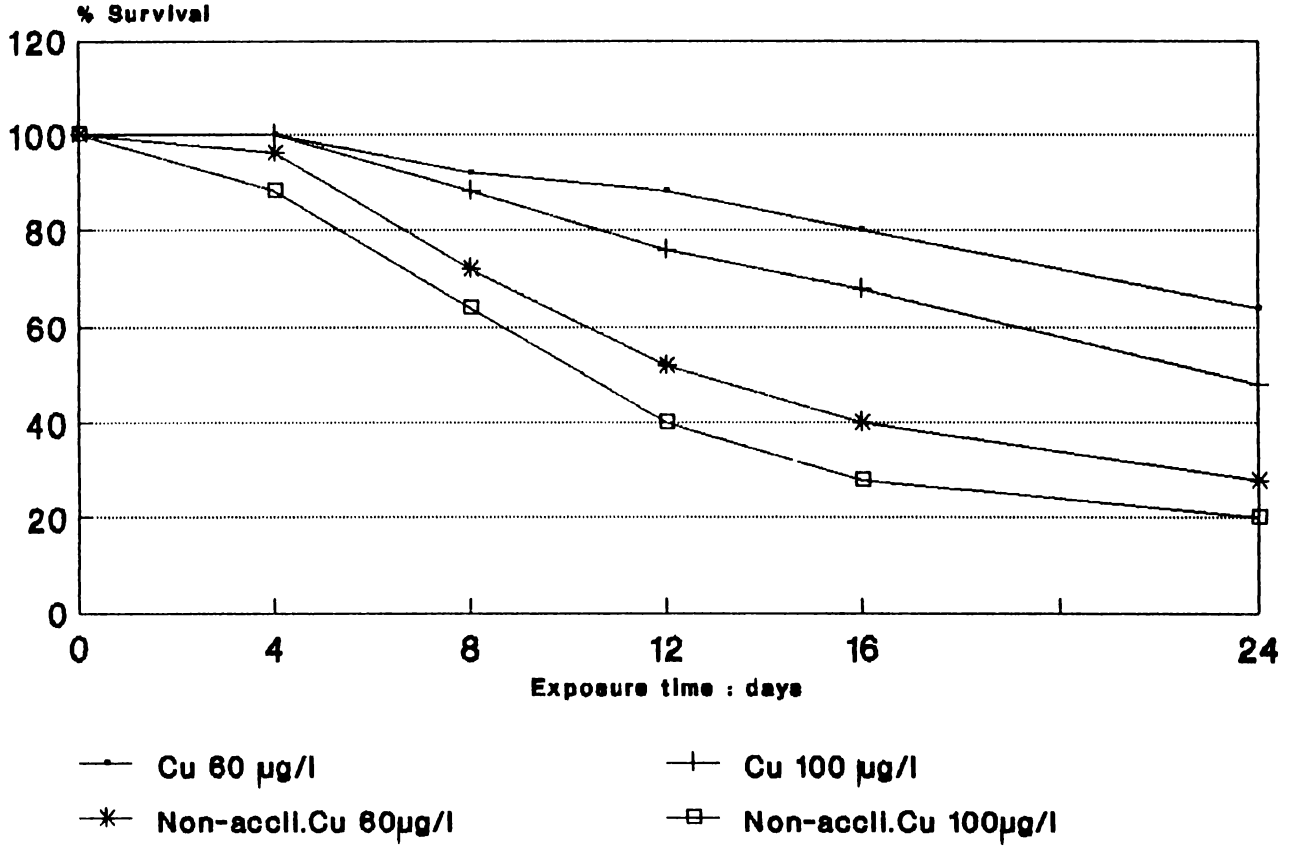
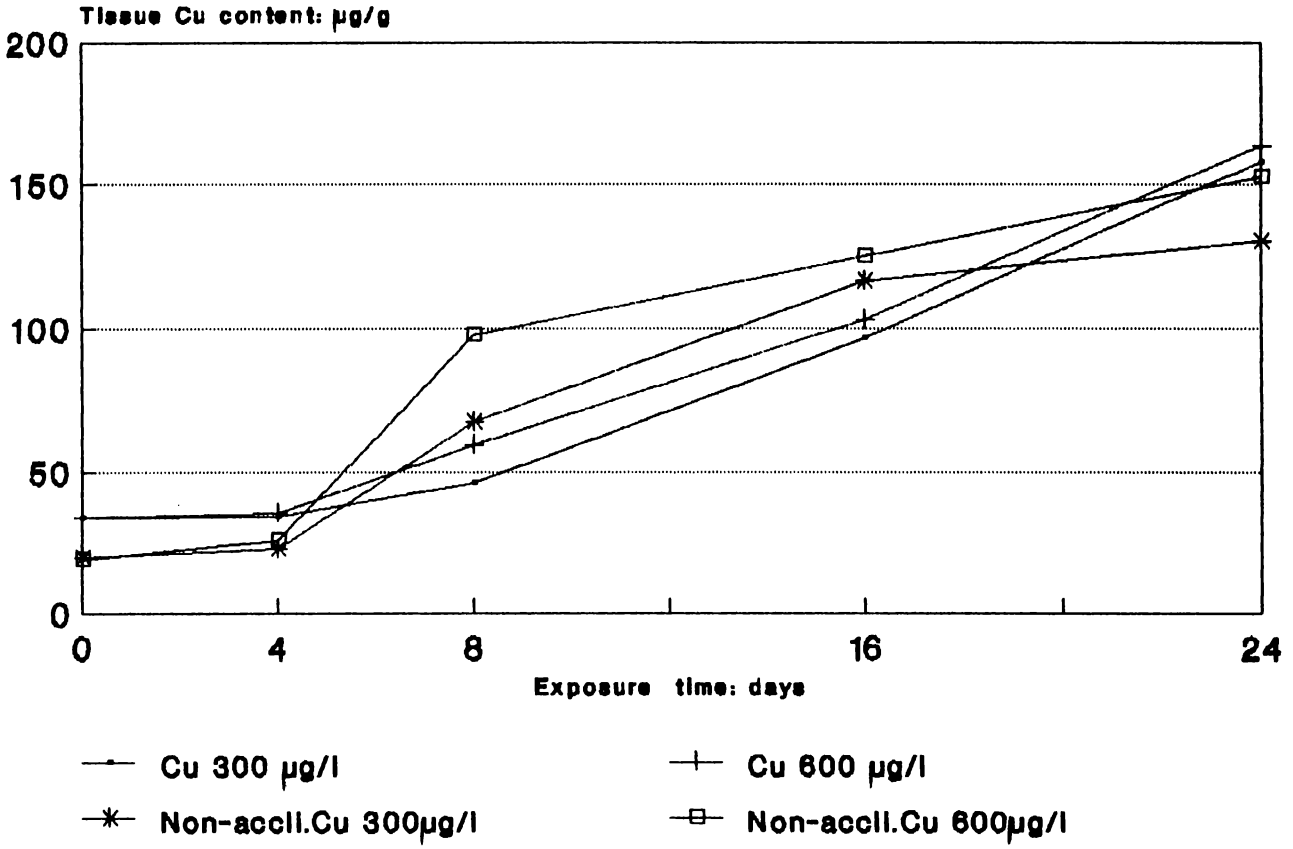


Fig 20:Cu accumulation in the clam after acclimation to Cu:Salinity 13 ppt



1 of non-accli. animals from Table 4)

Fig 21:Cu accumulation in the clam after acclimation to Zn:Salinity 13 ppt

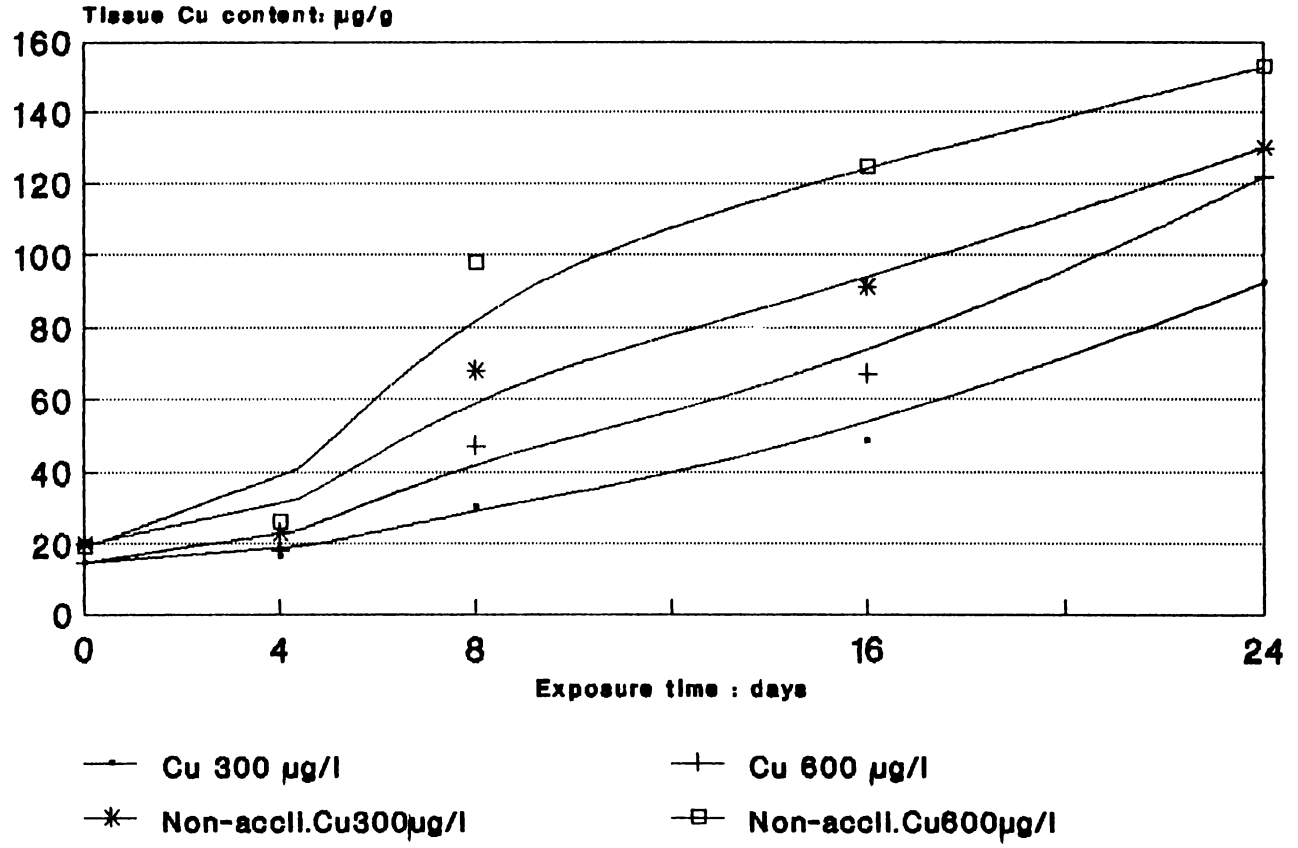
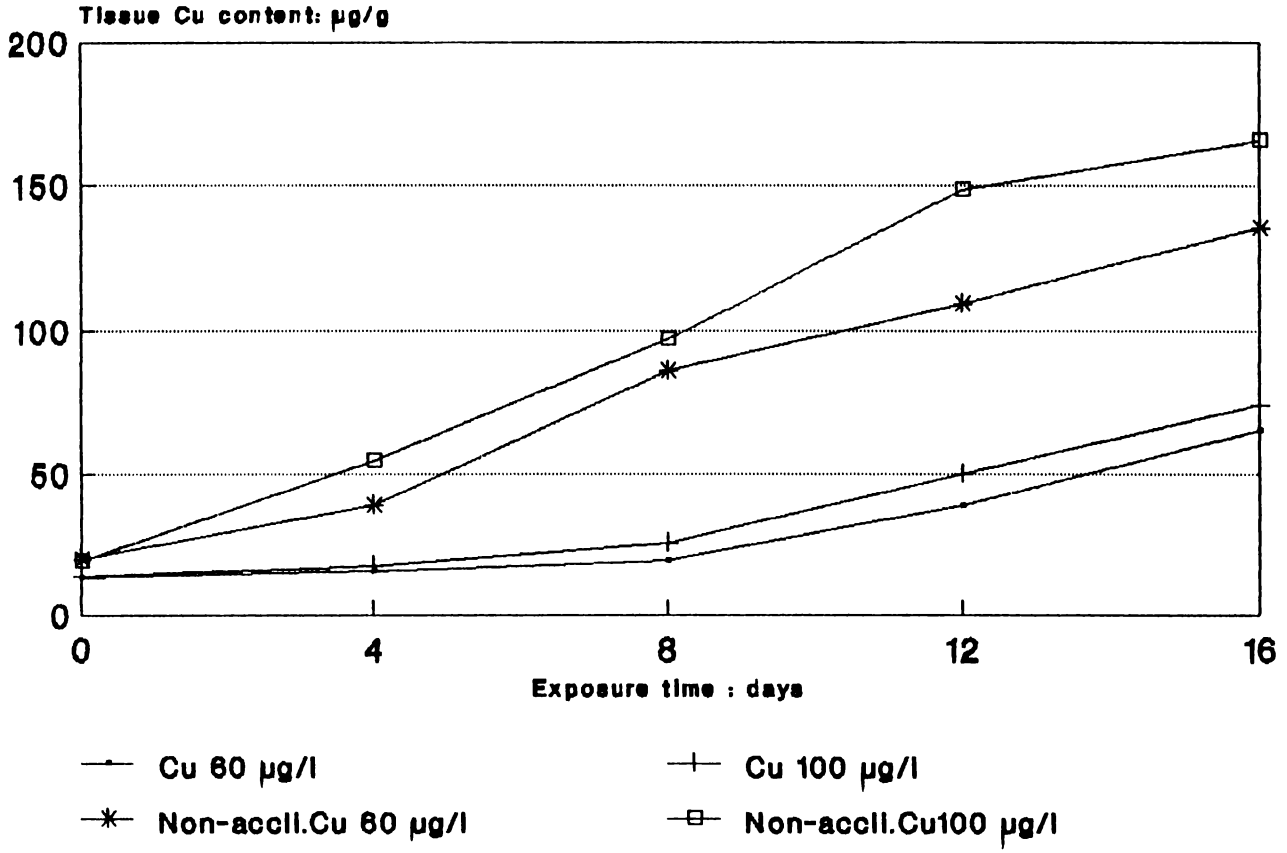


Fig 22:Cu accumulation in the clam after acclimation to Zn:Salinity < 1 ppt



5.4.1. Acclimation to Copper and Zinc

5.4.1.1. Percentage Survival of Acclimated Organisms

The percentage survival of Cu acclimated organisms on all days of post exposure to ambient levels of Cu at salinity $13x10^{-3}$ was significantly ($P<0.01$) higher than that of the non- Cu acclimated organisms (Table 17). The percentage survival on 4,8,16,24, and 32 days in 300 µg/l Cu of 10 µg/l Cu pre-exposed organisms increased to 100,100,92,80, and 68, respectively from 96,88,56,44, and 36, the latter values representing the survival rate of non-acclimated organisms(Table 2).Similarly, the percentage survival in 600 µg/l post-exposure to Cu was 100, 92,84,64, and 56 against 92,76,44,28,and 20, respectively, of non-Cu acclimated organisms (Compare Table 2 and 17).No significant difference was observed in percentage survival values of 0,4,and 8 days during post-exposure of Cu acclimated organisms, but the values of 16,24, and 32 days were significantly lower.

Significant increase ($P<0.01$) in survival rate of 60 µg/l Zn pre-exposed organisms in Cu dosed media was also noticed when compared with non-pre exposed organisms. For example, the survival percentage improved to 100,100,92,76,and 60, respectively, from 96,88,56,44,and 36 % in 600 µg/l Cu post exposure(Table 2 and 18 compared). There was no significant

difference in percentage survival values of 0, and 4 days. Similarly there was no significant difference between the percentage survival values of 12, and 16 days, though the values were significantly lower than those of 0 and 4 days. The main difference noticed between acclimation of Cu and Zn was that Zn acclimation increased the lethal tolerance of the organisms considerably even at lower salinity.

Approximately 1.5 to 2 times increase in lethal tolerance (ratio of acclimated LC_{50} to control LC_{50}) was observed in 21 day Cu and/or Zn pre-exposed organisms relative to non pre-exposed organisms. The onset of mortality was delayed, and the slope of the survival curves was also different (Table 17,18,19; Fig. 17,18,and 19). In 14 day acclimated organisms no significant change in lethal tolerance (as denoted by % survival) was noticed. Surprisingly, the increase in lethal tolerance to Cu of Zn acclimated organisms was more than that of Cu pre-exposed organisms (both relative to non-pre exposed organisms).Pre-exposure of even mild (0.5 ug/l) concentration of Cu to the organisms in freshwater (salinity $<1x10^{-3}$) sensitized the organism *i.e.*, tolerance to the metal decreased drastically.

The physiological responses of the acclimated organisms were also different compared to non-acclimated organisms. The classical metal stress responses; the alarm reaction (the

period of testing) seems to be absent, and the metabolic rate remained almost steady till the initial 96 h period. Depression in metabolic rate consequent to metal stress was also less compared to non pre-exposed animals at all time intervals (Tables and figures not presented).

5.4.1.2.Tissue Copper Content in Acclimated Organisms

Tissue residue analysis showed some interesting trends. Pre-exposure to Cu and Zn thoroughly changed the accumulation/retention pattern of Cu,during subsequent exposure of the organisms to higher concentrations of the metal. These altered rates of Cu uptake/or elimination were different for both metals. Statistical analysis of Table 20 revealed that there was significant difference ($P<0.05$) in the accumulation of Cu between 10 µg/l Cu acclimated, and non-acclimated organisms.In acclimated organisms no significant difference in values was obtained between days 0,4 and 8, but the values on 16 and 24 days were significantly higher when compared with the values on 0,4,and 8 days. No significant difference between concentrations of post-exposure was also noticed. The initial rate of uptake of the metal in 10 µg/l Cu exposed organisms seems to be less compared to non-acclimated organisms indicating some regulatory effect in the former. Tables 21 and 22 (compared with Tables 3 and 5) reveal that accumulation of Cu in Zn pre-exposed organisms was

significantly ($P< 0.05$) reduced or suppressed on all days relative to non-acclimated organisms.No significant difference was observed between the values on 0,4, and 8 days, but the values recorded on further exposure were significantly different. While acclimation to Cu equipped the organisms to survive for longer periods with increased Cu body burden, acclimation to Zn seems to suppress the uptake of the toxic metal. It may be noted that pre-exposure to Zn at both the salinities reduced even the control Cu content of the organism (though insignificant) (Tables 21 and 22). Zn acclimated organisms accumulated Cu slowly compared to control, and Cu acclimated organisms (Fig. 21 and 22). Hence, the BCF values of Zn acclimated organisms were significantly reduced when compared to non-acclimated organisms.

5.5. DISCUSSION AND CONCLUSION

Increased protein production could represent one facet of the mechanism for Cu/Zn acclimation (Dixon and Sprague,1981a; Bradley et al., 1985). Assuming that it binds Cu, the protein induced by pre-exposure would be available to do this during lethal exposure, thus reducing the effect of a given Cu dose. The presence of metallothionein-like proteins in organisms from metal- contaminated environments has been implicated in both the elevated resistance and metal body burden of those organisms.

The metallothioneins,a class of soluble low molecular weight proteins (mol.wt about 6800-7000) which is usually synthesised during heavy metal accumulation in organism may be playing its indispensable physiological role in the detoxification process and induction of enhanced tolerance (Viarengo, 1985; Hilmy et al., 1987 c).

Metallothioneins (MT) normally occur as a Zn or Zn and Cu containing protein (Funk et al., 1987). The physiological role of Metallothioneins could be related to the control of Cu and Zn metabolism, either through the binding of the excess quantity of divalent cations that penetrate into the cell or by permitting a redistribution of these essential metals among the appropriate apoenzymes (Viarengo, 1985). The binding capacity of MT is Zn<Cd<Cu<Hg. The extensive capacity of MT to bind a variety of physiological metals under *in vitro* conditions has been well demonstrated (Nielsen et al., 1985). Metallothioneins appears to be involved in metal homeostasis and detoxification. The biological functions of these proteins are not yet fully investigated. A recent study of Cu metallothioneins from an aquatic organism (after purification of metal free MTs by reverse phase HPLC, and amino acid analysis) showed that each isoform of Cu treated animals had a unique amino acid composition indicating the presence of three different classes of metallothioneins with different functional implications (Schlenk and Brouwer, 1991).

Since the ability of exogenous metals to bind to pre-existing metallothioneins with displacement of Zn *in vivo* and *ex vivo* during short time periods of exposure has been clearly established (Day et al., 1984) it may not be surprising to see in the present study that pre-exposure to Zn enhanced the lethal tolerance of Cu. MT provides a means of scavenging and storing Zn (Hodson, 1988). It has to be mentioned that metallothionein induction by Zn was reported to be associated with an accumulation of Zn not bound to MT also (Hilmy, 1987 a, c).

While these studies suggest a strong role for metallothionein in the metabolism of metals no proven function as yet has been established (Hodson, 1988). The most common theory is that MT acts to detoxify heavy metals through competitive binding and transport for excretion. The detoxification of metals in aquatic organisms was expressed by Brown and Parsons (1978) as a "spillover" hypothesis. The "spillover" hypothesis states that metallothionein acts as a 'mop' for free metals in the cytosol. As metal exposure increases, increasing amounts of MT maintains low levels of free metals in cytosol. It can also be assumed that MT detoxify metals through de novo synthesis, since MT always exist in a metal saturated form (Brown and Parsons, 1978). It was also reported that the reverse order of binding affinities of metals to MT is the order of the degree of acclimation of metals.

Recent work by Funk et al. (1987) has demonstrated the dynamic ability of the metals on MT to shuttle between and within clusters both inter and intra molecularly. The driving force for this movement may be the relative thermodynamic instability of the three metal clusters. Excess of Zn when present in hepatic MT may titrate the exogenous Cu with or without Cu being wedded for elimination (Funk et al., 1987).

To a certain extent the organisms can synthesise more of the metal binding protein to cope up with the additional metal ingested (Waldichuk, 1985). It may be assumed that Zn or Cu acclimation processes would have inducted additional metallothioneins to provide protection to the toxic metal Cu. The sensitization during pre-exposure to Cu in freshwater implies that induction did not occur, and that some deleterious effects of pre-exposure was carried over, contributing to the impact of the subsequent lethal concentration. While a hepatic Cu-binding protein could exert an intracellular protective effect against Cu, excretion of Cu could have to occur in order for the organisms to survive for extended periods. Moreover, the detoxification mechanism may have to get saturated in order to elicit enhanced tolerance to further exposure. During pre-exposure to Zn,even in freshwater, this would have probably happened resulting in reduced ventilation preventing effectively the toxic influx of Cu into the tissues and warding off the accumulated Cu. The reduction in toxicity to metal

acclimated organisms may also be due to the depression in the rate of uptake of the toxic metal into tissues, since it is known that the rate of uptake of a toxicant rather than the actual amount accumulated is the deciding factor determining toxicity of a solution (Mc Lusky and Philips, 1975).

The preferential apetite of Zn for this organism (the higher natural tissue concentration of this organism is already mentioned elsewhere) and its undefined role in providing apparent protection to Cu may seem complimentary to each other. The known cellular functions of Zn, which distinguishes it from the toxic action of Cu, is its stabilising effect of the lysosomal membranes.Cu ions cause destabilization of the lysosomal membrane and/or trigger hypersynthesis of the lysosomal enzyme (Suresh and Mohandas, 1990). Since this subcellular organelles are known to be one of the most interesting targets of heavy metals and important sites of metal compartmentation in the cell (Moore et al., 1982; Viarengo 1985) much work is needed to explore such cellular functions and to establish the exact mechanism underlying the detoxication and/or acclimation phenomena.

It is also reasonable to assume that Zn is entering gill and liver tissues to elicit the physiological responses of enhanced tolerance and metallothionein levels (Bradley et al., 1985).The concentration of Zn in cells governs many metabolic

processes specifically carbohydrate, fat, and protein metabolism, and nucleic acid synthesis or degradation-through initiation and\or regulation of the activity of these enzymes (Leland and Kuwabara, 1985).Correct ratios between Zn in the external medium, Zn bound to the membranes, and Zn in the internal environment are essential in the maintenance of the structure and function of cell membranes and the detoxification mechanisms to be effective (Viarengo, 1985). Zn ions stabilize the plasma and internal membrane either by binding to structural components or by preventing metal catalysed lipid peroxidation (Viarengo, 1985). It may be presumed that during pre-exposure to Zn the organisms become physiologically more competent and elicit defensive responses to invading toxicant. Zn was found to ameliorate the toxic effect of Cd on lipid peroxidation in liver and testis of male mice (Khan et al., 1991). They reported that in male mice pre-treated with Zn prior to Cd administration, significant decrease in lipid peroxidation in liver was observed. They argued that as Zn is an integral part of superoxide dismutase, the decrease in lipid peroxidation in liver tissue could be related with increased enzyme synthesis resulting in distribution of O2. Higher decomposition of H_2 O_2 can also be suspected as the reason for reduction in toxicity consequent to pre-exposure to Zn. The competitive interactions among heavy metals externally and internally are complex, and have little been studied.However, there are evidence from short term

experiments that absorption and retention of Zn and Cu are markedly influenced by the presence of either of the metal both in animals and plants (Misra and Mani, 1992).

The effect of pre-exposure to Cu and Zn, and the resultant biological response depict interaction, variability in tolerance, and changes in capacity for adaptation. The reaction of the animal facing exposure to the more toxic metal after exposure to the less toxic metal may be assumed to trigger the phenomena of capacity adaptation in an undefined way. The reasons for this could be multifarious. Selective absorption of the metal to reactive ligands, enhancement of detoxifying mechanisms, impediment/disruption on the transport of the metal ions after entry into the cells, and reaction of both the metal ions on the same site in the cell, could be the factors involved. The depurative process may also be affecting in such a way to ward off the more toxic metal, retaining the less toxic metals in cells.

The altered dynamics of metal uptake may be explained by the 'turn over' hypothesis of organic ligands. The cell contains limited amount of suitable ligands and the system is inducible, and there is turnover of the metal-ligand complex,and competition between metals for binding. The metals Cu and Zn are involved in quite different systems of ligand binding.Simkiss and Mason (1984) have demonstrated four

simple models depicting the metal-ligand systems within the cells.The models also predict the way the total metal content of the cell will change with time. Thus, if an organism is introduced into a metal containing environment, the metal will initially enter the cell and become bound to the available intracellular ligands so that direct proportionality will be maintained (the basis for the phenomena discovered in environmental surveillance programmes). The system becomes more complex if induction, ligand turnover, or metal-metal interaction become significant.Each of these systems may ultimately lead to a steady state concentration at any one time but one should need to know more about the kinetics of the uptake systems of the cells, and the chemistry of the intracellular ligands in order to predict,what the exact mechanisms will be(Simkiss and Mason, 1984). The processes by which metals shuttle into and out of metallothioneins under cellular conditions remain an area of interest for those researchers concerned with establishing more clearly the physiological function of metallothioneins (Neilsen et al., 1985).

Slower metal accumulation in metal exposed organisms (animals inhabiting in metal polluted environment) from field has been reported in a few studies. Bryan and Hummerstone (1973), and Bryan (1979) reported that worms inhabiting in Zn and Cu contaminated environment have shown higher tolerance and

reduced accumulation rates for the metals. This may be treated as an example for genetic adaptation via selection.Beattie and Pascoe (1978) found that rainbow trout alevins, pre-exposed to Cd as embryos, showed increased resistance and contained less body Cd after 320 h of exposure, than did fish which were first exposed as alevins. This also implies a slower rate of accumulation of that metal in the pre-exposed organisms. It was also found possible that a metal exposed female fish could incorporate metallothioneins or other metal binding compounds into her eggs, thus making them more resistant to metal toxicity throughout their development (Weis et al. 1981). It is likely that both genetic and physiological mechanisms are involved in such cases.

Higher tolerance (in LC_{50} values and respiration rates)was observed in *Artenia salina* after pre-exposure to oil and oil dispersant (Apostolopoulou et al., 1986). They demonstrated that higher pre-exposure concentrations lead to rapid induction of acclimation phenomena, but the higher resistance is partly lost when animals are transferred to clean water. And exposure to low concentration of toxicants induces a slow appearance of adaptation phenomena, but it was sustainable.

The ability of a species to limit non-specific binding of metals to vulnerable sites is a major factor in determining the accumulation of a metal and its ultimate toxicity (Jenkins and Mason, 1988). Many organisms have been reported to have

the capacity to regulate the distribution of both essential and non essential metals at the tissue, cellular, and sub cellular levels (Mason et al., 1984). Even though metals like Cu, Zn, Cd,and Hg may exist in sea water in a form that enables them to enter cells directly by a process that is only concentration dependent, it was noticed that the general sequence for uptake of metals into tissues was reversed or disrupted when the metals were combined or the organisms were inhabited in a mildly contaminated environment of one or the other metals. For example, when animals (*Littorina littorea*) were maintained in sea water containing Zn (^{65}Zn isotope), a linear accumulation of the metal into the whole soft body parts of the animal was observed over a 30-day period. The addition of Cd into the system induced a nine fold reduction in the accumulation of Zn and a loss in linearity (cited from Mason et al., 1984). Similar competitive interactions between Zn and Cd have been shown for *Mytilus edulis*, *Mytilus galloprovincialis*, and *Mulina lateralis* and the uptake of one metal was found to be significantly reduced. The Cu-Zn interactions which resulted in the reduction of the uptake of Cu and the loss of linearity were already described in Chapter 3.

It has to be assumed that the organism under study has specific cells which contain high capacity ligands that will bind a variety of metals.. So, a wide range of cellular

activities may be involved in the response of the organism to environmental metals, with wide range of reactivities including inducible systems.

One of the many implications of the modifying factors like the phenomenon of higher tolerance induction due to pre-exposure is in prescribing the quality criteria for different chemicals. The use of application factors ranging from 0.1 to 0.001 of the 96 h LC_{50} in the estimation of "no effects levels" and ultimately in the derivation of water quality standards reflects the uncertainity introduced by modifying factors such as the ones mentioned above. Hopefully, greater attention to these, in both acute and chronic tests, will go some way to reducing our reliance on application factors (which can prove expensive and sometimes unnecessarily over protective) in the formulation of water quality standards for environmental protection (Pascoe and Edwards, 1989). The validity of the surveillance programmes like `Mussel Watch' will be at stake unless corrective measures are taken and allowances are incorporated in each observation.

SUMMARY

SUMMARY

Apart from mechanical and amenities aspects, most pollution problems, in final analysis, are toxicological. The general acceptance of the advantages inherent in the use of bioindicators to monitor aquatic pollution has given rise to the establishment of national and international programmes employing such species in many parts of the world over the last decade. However, if the uptake of a given pollutant depends not only on its own ambient concentration but also on the presence, absence, or precise concentration of a second contaminant, and if the organisms metabolically regulate or physiologically compensate the bioindicator concept finally breaks down. This question is increasingly addressed by many, and this thesis is also a small step in this direction, although other aspects, as given elsewhere, are also included.

Eventhough much work has been carried out on the biology, and biochemical characteristics of molluscs in Cochin backwaters, no detailed attempt has been made to study the metal-metal interactions in this commercially important group of organisms. Hence, it was thought worthwhile to ascertain the metal-metal interaction in the clam *Villorita cyprinoides* (Hanley). No attempt, however, has been made to study the biology of the organism, and no distinction has been

made between males and females, since these are irrelevant as far as the main objectives of the present investigations are considered.

The thesis is presented in five chapters. Chapter 1 contains general introduction which briefly explains the significance of aquatic toxicity tests, the status of the backwaters, clam resources of Kerala, and the state of the art of heavy metal toxicity studies. Chapter 2 describes the materials and methods used in the study. The collection site of the organisms, acclimation procedures, and the general experimental protocols are also narrated here. The methods followed to determine the metabolic rate of the organisms, the analytical methodologies employed for the estimation of glycogen, lactic acid, and trace metals are also described in this chapter.

The interactive effects of the metals, Cu and Zn, particularly the influence of one metal on the accumulation, and retention kinetics of the other are described in Chapter 3. No attempt was made to quantify the mixture toxicity data by conventional models, and the reasons for the same are also explained. The toxicity evaluation and comparison are effected using Dose-Survival curves of the organisms exposed to individual and binary mixtures of the metals for periods extending up to more than one month. Comparison of the dose mortality/survival curves showed that there is significant

difference in the toxic action of the metals Cu and Zn when applied singly, and in binary combination. The slopes of dose mortality curves for all time intervals were not similar, which suggests that the toxic action of Cu did change with time and concentration. This again confirms that short period (96h) toxicity studies need not provide meaningful organismic toxic responses, or trends.

Significant changes in the accumulation pattern of Cu in the organism were noticed when the organisms were subjected to combined exposure (Cu and Zn in different permutations and combinations of concentrations in the medium). The body Cu burden was found to be less on all days of exposure when compared to that of organisms exposed to Cu alone. Zn uptake by the animals seems rather enhanced when the medium contained Cu even in lower amounts. A breakdown in the quantitative relationship between Zn in the ambient medium and the body was evident in such an environment.The kinetics of metal loss of the organism was found to be affected by the presence of the other metal. Copper was found to be lost at a faster rate when the tissue contained higher amounts of Zn. On the other hand, the body tried to retain higher amounts of Zn, when Cu residue in the tissues was in excess.

From the results it is assumed that the degree of toxic action of the metals is different, higher toxic responses of

Cu and less toxic responses of Zn. The multifarious reasons for this is explained briefly. The general concept of inorganic chemistry, that the higher the electronegativity and PK_s values of the metal sulphides and lower the ionic radius of the metal ion the more toxic the metal would be, seems valid in this context. The reduced toxicity of Cu to the organism during combined exposure may be due to the reduced uptake of Cu into the tissues in presence of Zn. Zn , in this context, may be acting as an ameliorating agent as far as Cu toxicity to the organism is considered. After briefly explaining the different accumulation and detoxification strategies of invertebrates, and the relavant/related observations of other authors the chapter is concluded by commenting on the *in situ* environmental implication of the observations.

Sublethal metal stress and physiological adaptive responses of the organism are discussed in Chapter 4. The changes in metabolic rate, variations in tissue glycogen, lactic acid, and carotenoid contents in clams exposed to Cu for various periods of time are given in this chapter. There was significant difference in metabolic rate of the organisms exposed to different metal concentrations on different exposure days at both the salinities when compared to the control. The decrease in metabolic rate on all days of exposure was less pronounced in combined metal exposure than in single exposure (Cu alone). An abrupt depression in metabolic rate

was noticed within 48 hours in the metal exposed organisms at both the salinities. The depression was more sharp at higher salinity. This sharp depression in metabolic rate observed within 48 hours of exposure to metal ions was not so conspicuous in combined exposure. The physiological dysfunction also varied depending upon the concentration of the metal ion in the medium, and the salinity.But after 72 hours, a temporary recovery in oxygen uptake was noticed in organisms maintained at both salinities. The change in metabolic rate was found to be inversely proportional to the carotenoid content. Significant variations in glycogen and lactic acid contents of the organisms were noticed during metal exposure. The muscle glycogen stores generally depleted fast during Cu exposure. Lactic acid content, however, increased during exposure period.

The results indicate that Cu affects the normal physiological and biochemical functions, including metabolic activity and energy budget. The animals can be assumed to have passed through two classical stages of stress response; the alarm reaction and the stage of resistance. The organisms were able to adjust to the initial stress, or may have partly released the stress. In higher salinity regime(optimum salinity) also the animals after showing symptoms of resilience went into a state of no return, and finally died. The physiological functions and the role of carotenoids in

tolerance of molluscs to metallic contaminants deserve further study.

It is pressumed that since this organism is typically euryhaline, it would be in overall osmotic balance at an intermediate salinity, and at this balance point the organism would be physiologically more effective and a flexible response to salinity change may be advantageous to overcome even the extra contaminant load in addition to fluctuating salinity regimes. But to what extent this will be effective is to be observed in natural environment itself. This chapter is also concluded with the *in situ* environmental implications of the observations.

The effect of pre-exposure (acclimation) to low concentrations of Cu and Zn, and the phenomenon of enhanced tolerance induction in the organism are discussed in Chapter 5.

Two sets of separate tests with the metal ions Cu and Zn were run on tolerance experiments at two salinities (< 1 x $10^{-3}$and 13 x 10^{-3}). The percentage survival of Cu and Zn acclimated organisms on all days of post-exposure to ambient levels of Cu at salinity $13x10^{-3}$ was significantly higher than that of the non - acclimated organisms.The main difference noticed between the acclimation of Cu and Zn was that Zn

acclimation increased the lethal tolerance of the organisms considerably even at the lower salinity.

In 14- day acclimated organisms no significant change in lethal tolerance (as denoted by % survival) was noticed. Surprisingly, the increase in lethal tolerance to Cu of Zn acclimated organisms was more than that of Cu acclimated organisms. Pre-exposure of even mild concentration of Cu to the organisms in freshwater sensitized the organism . The physiological responses of the acclimated organisms were also different compared to non-acclimated organisms. The alarm reaction (the period of testing) seems to be absent and the metabolic rate remained almost steady till the initial 96 h period.

The tissue residue analysis showed some interesting trends. Pre-exposure to Cu and Zn thoroughly changed the accumulation/retention pattern of Cu,during subsequent exposure of the organisms to higher concentrations of the metal. These altered rates of Cu uptake/or elimination were different for both metals. While acclimation to Cu equipped the organisms to survive for longer periods with increased Cu body burden, acclimation to Zn seems to suppress the uptake of the toxic metal.

Increased protein production is expected to represent one facet of the mechanism for Cu/Zn acclimation. The role of metallothionein in this respect is described in detail in this chapter. Metallothionein acts to detoxify heavy metals through competitive binding and transport for excretion. It is assumed that Zn or Cu acclimation processes would have inducted additional metallothioneins to provide protection to the toxic metal Cu. It is presumed that during pre-exposure to Zn the organisms become physiologically more competent and elicit defensive responses to invading toxicant. Selective absorption of the metals to the reactive ligands/ vulnerable sites and changes in the depurative process may be effective in such a way to ward off the more toxic metal and retaining the less toxic metal in cells. A wide range of cellular activities is assumed to be involved in the acclimation phenomena.

As concluding remarks the following may be mentioned. A question to be addressed is whether criteria for maximum allowable pollutants concentrations be universally established, or whether local/regional decisions be made based on environmental conditions and pollutants. The latter approach seems to be more reasonable and cost effective. Regarding the concept of sustainable development and long term environmental protection, GESAMP(1991) views may be fully endorsed and it may be suggested that Biological Monitoring should be integrated with Chemical Monitoring to fulfill the goals of marine

environmental protection. Those chemicals accumulated in the tissues of organisms, particularly those harvested for human consumption, may be monitored occassionaly to evaluate the concentrations found in the context of relevant acceptable daily intakes. Environmental models that represent the various inputs and the pathways by which critical target organisms, including man, are exposed to xenobiotics should be developed by environmentalists /local environmental managers which will provide them with rapid predictions of environmental distributions at low cost.

REFERENCES

Abdullah, A.M and Ireland,M.P (1986) Seasonal studies on Cadmium concentrations and toxicity, oxygen consumption, digestive gland glycogen and lipid in Dog whelks collected at Aberystwyth,Wales.Mar.Poll.Bull., 17, 562-566.

Andersen, A.T and Foyn Jr (1969) Common methods for chemical analysis of Sea water. In: Chemical Oceanography. Lange R, (ed.)., Scand.Univ.Press, Boston, 112-121.

Anderson, G (1977) The effects of parasitism on energy flow through shrimp populations. Mar.Biol., 42., 239-251.

Anderson,P.D and D'Apollonia (1978) Aquatic animals. In: Priciples of Ecotoxicology,12, Butler,G.C (ed), John Wiley and Sons, New-York.

Ahsanullah, M, Negilski, D.S and Mobley, M.C (1981) Toxicity of zinc,cadmium, and copper to the shrimp *Callianassa australiensis*. 111. Accumulation of metals. Mar.Biol., 64, 311-316.

Apostolopoulou, M.M, Verrioulos, G and Karakassis, I (1986) Effects of pre-exposure on the tolerance of *Artemia salina* to oil and oil dispersant. Mar.Poll.Bull., 17, 72-76.

Baby, K.V (1987) Combined toxicity of heavy metals and petroleum hydrocarbons on selected marine organisms. Ph. D Thesis, Cochin University of Science and Technology, Cochin, India

Barker, S.B (1957) Preparation and colorimetric determination of lactic acid. In:Methods in Enzymology Vol 3 Colowick SP and Kaplan N.O (eds), Academic Press, New York. 241 - 245.

Barton, B.A and Schreck (1987) Metabolic cost of acute physical stress in juvenile steel head. Trans.Am.Fish.Soc., 116, 257-263.

Bayne, B.L (1975) Aspects of physiological condition in *Mytilus edulis* L with respect to the effects of oxygen tension and salinity. Proc.Ninth Europ. Mar.Biol.Symp., 213 - 238.

Bayne, B.L (1976) Watch on mussels. Mar.Poll.Bull.,7, 217-218.

Bayne, B.L (1985) Cellular and physiological measures of pollution effects. Mar.Poll.Bull., 16, 127 - 129.

Beattie, J.H and Pascoe (1978) Cadmium uptake by rainbow trout, *Salmo gairdneri* eggs and alevins. J. Fish. Biol., 13, 631 - 637.

Bingham, F.T, Sposito G and Strong J.E (1984) The effect of chloride on the availability of Cd. Environ. Qual., 13, 71-74.

Boisson, F, Barelli M.G, Chiaverini J and Romeo M (1989) Effect of selenium on the uptake of cadmium by the marine microalga *Hymenomonas(cricosphaera)elongata*. Mar.Environ Res., 28, 465 - 469.

Boesch, D.F, and Rosenberg, R (1981) Responses to stress in marine benthic communities. In: Stress effects on natural ecosystems. Barrett, G.W and Rosenberg, R (eds), John Wiley & Sons, New York.,895 p.

Boudou,A and Ribeyre,F (1989) Aquatic Toxicology In:Fundemental concepts and methodologies 2. Boudou,A and Ribeyre, F(eds), CRC Press Inc., Florida. 127-162.

Bradley, R.W, Du quesnay C and Sprague J.B (1985) Acclimation of rainbow trout,*Salmo gairdneri* Richardson to zinc : kinetics and mechanism of enhanced tolerance induction J. Fish. Biol., 27, 367 - 379.

Breittmayer, J.Ph and Gutierrez Galindo E.A (1981) Acute toxicity from the combination of mercury & zinc with regard to the mussel *Mytilus edulis*. Chemosphere. 10, 795 - 798.

Britton, G (1985) General carotenoid methods. In: Methods in Enzymology 3, Colowick, S.P and Kaplan, M.O (eds)., Academic Press, New York. 113-149.

Brooks,R.R and Rumsby (1967) Studies on the uptake of cadmium by the oyster, *Ostrea sinuata* (Lamark). Aust. J. Mar. Freshwat. Res., 15, 53-61.

Brown,B.E (1977) Uptake of copper and lead by a metal tolerant isopod *Asellus meridianus*, Rac.Fresh.wat.Biol., 7, 235-44.

Brown,D.A and Parsons,T.R (1978) Relationship between cytoplasmic distribution of Hg and toxic effects to zooplankton and chum salmon (*Oncorhynchus keta*) exposed to Hg in a controlled ecosystem. J.Fish.Res.Bd. Canada., 35, 880-884.

Brown,B.E (1982) The form and function of metal containing granules in invertebrate tissues. Biol.Rev.Cam.Philos.Soc., 57, 621-667.

Brown,T.L and Lemay,H.E Jr (1985) Basic concepts and chemical bonding In : Chemistry, the central Science. Brown and Lemay Jr (eds), 3rd ed. Prentice-Hall Inc. N.J. 182-218.

Bryan, G.W and Hummerstone, L.G (1973) Adaptation of the polychaete *Nereis diversicolor* to estuarine sediments

containing high concentrations of zinc and cadmium. J.Mar.Biol.Assoc. U.K., 53, 839-857.

Bryan, G.W (1974) Adaptation of an estuarine polychaete to sediments containing high concentrations of heavy metals. In:Pollution and physiology of marine organisms. Vernberg J and Vernberg W (eds). 123 - 135.

Bryan, G.W (1976a) Some aspects of heavy metal tolerance in aquatic organisms. In: Effects of Pollutants on aquatic organisms. Lockwood A (ed). Cambridge Univ.press, Cambridge. 7- 34.

Bryan,G.W (1976b) Heavy metal contamination in the sea. In: Marine pollution , Johnson,R(ed). Academic Press, London. 185-302.

Bryan, G.W (1979) Bioaccumulation of marine pollutants. Phil.Trans.R.Soc.London., 286, 483 - 505.

Bryan, G.W (1984) Pollution due to heavy metals and their compounds In:Marine Ecology, 5. Kinne, O (ed), John Wiley & Sons Ltd. New York. 1311 -1393.

Carrier, R and Beitinger (1988) Resistance of Temperature tolerance ability of Green sunfish to Cd exposure. Bull. Environ. Contam.Toxicol., 40, 475 -480.

Caulcutt, R, and Boddy, R (1983) Statistics for Analytical Chemists. Chapman and Hall Ltd, New York. 253p.

Chan, H.M (1988) Accumulation and tolerance to Cd, Cu, Pb & Zn by the green mussel *Perna viridis*. Mar.Ecol. Progr., 48, 295-303.

Chapman, (1985) Acclimation as a factor influencing metal criteria.Aquatic toxicology and hazard assesment. Eighth symposium ASTM STP,891. 119 - 136.

Chvapil, M (1973) The chemistry, biochemistry and biological role of zinc : a stabilizer of macromolecules and biological membranes. Life Sci. 13., 1041 - 1049.

Clesceri, L.S, Greenberg, B.E ,and Trussel, R.R (1985) Toxicity test methods for the examination of water and waste water. APHA, 16th edition., Washigton DC. 1268 p

Clesceri, L.S,Greenberg, A.E and Trussel, R.R (1989) Toxicity test methods for the examination of water & waste water., APHA 17thedition., Washington DC 8.1 - 8.38.

Connell, D.W and Miller, G.W(1984) Interations between pollutants and multiple toxicity,conceptual aspects In : Chemistry and Ecotoxicology of Pollution. Connell and Miller (eds), John Wiley & Sons New York. 309p

Conklin, D.J, Mowbray, R.C, and Gingerich (1992) Effects of chronic exposure to soft, acidic water on gill development and chloride cellnumbers in embroyo-larval brook trout, *Salvelinus fontinalis*. Aqua. Toxicol., 22, 39-52.

Coombs, T.L (1972) The distribution of zinc in the oyster *Ostrea edulis* and its relation to enzymatic activity and to other metals. Mar.Biol., 12, 170 - 178.

Coombs,T.L (1974) The nature of zinc and copper complexes in the oyster *Ostrea edulis*. Mar.Biol.,9., 347-54.

Cotton, F.A and Wilkinson, G.W (1979) Biochemical activity. In :Advanced Inorganic chemistry . 3rd ed. Wiley & Sons Inc.,

New York. 921 p

Da Silva, J.J.R.F (1978) Interaction of the chemical elements with biological systems. In : New Trends in bioinorganic chemistry. Williams JP. and Da Silva J.J.R.F (eds). Academic Press,London 484p.

Davenport, J, Busschots, P.M.C.F, and Cawthorne, D.F (1980) The influence of salinity on behaviour and oxygen uptake of the hermit crab *Pagurus Bernhardus* L. J.Mar.Biol.Assoc.U.K., 60, 127-134.

Davis, A.G (1976) An assessment of the basis of Hg tolerance in *Dunaliella teritolecta*. J.Mar.Biol.Assoc.U.K.56, 39-57.

Dawson, R, Bewers, J.M, Andersen, N.R and Kullenberg, G (1988) Global strategies for the assessment of pollution in marine environment and progress achieved in the IOC-GIPME programme. Aqua.Toxicol., 11, 345-356.

Day, F.A, Funk, A.E and Brady, F.O (1984) *In vivo* and *ex vivo* displacement of Zn from metallothionein by cadmium and by mercury. Chem - Biol Interact., 50, 159 - 174.

Denton, G.R.W and Jones, C.B (1981) Influence of temperature and salinity on the uptake, distribution and depuration of mercury, cadmium and lead by the black-lip oyster *Saccostrea echinata*. Mar. Biol., 64, 317-326.

Depledge, M.H (1987) Enhanced copper toxicity resulting from environmental stress factor synergies. Comp. Biochem. Physiol., 87, 15-19.

Dillon, T.M and Lynch,M.P (1981) Physiological Responses as determinants of stress in marine and estuarine organisms. In:

Stress effects of Natuaral Ecosystems. Barrett, G.W and Rosenberg, R (eds)., John Wiley and Sons Ltd. pp 227-241.

Dixon, D.G and Sprague, J.B (1981a) Copper bio accumulation and hepatoprotein synthesis during acclimation to copper by juvenile rainbow trout. Aqua.Toxicol., 1, 69-81.

Dixon, D.G and Sprague, J.B (1981b) Acclimation to copper by rainbow trout-a modifying factor in toxicity. Can.J.Fish.aquat.Sci., .38, 880-888.

Donaldson, E.M and Dye, H.M (1975) Corticosteriod concentrations in sockeye salmon exposed to low concentrations of Cu. J.Fish.Res.Bd. Canada., 32, 533-539.

Duncan, D.A and Klaverkamp (1983) Tolerance and resistance to cadmiumin white suckers (*catostomus commersoni*) previously exposed to Cd, Hg and Zn or Se. Can.J.Fish.Aquat.Sci., 40, 128-138.

Eisler, R and Weinstein (1967) Changes in metal composition of the quahang clam, *Mercenaria mercenaria,* after exposure to insecticides. Chesapeake. Sci., 8, 253-258.

Eisler, R and Gardner (1973) Acute toxicity to an estuarine teleost of mixtures of Cd, Cu and Zn salts. J.Fish.Biol., 5, 131-142.

Eisler, R (1977) Toxicity evaluation of a complex metal mixture to the soft shell clam *Mya arenaria*. Mar.Biol., 43, 265-276.

Eisler, R (1981) Mollusca. In: Trace metal concentrations in marine organisms Pergamon press, New York.

Engle, D.W, and Fowler, B.A (1979) Copper and cadmium induced changes in the metabolism and structure of molluscan gill tissue. In: Marine Pollution-Functional response.Vernberg, W.B, Calabrese, A, Thurberg, F, Vernberg, F.J (eds), Academic Press, London. 239-255.

Ernest, W (1985) Accumulation in aquatic organisms In: Appraisal of Tests to predict the environmental behaviour of chemicals. Sheehan, P, Korte, F, Klein, W, and Bourdeaw Ph (eds). John Wiley and Sons 243-255.

Febry, R and Lutz, P (1987) Energy partioning in fish,the activity related cost of osmoregulation in a euryhaline cichlid. J. Exptl. Biol., 128, 63-85.

Forstner, U, and Wittmann, G.T.W (1979) In:Metal pollution in the aquatic environment. Springer-Verlag. New York, 1-486.

Fowler, B.A, Wolfe, D.A and Hettler, W.F (1975) Mercury and Iron uptake by cytosomes in mantle epithelial cells of quahog clams (*Mercenaria mercenaria*) exposed to Hg. J. Fish. Res. Bd. Canada, 32, 1767-1775.

Franz, E.H (1981) A general formulation of stress phenomena in ecological systems. In: Stress effects on natural ecosystems. Barret G.W and Rosenberg (eds), John Wiley and Sons Ltd., New York.

Frazier, J.M and George, S.G (1983) Cadmium kinetics in oysters : a comparative study of *Crassostrea gigas* and *Ostrea edulis*. Mar.Biol., 55, 615-61

Fry, F.E.J (1971) The effect of environmental factors on the physiology of fish. In: Fish Physiology 6,. Hoar W.S and Randall (eds), Academic Press, New York. 1-98.

Funk, A.E, Day,F.A and Brady, F.O (1987) Displacement of zinc and copper from copper induced metallothionein by cadmium and by mercury: *in vivo* and *ex vivo* studies. Comp.Biochem.Physiol., 86, 1-6.

Gaudy, R and Sloane, L(1981) Effect of Salinity on oxygen consumption in post larvae of the penaeid shrimps *Penaeus monodon* and *P.stylirostris* and without and with acclimation. Mar.Biol., 65, 297-301.

George, S.G, Pirie, J.S, Chupu, A.R, Coombs, T.L and Grant, P.T (1978) Detoxification of metals by marine bivalves : An ultra structural study of the compartmentation of Cu and Zn in the oyster *Ostrea edulis*. Mar.Biol., 45, 147-156.

George, S.G and Pirie, B.J.S (1980) Metabolism of zinc in the mussel, *mytilus edulis* (L) :a combined ultra structural and biochemical study. J. Mar.Biol.Assoc. U.K., 60, 575-590.

George, S.G (1983) Heavy metal detoxification in the mussel *Mytilus edulis* L; composition of cadmium containing kidney granules (tertiary lysosomes). Comp.Biochem.Physiol., 76, 49-58.

GESAMP (1991) Global strategies for marine environmental protection. GESAMP Reports and Studies. No.45, IMO. London.

Giesy, J.P, Bowling,J.W and Kania, H.J (1980) Cadmium and Zinc accumulation in fresh water cry fish. Arch. Environ. Contam. Toxicol., 9, 683-697.

Graney, R.L,Jr,Cherry, D.S and Cairs, J,Jr (1984) The influence of substrate, pH, diet and temperature upon cadmium accumulation in the asiatic clam (corbicula fluminea) in laboratory artificial streams. Wat. Res., 18, 833-842.

Green, D.W, Williams, K.A, Hughes, D.R, Gulnazbibi, A.R.S and Pascoe, D (1988) Toxicity of phenol to *Asellus aquaticus* (L) - effects of temperature and episodic exposure. Wat. Res., 22, 225.

Halstead, B.W (1972) Toxicity of marine organisms caused by pollutants In: Marine pollution and Sea Life. Ruivo (ed), F A O, Fishing News (Books) Ltd. England. 1-585.

Hassid, W.Z and Abraham, S (1957) Chemical procedure for analysis of polysaccharides. In: Methods in Enzymology 3. Colowick, S.P and Kaplan, N.O (eds), 34-35.

Herbert, D.W.N and Wakeford, A.C (1964) The susceptibility of Salmonid fish to poisons under estuarine conditions. 1. Zinc sulphate. Int. J. Air Wat. Poll., 8, 251-256.

Heslop, R.B, Jones, K (1976) Reactions of metal complexes. In: Inorganic chemistry. A guide to advanced study. Elsevier Sci. Publ.Co, New York. 580-739.

Hilmy, A.M, El-Domiaty, N.A, Daabees, A.Y and Taieb, A.E.M (1986) Studies of Zn, Mg, Cu, Ca and Fe in Cd poisoned Toads before and after treatment with EDTA. Comp. Biochem. Physiol., 85, 167-170.

Hilmy, A.M, El-Domiaty, N.A, Daabees, A.Y and Latif, H.A.A (1987a) Some physiological and Biochemical indices of zinc toxicity in two fresh water fishes,*Clarias lazera and Tilapia zilli*. Comp. Biochem. Physiol., 87, 297-301.

Hilmy, A.M, El-Domiaty, N.A, Daabees, A.Y and Latife, H.A.A (1987b) Toxicity in *Tilapia zilli* and *Clarias lazera* (pisces)induced by zinc, seasonally. Comp. Biochem. Physiol.86, 263-265.

Hilmy, A.M, El-Domiaty, N.A, Daabees, A.Y and Alsarha, A (1987c) The toxicity to *Clarias lazera* of copper and zinc applied jointly. Comp. Biochem. Physiol., 87, 309-314.

Hoar,W.S (1987) The animal and its environment. In: General and Comparative Physiology 3. Prentice Hall,New Delhi 3-7

Hochachka, P.W (1985) Theorems of Environmental adaptation. C.M.F.R.I. Cochin,Special Publication. 26, 1-51.

Hodson, P.V (1986) Water quality criteria and the need for biochemical monitoring of contaminant effects on aquatic ecosystems. In: Water quality management : Fresh water ecotoxicity in Austalia. Hart, B.T (ed), C I T, Melbourne. 3-9.

Hodson,P.V (1988) The effect of metal metabolism on uptake, disposition and toxicity in fish. Aqua. Toxicol., 11, 3-18.

Ivanovici, A.M and Wiebe, W.J (1981) Towards a working defanition of 'stress'. A review and critique. In: Stress effects on natural ecosystems. Barrett, G.W and Rosenberg, R (eds), John Wiley and Sons. 1-200.

James,R, Sampath, K, and Ponmani, K.P (1992) Effect of metal mixtures on activity of two respiratory enzymes and their recovery in *Oreochromis mossambicus* (Peters). Ind. J. Exptl. Biol., 30, 496-499.

Jenkins, K.D and Mason, A.Z (1988) Subcellular distributions of cadmium and peturbations in reproduction in the polychaete *Neanthes arenaceodentata*. Aqua. Toxicol., 12, 229-244.

Johns (1973) Influence of salinity and temperature on the toxicity of mercury to marine and brackish water isopodes

(crustacea). Est. Coast. Mar. Sci., 1, 425-431.

Karnaukhov, V.N (1971) The carotenoids in oxidative metabolism of molluscoid neurons. Exptl. Cell. Res. 64, 301-306.

Karnaukhov, V.N, Milovidova, N.Y, Kargopolova, I.N (1977) On a role of carotenoids in tolerance of sea molluscs to environmental pollution. Comp. Biochem. Physiol. 56, 189-193.

Katticaran, C.M, and Salih (1992) Copper induced metabolic changes in *Sunetta Scripta* (Bivalve): Oxygen uptake and Lactic acid production. Bull.Environ.Contam.Toxicol., 48, 592-598.

Kay, J, Thomas, D.G, Brown, M.W, Cryer, A, Shurben, D, Solbe, J.F and Garvey (1986) Cadmium accumulation and protein binding patterns in tissues of the rainbow trout, *salmo gairdneri*. Environ.Health.Perspect., 65, 133-139.

Khan, S, Khan, M.A, Bhatnagar, D, Poonam, Y and Sarkar, S (1991) Zinc protection against lipid peroxidation from Cadmium. Ind. J. Exptl. Biol., 29, 823-825.

Kinne, O (1984) Pollution due to heavy metals and their compounds. In: Marine ecology. 5, John Wiley and sons Ltd. 1103p.

Klaverkamp, J.F, Macdonald, W.A, Duncan, D.A and Wagemann, R (1984) Metallothionein and acclimation to heavy metals in fish : a review In: Contaminant effects on fisheries. Cairns, V.W, Hodsonn, P.V and Nriagu, J.O(eds) John Wiley and Sons., New York. 99-113.

Klerks, P.L and Levinton, J.S (1981) Rapid evolution of metal resistance in a benthic oligocliaete inhabiting a

metal-polluted site. Biol. Bull., 176, 135-141.

Klerks, P.L and Bartholomew, P.R (1991) Cadmium accumulation and detoxification in a Cd-resistant population of the oligochaete *Limnodrilus noffmeisteri*. Aqua. Toxicol., 19, 97-112.

Krantzberg, G and Stokes, P.M (1989) Metal regulation, Tolerance and body burdens in the larvae of *Genus chironomus*. Can. J. Fish. Aquat. Sci., 46, 389-397.

Krishnakumar, P.K, Damodaran, R and Nambisan, P.N.K (1987) Carotenoid content and tolerance of green mussel *Perna viridis* (Linnaeus) to acute mercury and Zn toxicity. Ind. J. Mar.Sci., 16, 155-157.

Lakshmanan, P.T (1982) Investigations on the chemical constituents and trace metal interactions in some bivalve molluscs of the Cochin backwaters. PhD Thesis, Cochin University of Science and Technology, Cochin, India.

Lange, R (1969) Marine Biology and Chemistry In: Chemical Oceanography, an introduction. Scand. Univ.Books, Stockholm. 35-46.

La Point, T.W, Fairchild, J.F, Little, E.E and Finger, S.E (1989) Laboratory and field techniques in ecotoxicological research :strength and limitations. In: Aquatic Ecotoxicology 2. Boudou, A and Ribeyre, F (eds) C.R.C Press Inc., Florida. 239-255.

Lavie, B and Nevo, E (1986) The interactive effects of Cd and Hg pollution on allozyme polymorphisms in the marine gastropod *Cerithium scabridum*. Mar. Poll. Bull., 17, 21-23.

Leland, H.V and Kuwabara, J.S (1985) Trace metals. In: Fundementals of Aquatic Toxicology. Rand, G.M and Petrocelli, S.R (eds) Hemis. Publ. Corp. Washington, 374-404.

Little, E.E, Flerou, B.A and Ruzhinskaya, N.N (1985) Behavioural approaches in aquatic toxicology investigations:a review. In: Toxic substances in aquatic environment. Mehrle, P.M, Gray, R.H and Kendall, R.L (eds), Amer.Fish.Soc. Bethesda. 72-98.

Lobel, P.B (1987) Inherent variability in the ratio of zinc to other elements in the kidney of the mussel *Mytilus edulis*. Comp. Biochem. Physiol., 87, 47-50.

Luoma, S.N and Jenne, E.A (1977) The availability of sediment bound cobolt, silver and zinc to a deposit feeding clam. In: Biological implications of metals in the environment. Drucker,H and Wildung, R.E (eds), U.S.N.T.S, Conf, 213-231.

Luoma, S.N (1977) Detection of trace contaminant effects in aquatic ecosystems. J. Fish. Res. Bd. Canada., 34, 436-439.

Luoma, S.N and Bryan, G.W (1978) Factors controlling the availability of sediment-bound lead to the estuarine bivalve *Scrobicularia plana*. J. Mar.Biol. Assoc. U.K., 58, 793-802.

Luoma, S.N and Bryan, G.W (1982) A statistical study of environmental factors controlling concentrations of heavy metals in the burrowing bivalve *Scrobicularia plana* and the polychaete *Nereis diversicolor*. Estuar. Shelf. Sci., 15, 95-108.

Mackinney, G (1968) Carotenoids and Vitamin. A. In: Metabolic path ways ii Greenberg, D.M (ed). Accademic Press. London. 311 p.

Malins, D.C and Jensen, A (1981) Editorial. Aqua.Toxicol. 1, 1-2.

Mangum, C.F, Miller, K.I, Scott, J.L, Holde, K.E.V and Morse, M.P(1987) Bivalve Hemocyanin : Structural, functional, and phyllogenetic relationships. Biol. Bull., 173, 205-221.

Manley, A.R and Davenport, J (1979) Behavioral responses of some marine bivalves to heightend sea water copper concentrations. Bull. Environ. Contam. Toxicol., 22, 739-744.

Mantuora, R.F.C, Dickson, A and Riley, J.P (1978) The complexation of metals with humic materials in natural waters. Estuar. Coast. Mar. Sci., 6, 387-408.

Marking, L.L (1985) In: Fundementals of Aquatic Toxicology. Rand, G.M and Petrocelli, S.R (eds),Hemisph. Publ. Corpn. Washington, 165-166.

Martincie, D Nurnberg, H.W, Steoppler, M, Branica, M (1984) Bioaccumulation of heavy metals by bivalves from Lim Fjord (North Adriatic sea). Mar. Biol., 81, 177-188.

Mason, A.Z, Simkiss, K and Ryan, K (1984) Ultrastructural localization of metals in specimenns in *Littoria littorea* (L) from polluted and non polluted sites. J. Mar. Biol. Assn. U.K., 64, 699-720.

Mc carter, J.A and Roch, M (1983) Hepatic metallothionein and resistance to copper in juvenile coho salmon. Comp. Biochem. Physiol., 74, 133-138.

Mc Leese, D.W (1956) Effects of temperature, salinity and oxygen in the survival of the american lobster. J. Fish. Res. Bd. Canada., 13, 247-272.

Mc Lusky, D.S, and Philips, C.N.K (1975) Some effects of Cu on the polychaete *phyllodace maculata* . Est. Coast. Mar. Sci., 3, 103-108.

Mc Lusky, D.S and Hugerman, L (1987) The toxicity of chromium,nickel and zinc: effects of salinity and temperature and the osmoregulatory consequences in the mysid *Praunus flexuosus*. Aqua. Toxicol., 10, 225-238.

Meador, J.P (1991) The interaction of pH, dissolved organic carbon, and total copper in the determination of ionic copper and toxicity. Aqua. Toxicol., 19, 13-32.

Meite, S and Louis (1963) Introduction. In: Hand book of Analytical Chemistry. Mc Graw-Hill Book company, New York. 1-19.

Millington, P.J and Walker, K.F (1983) Australian fresh water mussel *Velesunio ambiguus* (Philippi) as a biological monitor for Zn, Fe and Mn. Aust. J. Mar. Fresh. Wat. Res., 34, 873-892.

Misra, S.G and Mani, D (1992) Uptake of heavy metals by plants and fish. In: Metallic pollution. Ashish Publ.house, New Delhi. 1-161.

Morgan, J.D and Iwama, G.K (1991) Effects of salinity on growth, metabolism, and ion regulation in juvenile Rainbow and steelhead Trout (*Oncorhynchus mykiss*) and Fall chinook salmon (*Oncorhynchus tshawytscha*) . Can. Fish Aqut. Sci., 48, 2083-2094.

Moore, M.N, Pipe, R.K and Farrar, S.V (1982) Lysosomal and microsomal responses to environmental factors in *Littorina littorea* from Sullom voe. Mar. Poll.Bull., 13, 340-345.

Moulder, S.M (1980) Combined effect of the chlorides of mercury and copper in sea water on the euryhaline amphipod *Gammarus duebeni*. Mar. Biol., 59, 193-200.

Münzinger, A and Guarducci (1988) The effect of low zinc concentrations on some demographic parameters of *Biomphalaria glabrata* (Say), mollusca : Gastropoda. Aqua.Toxicol., 12, 51-61.

Narasimham, K.A, Rao, G.S and Achari, G.P.K (1986) The clam resources. R & D series for marine fishery resources management, CMFRI 9.

Nath, K and Kumar, N (1988) Hexavalent Chromium : Toxicity and its impact on certain aspects of carbohydrate metabolism of the fresh water teleost, *Colisca fasciatus*. The. Sci. Tot. Environ., 72, 175-181.

Negilski, D.S, Ahsanullah, M and Mobley, M.C (1981) Toxicity of zinc, cadmium and copper to the shrimp *Callianassa australiensis*. ii Effects of paired and triad combinations of metals. Mar. Biol., 64, 305-309.

Newell, R.E (1971) The global circulation of atmospheric pollutants. Sci.Ame., 224, 37-42.

Newman, N.C and Mitz, S.V (1988) Size dependence of Zinc elimination and uptake from water by mosquito fish *Gambusia affinis* (Baird and Girard). Aqua. Toxicol., 12, 17-32.

Nielsen, K.B, Atkin, C.L and Winge, D.R (1985) Distinct metal-binding connfiguration in metallothionein. J.Biol. Chem., 260, 5342-5350.

Nicholson, J.K, Osboru, D and Kendall, M.D (1984) Comparative distribution of zinc, cadmium and mercury in the tissues of experimental mice. Comp. Biochem. Physiol. 77, 249-256.

Nriangu, J.O (1989) Preface. In: Aquatic Toxicology. Fundemental concepts and methodologies. 1. Boudou, A and Ribeyre, F (eds)., C.R.C. Press Inc. Florida. 314 p.

O'Connor, J.M, Huggett, R.J (1988) Aquatic pollution problems. Aqua. Toxicol., 11, 163-190.

Oeheme, F.W (1976) Mechanism of heavy metal inorganic toxicities. In: Toxicity of heavy metals in the environment. 1. Marcel Decker Inc. New York. 1-86.

Pascoe, D , Edwards, R.W (1989) Single species toxicity tests. In:Aquatic Toxicology:Fundemental concepts and methodologies, 11. Boudou A, Ribeyre, F (eds). CRC press Inc., Florida. 93-126.

Pauling, L (1975) The nature of the chemical bond. 3. Cornell Univ.Press, New York. 644 p.

Payne, J.F, Fancey, L.L, Rahimtula, A.D, Porter, E.L (1987) Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. Comp. Biochem. Physiol., 86, 233-245.

Pearce, J.B and Patanjo, L.D (1988) A review of monitoring strategies and assessment of estuarine pollution. Aqua. Toxicol., 11, 323-343.

Phillips, D.J.H (1976) The common mussel *Mytilus edulis* as an indicator of pollution by zinc, cadmium and copper. Effects of environmental variables on uptake of metals.

Mar.Biol., 38, 59-69.

Phillips, D.J.H (1980) The preliminary selection of an indicator organism. In: Quantitative aquatic biological indicators, their use to monitor trace metals and organochlorine pollution. Appl.Sci.Publ.Ltd. London. 488p.

Phillips, D.J.H and Yim, W.W.S (1981) A comparative evaluation of oysters, mussels and sediments as indicators of trace metals in Hong Kong waters. Mar. Ecol. Prog. Ser. 6, 285-293.

Phillips, D.J.H and Segar, D.A (1986) Use of bioindicators in monitoring conservative contaminants . Programme design Imperatives. Mar. Poll. Bull., 17, 10-17.

Phillips, D.J.H (1989) Trace metals and organo chlorines in the coastal waters of Hong Kong. Mar.Poll. Bull., 20, 319-327.

Putte, V.I, Lubbers, J and Kolar, Z (1981) Effect of pH on uptake, distribution and retention of hexavalent chromium in rainbow trout (*salmo gairdneri*). Aqua.Toxicol., 1, 3-18.

Pymbroeck, S.L.C, Strips, W.J.J and Vanderborght, O.L.J (1982) The antagonism between selenium and cadmium in a fresh water mollusc. Arch. Environ. Contam. Toxicol., 11, 103-106.

Qasim, S.Z and Madhupratap. M (1979) Changing ecology of Cochin backwaters. In: Contributions to Marine Sciences. Sharma, G.S, Mohandas, A and Antony, A (eds). 137-142.

Rahel, F.J (1981) Selection for zinc tolerance in fish, results from laboratory and field populations. Trans. Am. Fish.Soc., 110, 19-28.

Rainbow, P.S and White, S.L (1989) Comparative strategies of heavy metal accumulation by crustaceans : zinc, copper and cadmium in a decapod, an amphipod and a barnacle. Hydobiologia. 174, 245-262.

Rainbow, P.S, Phillips, D.J.H and Depledge, M.H (1990) The significance of trace metal concentrations in marine invertebrates. Mar. Poll. Bull., 21, 321-324.

Rand, G.M (1985) In:Fundementals of aquatic toxicology.,Hemisphere Publ. Corpn., Washigton. 234-265

Rand, G.M and Petrocelli, S.R (1985) In:Fundementals of aquatic toxixology. Hemisphere Publ.Corpn. Washigton.,666p

Redpath, K.J, Davenport, J (1988) The effect of copper, zinc and cadmium on the pumping rate of *Mytilus edulis* L. Aqua. Toxicol., 13, 217-226.

Roberts, J.R and Mc Garrity, J.T (1985) Modelling of biotic uptake In: Appraisal of tests to predict the environmental behaviour of chemicals. Sheeham, P, Korte, F, Klein, W and Ph Bourdeau (eds) John Wiley and Sons. New York. 223 p.

Roch, M, Mc Carter J.A, Matheson, A.T, Clark, M.J.K and Olafson (1982) Hepatic metallothionein in rainbow trout (*Salmo gairdneri*) as an indicator of metal pollution in the campbell River system. Can. J. Fish. Aqua. Sci. 39, 1596-1601

Roesijadi, G (1981) The significance of low molecular wt metallothinein -like proteins in marine invertebrates : current status. Mar.Environ. Res., 4, 167-179.

Romeril, M.G (1971) The uptake and distribution of Zn^{65} in oysters. Mar. Biol. 9, 347-354.

Rutter, J.G Jr. and Morel, F.M.M (1981) The interaction between zinc deficiency and copper toxicity as it affects the silicic acid uptake mechanisms in *Thalassiosira pseudonana*. Limnol. Oceanogra., 26, 67-73.

Rygg, B (1986) Heavy metal pollution and log-normal distribution of individuals among species in benthic communities. Mar. Poll. Bull., 17, 31-36.

Sathyanathan, B, Nair, S.M, Chacko, J and Nambisan, P.N.K (1988) Sublethal effects of copper and mercury on some biochemical constituents of the estuarine clam *Villorita cyprinoides* var. *cochinensis* (Hanley). Bull. Environ. Contam. Toxicol., 40, 510-516.

Sathyanathan, B (1989) Coastal shell fisheries in India;Some ecological aspects. Proc. 3rd International conf. on Environmental Education (Goa).

Sathyanathan, B and Nambisan, P.N.K (1990) On the physiological adaptive responses of the estuarine clam *Villorita cyprinoides* to sublethal concentrations of some metal pollutants. Proc. 77th Indian Sci. Cong (Cochin) 3, 113-114.

Schlenk, D and Brouwer, M (1991) Isolation of three copper metallothioneins isoforms from the blue crab *Callinectes sapidus*. Aqua. Toxicol., 20, 25-34.

Schroeder, H.A, Nason, A.P, Tipton, I.H and Balassa, J.J (1967) Essential trace metals in man : Zinc relation to environmental cadmium. J. Chron. Dis., 20, 179-198.

Scott, D.M and Major, C.M (1972) The effect of copper(11) on survival, respiration and heart rate in the common mussel

Mytilus edulis. Biol. Bull., 143, 679-688.

Senus, P.V (1985) The effects of temperature, size,season and activity on the metabolic rate of the amphipod *Talorchestia capensis* (crustacea,Talitridae). Comp. Biochem. Physiol., 81, 263-269.

Shaffi, S.A (1978) Variation in tissue glycogen content, serum lactate and glucose levels due to copper intoxiication in three fresh water teleosts. Curr. Sci., 47, 954-956.

Simkiss, K and Mason, A.Z (1984) Cellular responses of molluscan tissues to environmental metals. In: Responses of marine organisms to pollutants. Stegeman, J.J (ed), Elsevier Appl. Sci. Publ. London. 103-118.

Singh, S.R and Singh, B.R (1979) Changes in oxygen consumption of a siluroid fish, *Mystus vittatus* put to different concentrations of some heavy metals salts. Ind. J. Exptl. Biol., 17: 274-276.

Snedecor, G.W and Cochran, W.G (1967) Statistical Methods.6thed. Oxford and I B H Publ. Co. New Delhi. 593 p.

Sprague, J.B and Ramsay, B.A (1965) Lethal levels of mixed Cu-Zn solutions for juvenile salmon. J.Fish. Res. Bd. Canada., 22, 425-432.

Sprague, J.B (1970) Measurement of pollutant toxicity to fish. 11 Utilizing and applying bioassay results. Wat. Res., 4, 3-32.

Sprague, J.B (1985) Factors that modify toxicity. In: Fundementals of aquatic toxicology.Methods and application. Rand, G.M and Petrocelli, S.R (eds)., Hemisphere Publ.Corp.

Washington. 124-163.

Steen, J.B (1971) Comparative physiology of respiratory mechanisms. In: Comparative physiology.. Academic Press. New York. 182 p.

Sternleib, I and Goldfisher, S (1976) Heavy metals in lysosomes. In: Lysosomes in biology and pathology 5. Dingle, J.T and Dean, R.T (eds). Elsevier, New York. 185-200.

Stocks,P and Davies R.I (1964) Zinc and copper contents of soils associated with the incidence of cancer of the stomach and other organs. Br. J. Cancer., 18, 14-24.

Strickland, J.D.H and Parsons, T.R (1972) A practical hand book of sea water analysis. Bull. Fish. Res. Bd. Canada., 1-21.

Suckling, K.E and Suckling, C.J (1980) In: Biological chemistry. The molecular approch to Biological systems. Cambridge Univ. Press. Great Britain. 1-381.

Suresh, K and Mohandas, A (1987) Hemolymph lactic acid levels in *Sunetta scripta* (Bivalvia) exposed to sublethal concentration of mercury and copper. J. Invertebr. Pathol., 49, 265-272.

Suresh, K and Mohandas, A (1990) Hemolymph acid phosphate activity pattern in copper-stressed bivalves. J. Invertebr. Pathol,. 55, 118-125.

Sutherland, J and Majors, C.W (1981) Internal heavy metal changes as a consequence of exposure of *Mytilus edulis*, the blue mussel to elevated external copper levels. Comp. Biochem.Physiol.,68, 63-67.

Swain, W.R (1988) Human health consequences of consumption of fish contaminated with organochlorine compounds. Aqua. Toxicol., 11, 357-377.

Thomas, D.G, Brown, D.W, Shurben, D, Solbe, J.F, Cryer, A and Kay (1985) A comparison of the sequestration of cadmium and zinc in the tissue of rainbow trout (*Salmo gairdneri*) following exposure to the metals singly or in combination. Comp. Biochem. Physiol., 82, 55-62.

Thorp, V.J and Lake, P.S (1974) Toxicity bioassays of cadmium on selected fresh water invertebrates and the interaction of cadmium and zinc on the fresh water shrimp *Paratya tasmaniensis* Riek. Aust. J. Mar. Fresh. Wat. Res., 25, 97-104.

Tomita, M, Heisey, R, Witkus, R and Vernon, G.M (1991) Sequestration of copper and zinc in the hepatopancreas of *Armadillidium vulgare* Latreille following exposure to lead. Bull. Environ. Contam. Toxicol., 46, 894-900.

Torres, P, Tort, L and Flos, R (1987) Acute toxicity of copper to mediterranean dog fish. Comp. Biochem. Physiol., 86, 169-171.

Train, R.E(1979) The philosophy of quality criteria. In: Quality criteria for water. USEP. Washington DC., 256 p

Turner, M.A and Swick, A.L (1983) The English - Wabingoon river system. Interaction between mercury and selenium accumulated from water borne and dietary sources by northern pike (*Esox lucius*) . Can. J. Fish. Aquat. Sci., 40, 2241-2250.

Underwood, E.J (1977) Trace elements in human and animal nutrition. In: Trace elements in human and animal nutrition.

Academic Press. New York. 1-63.

Underwood, E.J (1979) Interactions of Trace metals In: Toxicity of heavy metals in the environment.2. Oehme, F.W (ed), Marcel Dekker Inc. New York. 1-170.

Unlu, M.Y and Fowler, S.W (1979) Factors affecting the flux of arsenic through the mussel *Mytilus gallovincialis*. Mar. Biol., 51, 209-219.

Vallee, B.L (1959) Biochemistry, Physiology and Pathology of Zn. Physiol. Rev., 39, 443-490.

Vallee, B.L (1978) Zinc biochemistry and physiology and their derangements . In: New trends in bioinorganic chemistry. Williams R.J.P and Da Silva, J.R.R.F (eds)., Academic Press London. 11-57.

Varghese, G, Naik, P.S and Katdare (1992) Respiratory responses and blood sugar level of the crab *Barytelphusa cunicularis* (westwood), exposed to mercury, copper and zinc. Ind. J. Exptl. Biol., 30, 308-312.

Viarengo, A, Pertica, M, Mancinelli, G, Orunesu, M, Zanicchi, G, Moore, M.N and Pipe, R.K (1983) Possible role of lysosomes in the detoxification of copper in the digestive gland cells of metal exposed mussels. Mar. Environ. Res., 14, 469-470.

Viarengo, A (1985) Biochemical effects of trace metals. Mar. Poll. Bull., 16, 153-158.

Vijayaram, K and Vasugi, S.R (1989) Sublethal effects of pulp and paper mill effluents on the biochemistry of a fresh water fish, *Rasbora Daniconius*. Ind. J. Environ. Hlth., 31, 36-42.

Waldichuk, M (1985) Biological availability of metals to marine organisms. Mar. Poll. Bull., 16, 7-11.

Webb, M (1979) The chemistry, biochemistry and biology of cadmium . In: The chemistry, biochemistry and biology of cadmium. Elsevier, Amsterdam.1-25.

Weis, J.S, Weis, P, Heber, M and Vaidya, S (1981) Methyl mercury tolerance of kill fish (*fundulus heteroclitus*) embroys from a polluted vs non polluted environnment. Mar. Biol., 65, 283-287.

Widdows, J (1985) Physiological responses to pollution. Mar. Poll.Bull., 16, 129-134.

Williams, M and Lutz, R (1975) Carbohydrate metabolism in the crab *Carcinus maenas*. J.Mar.Biol.Ass.U K., 55, 665-669.

Winner, R.W and Farrell, M.P (1976) Acute and chronic toxicity of Cu to four species of Daphnia. J. Fish. Res. Bd. Canada., 33, 1885-1891.