HISTOPATHOLOGICAL STUDIES ON ZINC TOXICITY IN *PENAEUS INDICUS* H. MILNE EDWARDS

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C E R T I F I C A T E

This is to certify that this dissertation is a bonafide record of work carried out by **Kum. SHOBA VISWANATHAN,** under my supervision and that no part thereof has been presented before for any other degree.

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PREFACE

Aquaculture is, at present, undoubtedly one of the ideal solutions to the three basic problems posed before the developing civilization, namely

(1) augmentation of protein production in pace with the increasing nutritional demands of an ever-expanding population,

(2) effecting better utilisation of several natural resources, both, physico-chemical and biological, to boost production economy,

(3) conservation of natural fishery resources.

Aquaculture in India is a traditionally established practice constantly being exposed to new horizons of scientific, technical and economic advancement. Prawns, beung highly amenable to culture, form the mainstay of aquaculture in the marine and brackishwater sectors. Culture of prawns derives great emphasis from the immense demand for this commodity in international markets.

Among the commercially important marine prawns from the point of view of both, capture and culture fisheries, is the Indian White Prawn, Penaeus indicus. Culture techniques for this prawn has been established in India and commercial culture of the same on a large scale, in scientifically run semi-intensive and intensive culture systems, is being taken up widely in the coastal states of southern India.

With the growing need to boost our food production, it is necessary to develop strategies to promote aquaculture by improving upon existing practices,

propagation of culture prospects for new species and effecting proper management of important resources and viable culture systems.

The backwaters of Kerala are highly productive ecosystems supporting a rich brackishwater fishery and serving as nursery grounds for a variety of fish and shellfish. These backwaters offer considerable potential for aquaculture. The traditional culture systems and the reknowned "Pokkali" fields of Kerala thrive along these backwaters which also serve as the source of water for many semi-intensive culture systems. Ecological conditions in the backwaters are thus reflected on its own resources and on the life in culture systems supported by it. Juveniles of P. indicus inhabit these backwaters during their growing phase. Hazards arising from the discharge of pollutants by industries, which often contain high concentrations of heavy metals, are thus more than likely to have an impact on the P. indicus seed resources along these backwaters. Moreover, the contamination of culture systems through use of polluted water may either cause large-scale destruction of the crop, or result in bioaccumulation of toxic materials in the animals, which when consumed, can cause even fatal poisoning in humans.

In the face of such looming dangers, it is necessary to assess the extent of contamination in our waters and the effects of the contaminants on the biota, with particular reference to the commercially important cultivable species. The present study was carried out to assess the deleterious effects of the heavy metal zinc, which is a constituent of several industrial effluents, on P. indicus. The main aspects worked out in the course of the study were:

(1) Lethal toxicity of the metal to the animal.

(2) Sub-lethal manifestations as histopathological disorders.

investigations to assess the extent of histological variations induced by starvation, which is a stress commonly endured by the animals in both, culture systems and natural waters, were also carried out.

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Chapter I

I - INTRODUCTION

The growing awareness of the need for world-wide promotion of aquaculture development to meet the demands of an expanding population and its food requirements lays great emphasis on the need to integrate available resources and technologies with proper management of culture systems. Managerial measures tend to solve, directly or indirectly, major problems that, in any way, hinder the functioning of the culture systems at any point of time. One such problem which has become the subject of public concern today is pollution.

Aquatic pollution is undoubtedly a menace in both, natural waters and in enclosed culture systems. The world's aquatic ecosystems are among the most abused of all ecosystems, for the simple reason that man has been, over the ages, directing all ranges of effluents from his domestic, agricultural and industrial activities into nearby water bodies under the assumption that these natural ecosystems have an unending capacity to assimilate wastes, without themselves being significantly degraded. Constant monitoring of various aquatic ecosystems has shown that a tremendous amount of material, highly potential as pollutants, are being released into these systems, from sources within and without the same, in different forms and levels. The use of such polluted waters for aquaculture practices results in contamination of the cultured products.

The most endangered areas are those near the primary source of pollutant release, especially in the vicinity of industrial sites, estuaries and mudflats, which tend to trap, retain and accumulate pollutants in their sediments, and are thus, particularly vulnerable areas (Kinne, 1984). The effects of pollution

in these areas are often drastic since they play an important role in the recruitment and supporting of life in adjacent sea and land areas (Kinne, 1984). In India, where most of the aquaculture systems are located in the inshore waters and estuarine regions, pollution assumes a greater importance than that meets the eye.

Metal ions, on account of their environmental persistance and toxicity at low concentrations (Negilski, 1976), are considered to be serious pollutants in aquatic ecosystems. They can also be incorporated into food chains and concentrated by aquatic organisms to a level which affects their physiological state (Bryan, 1971; Negilski, 1976). Kinne (1984) has ranked heavy metals as potential hazards, second only to pesticides, particularly in estuaries and coastal regions. Heavy metals like iron, copper, cobalt, zinc, chromium, manganese, molybdenum, nickel and vanadium, though essential to living organisms in trace amounts, are toxic if present in excess (Hungspreugs, 1985). While rivers provide the most important input of metals to coastal areas under natural conditions, atmospheric input of metals into coastal waters is also of considerable significance (Bryan, 1984). However, metal input into coastal waters through anthropogenic activities, no doubt, remains the major cause for metal pollution in the aquatic environment. Nriagu (1979) listed the chief anthropogenic activities releasing metals into the surroundings as follows:-

(i) mining

(ii) primary and secondary non-ferrous metal production

(iii) iron and steel production

(iv) industrial applications

(v) combustion of coal, oil, wood and waste

(vi) phosphate fertilizer manufacture

(vii) miscellaneous activities

All stages of metal production are potential sources of metal contamination. Problems posed by acidic mine drainage waters have been stressed by Förstner and Wittman (1979). Smelting processes have caused contamination in many coastal areas. Owen (1977) reported the possibilities of recovering metals from deeper waters by dredging. Virtually, all industrial processes involving water are potential sources of metal contamination in estuaries and coastal areas, typical examples being manifold uses of copper, cadmium and zinc, particularly in plating and galvanising (Association Europeene Oceanique, 1977). Electricity power stations are a significant source of heavy metals. The burning of coal releases metals to the atmosphere (Bertaine and Goldberg, 1971) and the disposal of flyash is a common occurrence. Sewage disposal is a significant source of metals to the sea (Bryan, 1984). Contamination from ships, most obvious in docks and harbours, is due to the use of copper and mercury in anti-fouling paints and other metals, including lead, chromium and zinc, in preservative paints (Bellinger and Benham, 1978; Young et al., 1979). Through all these inputs, the concentration of heavy metals in coastal waters and estuaries can easily be increased to high levels which the aquatic organisms have not previously encountered (Bryan, 1975).

The importance of metals in the marine environment emerged from studies of radionuclides resulting from fall-out in the oceans during the 1950's and the

1960's (NAS, 1971a), when it became apparent that certain nuclides were accumulated in large concentrations, as in the case of cobalt-60 accumulated in the kidney of the Giant clam, Tridacna (Lowman, 1960). Metal accumulation has also been found to occur from industrial effluents. Waldichuck (1974) reported high concentrations of zinc and copper in oysters dwelling in waters receiving effluents containing these metals. Metals often cause stress to animals when they are present in excessive concentrations (Waldichuck, 1974).

Aquaculture farms located in inland/inshore and coastal waters are highly susceptible to contamination through domestic, agricultural and industrial wastes. Tsu-Chang et al. (1986) reported large scale mortality of larval shrimps in traditional culture areas in the coastal waters of Taiwan due to possible metal pollution. A study of the levels of copper and zinc from prawn culture fields in Cochin area has shown that the adjoining Cochin estuarine system plays a role as the source of pollutants to the culture systems (Subhash Chander, 1986; Joshi, 1970). In aquaculture, special care is required in rearing of larvae and post-larvae which are easily prone to metal effects. Elderfield et al. (1971), in a study of oyster culture involving rearing of the larvae of Ostrea edulis, concluded that heavy metals contamination can cause poor larval performance. The effects of zinc on the embryos of Crassostrea virginica was documented by Calabrese (1972) and Calabrese et al. (1973) while Brereton et al. (1973) reported retardation of development and growth in Crassostrea gigas subjected to zinc contamination.

From an ecological point of view, metal pollution presents the problems of accumulation and biomagnification of the metals at various trophic levels.

In this respect, it is often the more abundant metals like zinc and copper, which prove to be a great hazard (Bryan, 1984). Excessive intake of metal contaminated sea food may result in health problems in man (Kinne, 1984). Bryan (1984) has stressed upon the significance of the problems caused by metal pollution from the public health point of view.

Zinc, which enters the aquatic environment as a constituent of industrial effluents, though indispensable to life, can become toxic at high concentrations (Devineau and Amiard-Triquet, 1985). The presence of metals in industrial discharges and its toxic effects on icthyofauna have been well documented (Mount, 1968; Shaw and Brown, 1974; Solbe and Cooper, 1976; Pickering et al., 1977; Horning and Neiheisel, 1979).

Vallee (1959) and Ogino and Yang (1978; 1979) have commented on the necessity of zinc for enzyme activity, protein and carbohydrate metabolism and growth. However, the presence of an excess amount of zinc in the surrounding waters have been found to cause a retardation of growth in fishes (Crandall and Goodnight, 1962, 1963; Bengston, 1974; Watson and McKeown, 1976; Pierson, 1981). Crespo and Sala (1986) summarised the mechanisms by which an excess amount of zinc alters the physiology of animals by altering their osmoregulatory mechanisms by,

(i) inhibition of enzyme activity

(ii) altering ionic transport across ion-transporting epithelia

(iii) effecting changes in electrolytic plasma levels

(iv) causing proliferation of chloride cells

(v) causing an accelerated chloride cell turn-over

These conclusions support the findings of Hewitt and Nicholas (1963), who stated that exposure of aquatic organisms to low concentrations of heavy metals causes depressed or activated enzyme activity. May and Brown (1973) demonstrated the activation and inhibition of the enzyme Allantoinase (extracted from the polychaete, Eudistylia vancouveri, by zinc. Bargmann and Brown (1974) showed that zinc is capable of inhibiting the activity of alpha-glycerophosphate-dehydrogenase found in trout muscle tissue.

While the analyses of zinc in animals from principal marine phyla have shown that this element is nearly always present (Phillips, 1917; Bodansky, 1920; Severy, 1923), the concentrations have been found to vary from barely detectable amounts, as in the blood of the lobster, Palinurus, (Phillip, 1917), to values of several hundred ug/g of wet tissue, as in oysters.

One of the most important biological properties of metals is their tendency to be accumulated and zinc is no exception to this. Hiltner and Wichman (1919) showed variability in body zinc concentrations in oysters, and reported that the highest concentrations occur in oysters exposed to metal contamination. Waldichuck (1974) reported body zinc concentrations upto 17,000 ppm in oysters from zinc-contaminated waters. Greig et al. (1975) reported zinc concentrations of 97-900 ug/g dry weight in the oyster, Crassostera glomerata, from unpolluted waters, while specimens from polluted waters contained zinc concentrations of upto about 9000 ug/g dry weight.

Accumulation patterns of zinc in crustaceans have been widely documented (Bryan, 1964, 1968, 1971, 1976, 1979; Prosi, 1979; Jennings and Rainbow, 1979;

White and Rainbow, 1982; Rainbow, 1985; Weeks and Rainbow, 1991). Zinc being an essential trace element, it might be expected that aquatic animals have developed an ability to regulate body concentrations of the metal upto certain operational limits, beyond which regulation breaks down (White and Rainbow, 1982). There is increasing evidence to suggest that there has been the evolution of an ability in crustaceans, particularly decapod crustaceans, to regulate the internal concentrations of essential but potentially toxic metals to a constant body level, presumably atleast meeting the metabolic demands (Rainbow, 1985). Bryan (1964, 1966, 1968, 1971, 1976), Bryan and Ward (1965) and Martin (1974) have provided evidence of possible regulation of body levels of zinc, copper and manganese in the crab, Carcinus maenas, and the lobster, Homarus vulgaris. White and Rainbow (1982, 1984) and Nugegoda and Rainbow (1987) have shown that the decapod, Palaemon elegans, regulates body levels of zinc over a wide range of dissolved concentrations before the regulatory process breaks down under high metal exposure and net accumulation begins.

Bodansky (1920) and Severy (1923) reported zinc concentration in a majority of decapods to be of the order of 20 ug/g wet tissue. Mechanisms for regulation are developed when the animals acquire more than the required levels, the chief regulatory methods being temporary absorption and storage by hepatopancreas, loss across body surface, excretion through urine and excretion through faeces (Bryan, 1968). White and Rainbow (1982) reported that P. elegans regulates total body concentrations upto 100 ug/L of the metal. According to Bryan (1968), the animals living in clean waters, away from the coastal areas, show a tendency to absorb less pollutant than those living in polluted estuaries, however slight

the pollution may be. Though zinc absorption occurs mainly from the stomach, Bryan (1968) reported an increase in direct absorption across body surface when the animals are subjected to starvation. White and Rainbow (1984) reported that zinc is absorbed into the body at a rate which increases with the concentration of dissolved zinc to which the animal is exposed, the zinc uptake over the regulated exposure range being matched by increasing zinc excretion, thereby maintaining a constant body concentration of zinc. It is when the bioavailability of zinc exceeds a threshold and zinc excretion can no longer match zinc uptake, that regulation breaks down, giving way for net accumulation (Rainbow, 1968). The uptake of zinc from the surrounding medium has been found to be influenced by several environmental factors. White and Rainbow (1984) and Nugegoda and Rainbow (1988) discussed the physico-chemical variables affecting zinc uptake in prawns. One of the most important factors affecting heavy metal accumulation and toxicity in animals has been found to be salinity (Phillips, 1980; McLusky et al., 1986). Jones (1975) showed that zinc toxicity to six species of marine and estuarine isopods increased with decreasing salinity and Bryant et al. (1985) reported the increasing toxicity of zinc to the amphipod, Corophium volutator, with decreasing salinity over a range of 35 to 5 ppt. Temperature (White and Rainbow, 1984) and chelating agents (Nugegoda and Rainbow, 1988) have also been found to affect zinc uptake in the prawn, P. elegans. McKenney and Nelt (1979) found that the viability of the larvae of Palaemonetes pugio in elevated zinc concentrations was reduced by both, the individual effects of salinity and temperature and the interactions between the two factors outside the optimal salinity-temperature conditions. Nugegoda and Rainbow (1989) concluded that zinc uptake and regulation in decapods are affected by both, extrinsic physico-chemical parameters and intrinsic adaptations of the species concerned.

The toxicity of zinc to a wide range of marine, estuarine and freshwater animals has been worked out extensively. In general, the effects of toxic compounds on aquatic animals are considered to be direct, indirect or induced. Toxicity may be either acute or chronic; acute toxicity, which causes rapid death from short-term exposure of animals to lethal doses of pollutants, occurs rarely, and, in most cases, only accidently, in the natural environment. More often, aquatic animals are subjected to long-term stress caused by exposure to low but potentially harmful levels of pollutants - such chronic exposures cause sub-lethal changes in the animals, which may or may not account for death, eventually. Sub-lethal changes can occur from a single encounter or from continuous exposure over a long period (Mathew, 1990). Luckey and Venugopal (1977) came to the conclusion that toxic metals often change the biological structures of systems into irreversible and inflexible conformations, causing deformity or eventual death.

Most of the information documented on the toxic effects of pollutants on aquatic animals has been obtained from mortality studies. In contrast, very little is often known about damage to different internal organs and alterations in physiological and biochemical process induced by chronic exposure of organisms to pollutants. Consequently, there often exists a *lacuna* between knowledge of lethal and sub-lethal effects of pollutants on aquatic organisms and knowledge of the modes of action of these toxicants and causes of death in the poisoned organisms. To predict the potential harmfulness of various toxicants to them, it is necessary to overcome this lacuna and derive a better understanding of these mechanisms. Various means of investigating sub-lethal effects of pollutants

on aquatic animals under laboratory conditions, adopted by several workers over the years, include studies on growth, fecundity and brood survival, swimming activity and physiological, biochemical and histological alterations in animals subjected to pollutant stress.

Pathology is now a part of environmental monitoring programmes on pollution effects (Yevich and Barszcz, 1976; Balouet and Poder, 1981; Couch, 1985). Based on an easily reproducible technique, histopathologic studies yield basic information on tissue disorders related to the general state of the organisms and assess the host's susceptibility to infectious diseases and parasitic infestations. Some of these parameters may serve as indicators of the effects of xenobiotic contamination in marine animals (Sindermann, 1980). Histological, cytological and cytochemical responses, observable from animal tissue sections, form an important link between effects at the biochemical level and those measured in the whole organisms (Lowe, 1988). Standard histopathological approaches, which are useful in providing an overall picture of the degree of disturbance within the organ systems concerned (Moore, 1988) are thus, an integral part of studies on the effects of external stress on organisms.

The assessment of the effects of zinc toxicity in fishes has been done by several workers. Saiki and Mori (1955), Cairns and Scheier (1957), Joyner (1961), and Skidmore (1964) reported the possible absorption of zinc from surrounding waters through gills in fishes. According to Skidmore (1970), tissue hypoxia could be a probable cause of death in fish exposed to zinc sulfate. Crandall

and Goodnight (1963) and Kumar and Pant (1981) reported pathological alterations in the liver and kidneys of fishes exposed to different levels of lethal and sub-lethal concentrations of zinc. Histopathological alterations in the gills of fishes exposed to zinc have also been well documented (Carpenter, 1927; Jones, 1938; Lloyd, 1960; Bhatnagar, 1975; Kumar and Pant, 1981; Kodama et al., 1982). Benoit and Holcombe (1978), working on Fathead minnow life cycle exposure to various zinc concentrations, demonstrated that egg adhesiveness and fragility were the most sensitive indicators of zinc toxicity. Radhakrishnaiah et al. (1991) observed decreases in soluble, structural and total protein contents and increase in free amino-acid levels and activities of Proteases and Alanine/Aspartate amino-transferases in the osmoregulatory and non-osmoregulatory tissues in Cyprinus carpio exposed to zinc. Goel and Gupta (1985) reported alterations in haemato-biochemical characteristics in zinc-treated Heteropneustes fossilis. Sen et al. (1991) noted variations in cholesterol, protein and ascorbic acid levels in brain, liver and kidneys of fishes exposed to zinc.

The toxicity of zinc to crustaceans has been studied by some workers (Thorp and Lake, 1974; Ahsanullah, 1976; Eisler and Hennekey, 1977; Ahsanullah and Arnott, 1978; Ghate and Mulherkar, 1979; Ram Murti and Shukla, 1984; Liao and Chieh-Shih, 1989; Patil and Kaliwal, 1989; Weeks and Rainbow, 1991; Chan, 1992; Timmersons et al., 1992). Ahsanullah et al. (1981a) demonstrated that the toxicity of zinc to the shrimp Callianassa australiensis, increased with increases in exposure time and the shrimps were found to secrete substantial quantities of mucus. Liao and Chieh-Shih (1989) reported that the tolerance levels of various larval stages of the prawn, Macrobrachium rosenbergii, to zinc

reduced as development advanced, the toxicity of the prawn at different stages showing considerable difference. Chan (1992) reported high rates of zinc accumulation in the haemolymph of crabs living in highly saline conditions.

Trends in the accumulation and regulation of zinc levels by decapod crustaceans show the hepatopancreas and the gills to be the most important sites facing the impacts of contamination (Bryan, 1968). The hepatopancreas have been found to be highly sensitive to external contamination. Hiltibran (1971) and Brown et al. (1974) have reported that heavy metal toxicity tends to affect cellular respiratory metabolism of hepatic cells. Food is the main source of zinc in macrophagous animals and the hepatopancreas exhibit considerable structural and biochemical variations with increased zinc uptake (Cuadros et al., 1981). Bryan (1968) reported the tendency of decapod hepatopancreas to store excess zinc to facilitate regulation of body levels, the stored zinc serving as a reservoir for blood proteins which are not normally saturated. These findings are in agreement with the histopathological alterations observed in the liver of fishes treated with zinc (Crandall and Goodnight, 1963; Kumar and Pant, 1981) and other heavy metals which have been found to initiate hepatotoxic lesions of fatty infiltration, nuclear or general hypertrophy of hepatocytes, cytoplasmic vacuolation, cellular pleiomorphism bile/ceroid pigment deposition, loss of hepatic glycogen, hydrophic degeneration, coagulative hepatocyte necrosis sinusoidal and vascular congestion, loss of normal murarial architecture degeneration or necrosis of billiary epithelium and perivascular or periportal fibrosis. (Baker, 1969; Gardener and La Roche, 1973; Trump et al., 1975; Establier et al., 1978a,b,c; Gutierrez et al., 1978; Sastry and Gupta, 1978). Similar

variations due to contamination in the structure of digestive tubules have been reported in mussels (Rasmussen, 1982; Calabrese et al., 1984; Sunila, 1984), clams (Tripp et al., 1984; Couch, 1984) and scallops (Yevich and Yevich, 1985).

Gills, which are the sites of respiration and transport involved in osmoregulation, form the most permeable region in the body of an aquatic animal (Quinn and Lane, 1966; Bielawski, 1971). Physiological, histological and ultrastructural studies have shown that heavy metal ions interfere with respiration and osmoregulation by disrupting the structure of the gill cells in fishes and crustaceans (Baker, 1969; Eisler and Gardner, 1973; Jones, 1975; Bubel, 1976; Papthanassiou and King, 1983). Plonka and Neff (1969) suggested that excessive mucus secreted by fish gills in response to toxic levels of heavy metals and low pH results in death by suffocation as a result of mucus coagulation and precipitation with the metal ions on the gill surface. Death in fish with gills damaged by zinc sulfate toxicity or by other heavy metals has been reported by Skidmore (1970) to be caused probably by tissue hypoxia which appeared to be a major physiological change preceeding death, once the gas exchange process at the gills is no longer sufficient to satisfy the oxygen requirements of the fish (Burton et al., 1972). Lloyd (1960) and Skidmore and Torell (1972) have reported through histological investigations, the separation of gill epithelium from the basement membrane in fishes subjected to zinc poisoning.

Cuadros et al. (1981) reported the high uptake of zinc through gills in the hermit crab, Dardanus arrosar (Herbert), and a wide degree of variations were found to be initiated in the gills by this uptake. Nimmo et al. (1977)

reported the consistent development of blackened foci or melanized lamellae in the gills of the pink shrimp, Penaeus duorarum, exposed to cadmium in sub-acute and acute tests. Histological observations of these gills showed damaged gill processes, haemocyte infiltration, blackened lamellae, necrosis and sloughing off of individual lamellae and pycnosis. They reported similar changes in the shrimp, Palaemonetes vulgaris, also. Patil and Kaliwal (1989) reported that the degree of damage to the gill tissue in the prawn, Macrobrachium hendersodyanum increased with increasing zinc concentration and exposure time. They found gill lesions, necrosis, pycnotic nuclei, oedema and loss of cuticular lining of lamellae even at low concentrations and also noted the inhibition of gas exchange by distension of gill epithelium.

Only very little work has been done on the assessment of the effects of zinc on other organs and tissues of aquatic animals. Chan (1992) reported variations in haemolymph concentrations in zinc-treated crabs. Cuadros et al. (1981) have noted concentrations of zinc in the brain of the hermit crab, Dardanus arrosar, to be similar to those of the gills and the hepatopancreas. Changes in the biochemical constituents and enzyme activities in zinc-treated fish have been reported by some authors (Radhakrishnaiah et al., 1991; Sen et al., 1991).

Owing to the dynamic nature of their environment, aquatic organisms are subjected to a wide range of physical and chemical conditions through natural and anthropogenic influence, and are more or less, always under some form of

stress. One of the most important stress inducing factors is the scarcity of food, under which condition, the animal undergoes starvation. Starvation also occurs when the animal undergoes periods of voluntary fasting during moulting, as in crustaceans or when subjected to some other stress, extrinsic or intrinsic by nature. Starvation stress has been reported to influence metal intake by animals exposed to metal-contaminated waters. An increase in the direct absorption of zinc across the general body surface in decapod crustaceans was noted when the animals were subjected to starvation (Bryan, 1968).

Starvation studies gain an added importance in the light of the current advancements made in the aquaculture industry. One of the most important factors to be considered in intensive aquaculture being good nutrition (Vogt et al., 1985), it is important to have a knowledge of the effects of dietary stress and minimise it as far as possible. Storch (1984) reported that hepatocytes of vertebrates and mid-gut gland cells of invertebrates reflect the nutritional value of a diet even over shortly extended periods. Such variations have been reported in many taxa (Brown et al., 1976; Alberti and Storch, 1983; Storch and Anger, 1983; Senger and Moller, 1984). Although inadequate nutrition is one of the main limiting factors in aquaculture, very little is known about the influence of feed on the digestive tract cells of economically important crustaceans and only very little work has been documented on the effects of starvation and feeding on the mid-gut gland cells of decapod crustaceans (Pascual et al., 1983; Storch et al., 1984). The effect of different diets on the mid-gut gland cells have been reported in decapods (Storch and Anger, 1983), isopods (Storch, 1984) and amphipods (Storch and Burkhardt, 1984). Vogt et al. (1985)

reported the remobilisation of storage materials like lipid droplets and decrease and enlargement of mitochondria in Penaeus monodon, starved for five days. An earlier report by Schafer (1968) stating that proteins are catabolized last of all under starvation is substantiated by the findings of Vogt et al. (1985). Clifford and Brick (1983) found that an extension of starvation period in the prawn, M. rosenbergii, from four to eight days led to a reduction in the role of carbohydrate substrates, accompanied by a two-fold increase in the oxidation of lipid and protein Rosemark et al. (1980) noticed a general atrophy, especially of the absorptive and secretary cells in the hepatopancreas of starved juveniles of the lobster, Homarus americanus, after just four days of starvation. Refeeding of starved post-larvae of P. monodon established a food specific ultrastructure of the hepatopancreas in 24 to 48 hrs (Vogt et al., 1985). Storch et al. 1984b reported similar results in the case of milkfish fry, whereas it takes about twenty days for a complete reconstruction of liver cell ultrastructure in milkfish fingerlings. The authors came to the conclusion that after a certain period of starvation a diet cannot be utilised any more, although the animals ingest feed and live on for a few days. Extended starvation with subsequent feeding resulted in phenomenal changes, suggesting cellular damage. Starvation of the prawn, Metapenaeus dobsoni, produced extensive vacuolation in the hepatopancreatic cells, with autolysis setting in by the third week of starvation, while refeeding for a week produced rapid and intense proliferation of the cells (Kumar, 1991).

The Indian White Prawn, Penaeus indicus H. Milne Edwards, one of the most commercially important marine prawns in India contributes to a good share of the total prawn culture turn-over of the country. An attempt is made

in the present study to throw light on the level of tolerance of this prawn to the heavy metal, zinc. A study has also been made to note the effects of starvation on the animal, which could be of help in the assessment of dietary stress, if any, in culture systems.

Chapter II

II - MATERIAL AND METHODS

2.1 TEST MEDIUM

Fresh sea water collected off Cochin was transported to the laboratory in polythene carboys and used as test medium in all the experiments conducted in the course of the present study. The water was allowed to settle, filtered using standard grade phytoplankton net (No.24), diluted to 20±1 ppt salinity by adding the required quantity of filtered, dechlorinated tap water and aerated to saturation before use. The pH of the water ranged between 7.3 and 7.8 for various sets of experiments. All sets of experiments were conducted at room temperature (28±2°C).

2.2 TEST ANIMALS

Live juveniles of Penaeus indicus were collected from the culture ponds of the Fisheries Station of Kerala Agriculture University at Puthuvyppu and transported to the laboratory in storage bins. They were acclimatised in 30 L plastic tubs containing 25 L of well-aerated sea water of 20±1 ppt salinity. The animals were fed twice a day with raw clam meat. Faecal matter and left over feed were siphoned out every morning. Water exchange was done at the rate of 80% every day. The acclimatisation period lasted for five days after which the animals were transferred to the experimental tubs.

2.3 TEST CONTAINERS

Circular plastic tubs of 30 L capacity were used as test containers in all sets of experiments conducted during the study.

2.4 LETHAL TOXICITY TESTS

Toxicity tests were carried out to assess the acute lethal effects of zinc on juvenile P. indicus. The LC_{50} values were estimated from the acute toxicity tests.

2.4.1 TOXICANT

Zinc sulphate ($ZnSO_4.7H_2O$, M.W. 287.55 ; Analytical grade) was used as the source of zinc for the experiments. A stock solution of 1 ppm zinc was prepared by dissolving the required amount of the salt in double distilled water. Calculated amounts from this stock solution were added to different experimental tubs containing known volume of sea water to get the desired concentrations of zinc in terms of ppb metal ion in the medium.

2.4.2 BIOASSAY

Static Renewal Bioassay, with 100% water exchange once every 24 hrs, was conducted following the guidelines given by APHA (1980) and FAO (1987). Test concentrations were decided after performing range-finding tests. Ten animals (length 40-50 mm, measured from the tip of the rostrum to the tip of the telson) were exposed to 25 L of test solution in each tub, all concentrations being run in triplicate. A control in triplicate, with no toxicant, was also maintained. Only healthy animals were transferred to the experimental tubs using a hand net. Care was taken to offer only minimal stress to the animals during transfer

and daily water exchange. Uniform aeration was maintained in all the tubs throughout the experiment which was run for 96 hrs. The animals were not fed during this period.

Animal behaviour and mortality in each concentration were recorded every 12 hrs. Dead specimens were removed immediately; the criterion for death was taken as the lack of movement even on gentle prodding.

2.4.3 DATA ANALYSIS

The 96 hr LC_{50} value was estimated from Probit Analysis of the experimental data as given in the software MSTATC. The ET_{50} and toxicity curve were obtained through graphical representation.

2.5 CHRONIC EXPOSURE STUDIES

In the chronic exposure studies, test animals (length 40-50 mm, measured from the tip of the rostrum to the tip of the telson) were subjected to two sub-lethal concentrations (100 ppb and 300 ppb) of zinc for specific periods, following which they were sacrificed and processed for histopathological study. Each concentration was run in duplicate, with each replicate containing seven animals in 20 L of test solution. A control in duplicate, with no toxicant, was also maintained. The animals were fed with raw clam meat every evening. Faecal matter and left over feed were siphoned out daily and 100% water renewal was given

every 24 hrs. Uniform aeration was maintained in all the tubs throughout the experiment.

Animals in the intermoult stage were selected and sacrificed after periods of 10 and 20 days of exposure to the test solutions. Their tissues were dissected out and processed for histopathological observations.

2.5.1 HISTOPATHOLOGICAL STUDIES

The following tissues were selected to observe the histopathological manifestations of sub-lethal doses of zinc in the test animals:

(a) Hepatopancreas

(b) Gills

The tissues were dissected out from the live animals using sterilized instruments. All the tissues were fixed immediately in Aqueous Bouin's Fixative (ABF) for 24 hrs. The gills were left in decalcifying solution of 1.5% nitric acid in 70% alcohol for three days after thorough washing in 70% tert. Butyl alcohol. They were then dehydrated by washing in 80%, 90%, 95% and 100% tert. Butyl alcohol. The hepatopancreas, after fixation, were washed in running tapwater and dehydrated through a graded series of tert. Butyl alcohol (10%, 30%, 50%, 70%, 80%, 90%, 95% and 100%). After dehydration, all the tissues were cleared in Chloroform and left in a mixture of Paraffin wax (M.P. 58-60°C) for 30 minutes. Impregnation and embedding was done in molten Paraffin wax. The blocks prepared were cut on a rotary hand microtome to obtain sections of 7 u thickness. These

sections were spread on albumen coated micro-slides, deparaffinised in xylene, hydrated by passing through descending grades (100%, 95%, 90%, 70%, 50%, 30%) of tert. Butyl alcohol, distilled water and tap water, stained in Harris' Haematoxylin, washed in tap water and distilled water, dipped in acid-alcohol to remove excess stain, washed in tap water and ammonia water to remove acid, washed in tap water and distilled water, dehydrated through a graded ascending series (30%, 50%, 70%, 90%) of tert. Butyl alcohol, counter-stained in Eosin (Spirit soluble) and run through 90%, 95% and 100% tert. Butyl alcohol. The sections were finally cleared in xylene and mounted in DPX. The slides were allowed to dry before subjecting them to scrutiny under a light microscope. Photomicrographs depicting the histopathological manifestations were taken and compared with photomicrographs taken of sections obtained from the control specimens.

2.6 STARVATION STUDIES

Histological changes induced by starvation stress in juvenile *P. indicus* were studied by subjecting them to starvation for 10 days.

2.6.1 EXPERIMENTAL PROCEDURE

The experimental tubs containing 20 L of sea water, were stocked with healthy animals at the rate of one animal per tub (as a measure to avoid cannibalism). Each run was accompanied by a control in which the animal was fed *ad. libitum* during the entire period of study. Uniform aeration was maintained in all the tubs. Faecal matter and left over feed (in control) were siphoned

out daily, with 80% water exchange. The animals were sacrificed after 10 days, taking care to select only animals in the intermoult stage for this purpose. The hepatopancreas were dissected out from these animals and processed for histological study.

2.6.2 HISTOLOGICAL STUDIES

The hepatopancreas dissected out from the test animals were immediately fixed in Aqueous Bouin's Fixative (ABF) for 24 hrs and processed as per the procedure outlined under Section 2.5.1. The slides prepared were observed under a light microscope and photomicrographs were taken to present a comparison between the histology of the hepatopancreas of starved and fed animals.

Chapter III

III - RESULTS

3.1 LETHAL TOXICITY TESTS

Exposure of juvenile P. indicus to various concentrations of zinc for a period of 96 hrs revealed a marked difference in the response of the animals to low and comparatively higher concentrations. The animals exposed to 100 ppb of zinc remained more or less in a "resting" state at the bottom of the test containers. At the same time, animals exposed to 500 ppb and higher doses of zinc showed an almost immediate response by exhibiting highly irritated and rather unbalanced swimming. While the animals in 1350 and 1800 ppb solutions remained "resting" at the bottom after an irritated activity during the first 12 hrs of exposure to the toxicant, the animals in the higher concentrations (2400, 3200 and 4200 ppb) exhibited an irritated activity for almost 36 hrs, before they resorted to "resting" at the bottom.

A regular feature noticed during the experiment was the development of a distinct blackened appearance of the gills in the zinc-exposed animals (Fig. 5). The consistent development of black gills was noticed in the case of animals exposed to 1000 ppb and above, while in 500 ppb, the occurrence of black gills was less consistent. There was no blackening of the gills in any of the animals exposed to 100 ppb of zinc during the 96 hr exposure.

Mortality was first recorded in 3200 and 4200 ppb test concentrations, in just 12 hrs of exposure. No mortality was recorded during the 96 hr

assay in test solutions containing 100 ppb and 500 ppb of zinc. Morphologically, all the animals in 100 ppb zinc solution looked normal and showed an immediate and strong response to stimulus. At the end of 96 hrs, 10% of the animals in 500 ppb test solution had developed slightly blackened gills. The response of the animals in this concentration was also immediate. 30% mortality was recorded at the end of 96 hrs in 1000 ppb of zinc. Nearly 77% of the animals, including the ones that died, had developed black gills. Mortality recorded in 1350 and 1800 ppb test concentrations was 50% and 57% respectively, with almost 90% of the animals showing black gills. All the animals showed lesser response to external stimulus. 97% of the animals in 2400 ppb zinc solution exhibited deeply blackened gill lamellae after 96 hrs, with 70% mortality. The animals showed a tendency to remain dormant at the bottom of the container and responded very slowly to external stimulus. All the animals in 3200 and 4200 ppb solutions of zinc had developed blackened gills, with the blackening being very intense. At the end of 96 hrs, the mortality rates in these concentrations were 80% and 90% respectively. The animals which remained in a "resting" state during the experiment did not respond well to external stimulus and exhibited only a lethargic movement along the bottom of the container.

An interesting feature noticed during the assay was that all the dead animals in the higher concentrations had developed blackened gill lamellae. The time taken for the development of black gills varied even among animals exposed to the same concentration of the toxicant. However the intensity of blackening was greatest in 2400, 3200 and 4200 ppb of zinc while blackening

TABLE - 1 CUMULATIVE PERCENTAGE MORTALITY OF P. INDICUS EXPOSED TO VARIOUS ZINC ($ZnSo_4$) CONCENTRATIONS.

Zn conc. (ppb)	TIME (hrs.) 24	36	48	60	72	84	96
0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
500	0	0	0	0	0	0	0
1000	0	0	0	0	0	0	13.33
1350	0	3.33	16.66	20	26.67	33.33	50
1800	0	6.66	20	30	36.67	36.67	56.67
2400	3.33	10	26.67	40	50	56.67	70
3200	3.33	10	30	53.33	66.67	70	80
4200	6.66	13.33	36.67	56.67	70	76.67	90

TABLE - 2 ESTIMATION OF LC_{50} BY PROBIT ANALYSIS

Slope (b)	-3.3229
Intercept (a)	15.7071
Variance of Slope	0.2729
Chi-Square	6.5446
Probability	0.9810
DF	16
Log LC_{50}	3.2222
Variance of log LC_{50}	$1.0655036e^{-003}$
LC_{50}	1668.16

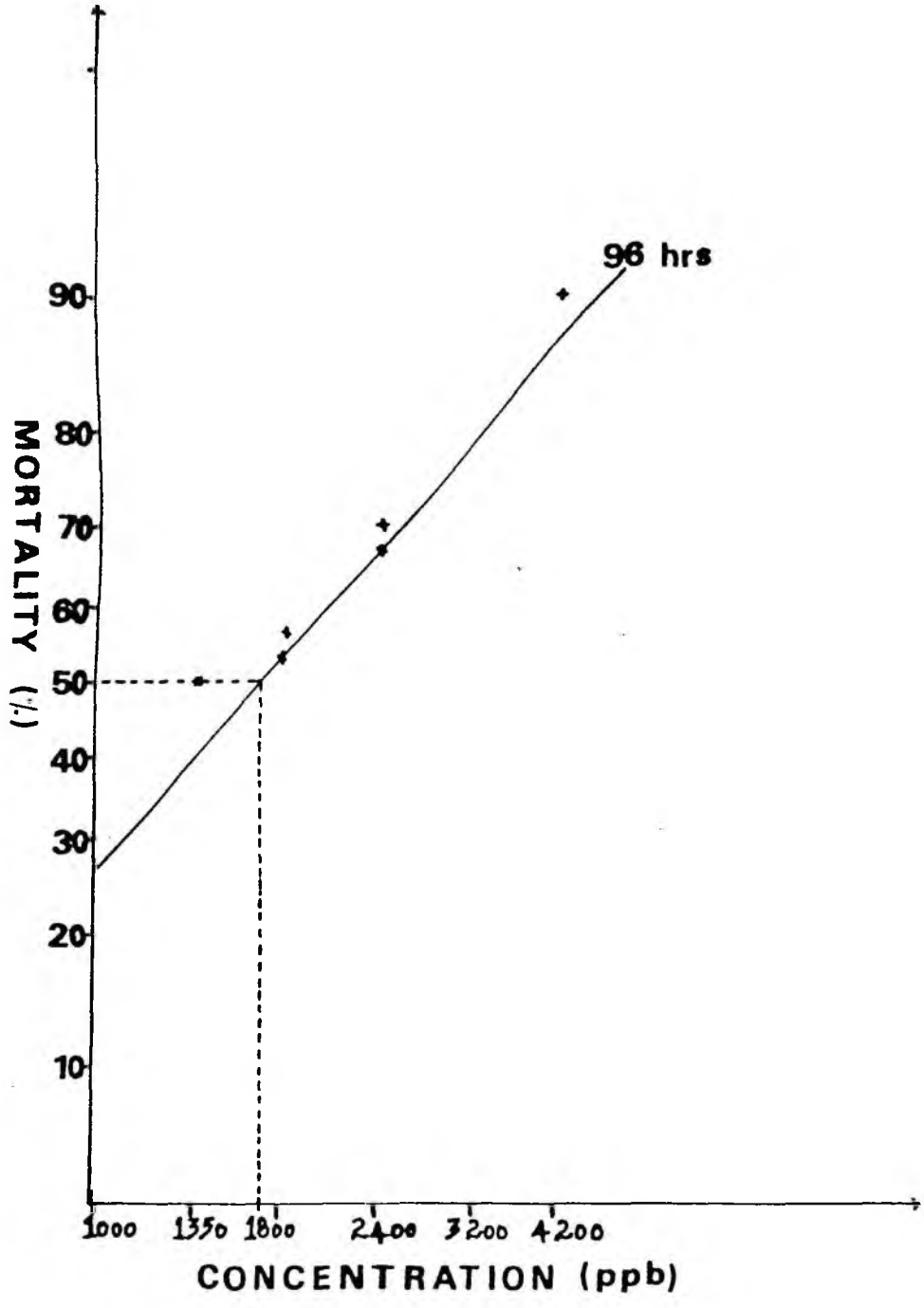

Fig. 1 GRAPHICAL REPRESENTATION OF 96hLC_{50} (PROBIT METHOD)

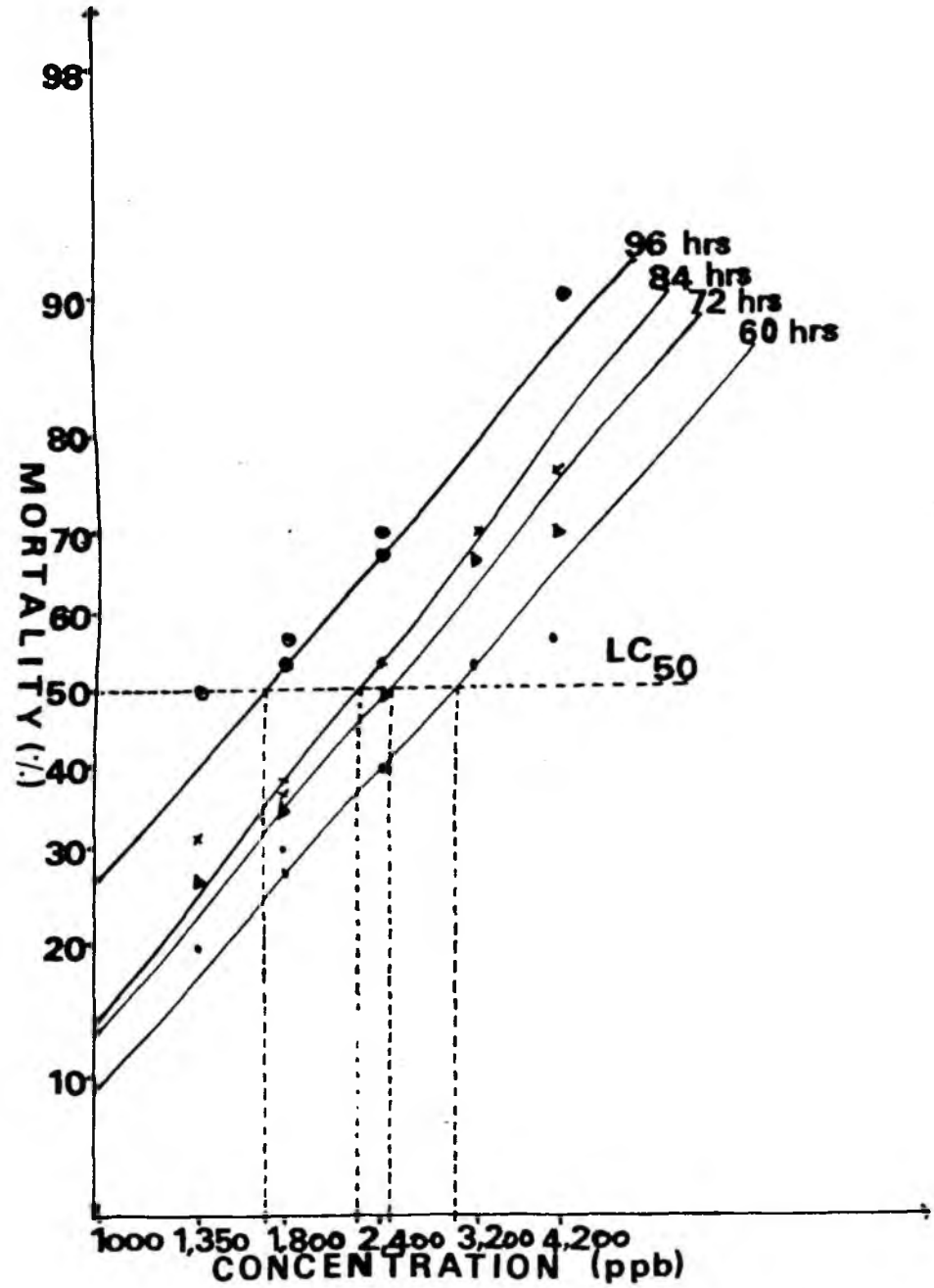

Fig. 2 LC_{50} VALUES FOR P. INDICUS EXPOSED TO VARIOUS ZINC ($ZnSO_4$) CONCENTRATIONS FOR DIFFERENT EXPOSURE TIME.

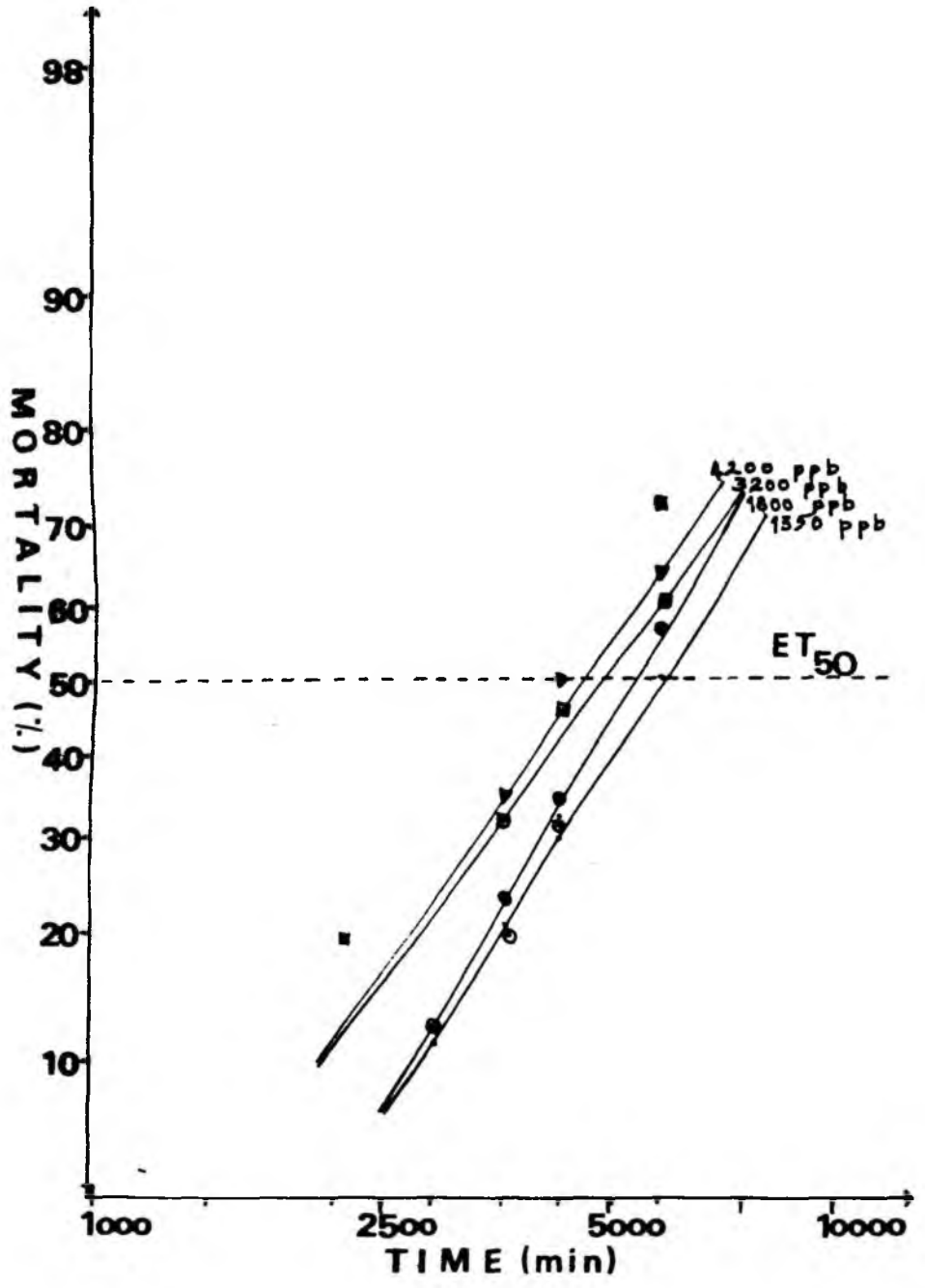

Fig. 3 ET_{50} VALUES FOR P. INDICUS EXPOSED TO VARIOUS SINC ($ZnSO_4$) CONCENTRATIONS.

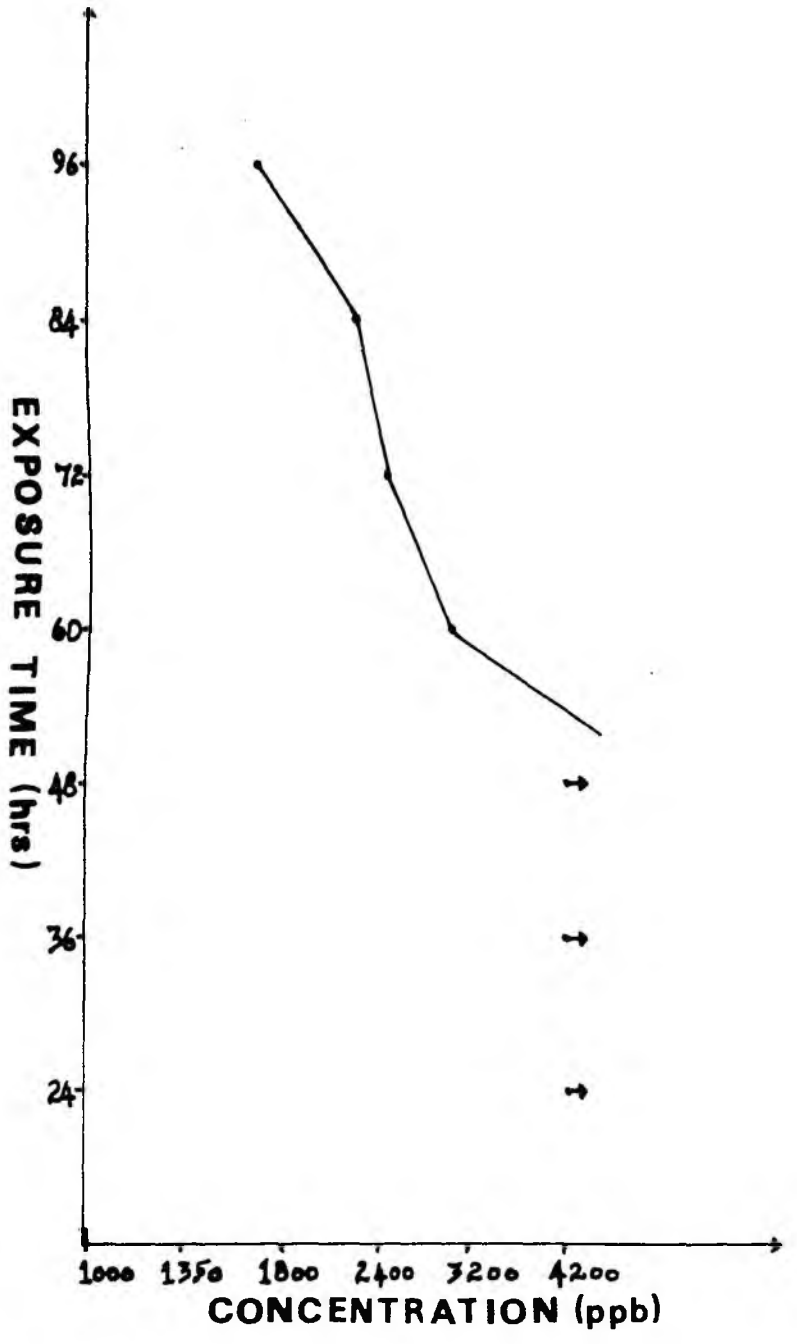

Fig. 4 TOXICITY CURVE

in 500 ppb test solution, in which there was no mortality, was depicted only by slightly brownish gills.

The cumulative percentage mortality of the animals in various test concentrations is given in Table 1. The 96 hr LC_{50} was worked out to be 1668.16 ppb by Probit method (Table 2 and Fig.1). The lines of best fit for mortality over different exposure times (60, 72, 84 and 96 hrs) at various test concentrations, with the corresponding LC_{50} values are depicted in Fig.2. The ET_{50} values for different test concentrations and the toxicity curve obtained are represented in Figs. 3 and 4, respectively.

3.2 CHRONIC EXPOSURE STUDIES

3.2.1 HISTOPATHOLOGY OF HEPATOPANCREAS

Histopathological alterations induced in the hepatopancreas of P. indicus exposed to 100 and 300 ppb of zinc for 10 and 20 days were assessed. The decapod hepatopancreas, in general, has been described to be composed of four different cell types, namely, the E-cells (Embryonalenzellen), the F-cells (Fibrillenzellen), the B-cells (Blazenzellen) and the R-cells (Restzellen). The E-cells, which are generally among the smallest of the hepatopancreatic cell types, are unspecialized cuboidal cells concentrated in the distal portion of the tubules, which is the area of proliferation. The F-cells, which appear striated because of an extensively developed rough endoplasmic reticulum, are secretory in function and are present in the mediodistal, medioproximal and proximal portions of the tubules. The B-cells, which are secretory and excretory in

function are the largest of the hepatopancreatic cell types, seen mainly in the proximal areas of the tubules. The R-cells, the most abundant of the four cell types, form about 75% of the total number of cells in a single tubule and are seen in the mediodistal and proximal areas of the tubules.

Exposure of the animals to zinc produced a lot of variations in the structural conformity of hepatopancreas. There was a nominal increase in the number of vacuolated cells, especially the B-cells. Delamination of epithelial cells, pycnotic nuclei and tubular rupture were of common occurrence.

The hepatopancreas of prawns exposed to 100 ppb of zinc for 20 days exhibited delamination of epithelial cells in several tubules; clumps of cells were seen in the lumen of the tubules in many cases. Figs. 8 and 9 show clusters of embryonic and absorptive cells detached from the basal lamina, in the lumen. Delamination was also seen in the hepatopancreas of animals exposed to 300 ppb of zinc for 10 days; at the same time, 20 days exposure resulted in a widespread occurence of cellular rupture and structural damage in the tubules (Fig.10). The increase in the number of vacuolated B-cells (Fig.11) following zinc treatment was especially high in the case of animals exposed to 300 ppb of the toxicant, with the size of the vacuoles showing a slight tendency to increase with concentration of the metal and exposure time. Vacuolar contents were sparce and mostly absent in the zinc treated animals (Fig.10). Excessive vacuolation often resulted in a reduction in the luminal space and distortion of tubular shape (Fig. 10). There was a slight increases in the number of F-cells in the case of animals exposed to 300 ppb of zinc for 20 days. The

Fig. 5. Penaeus indicus a. control. b. exposed to 300 ppb of zinc for 3 days. c. exposed to 300 ppb of zinc for 5 days; arrows indicate blackened gills.

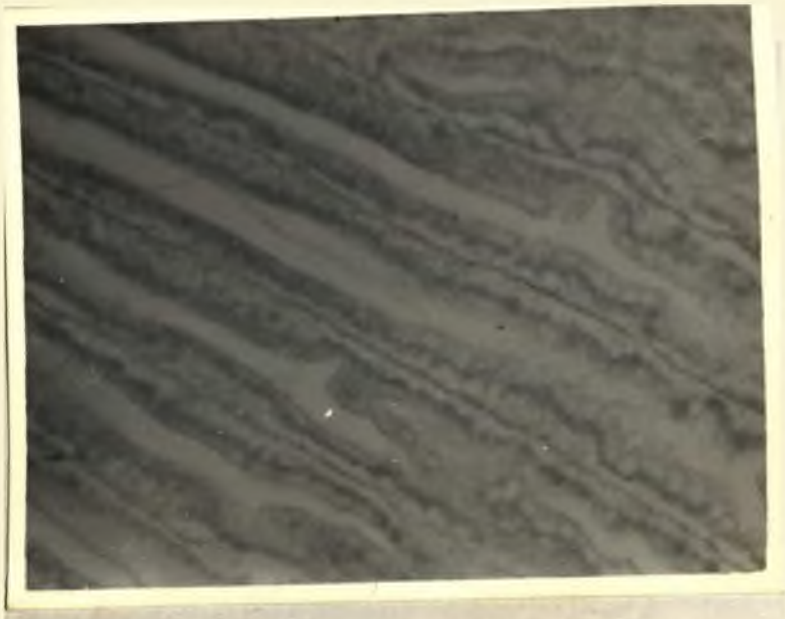

Fig. 6. L.S. of hepatopancreatic tubules of P. indicus maintained as control. 100X

Fig. 7. T.S. of hepatopancreatic tubules of P. indicus maintained as control. 100X.

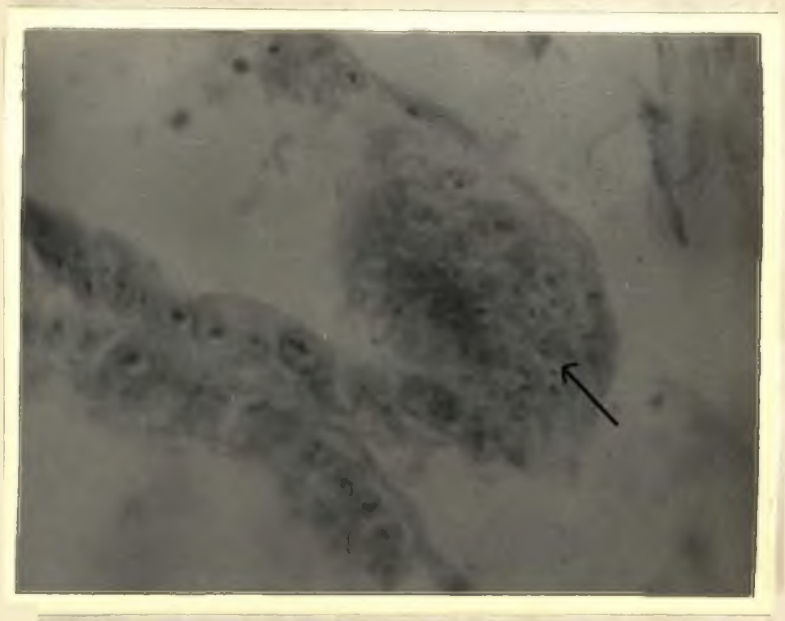

Fig. 8. L.S. of hepatopancreatic tubules of P. indicus exposed to 100 ppb of zinc for 20 days; arrow indicates clump of embryonic cells at distal end of the tubule. **400X**

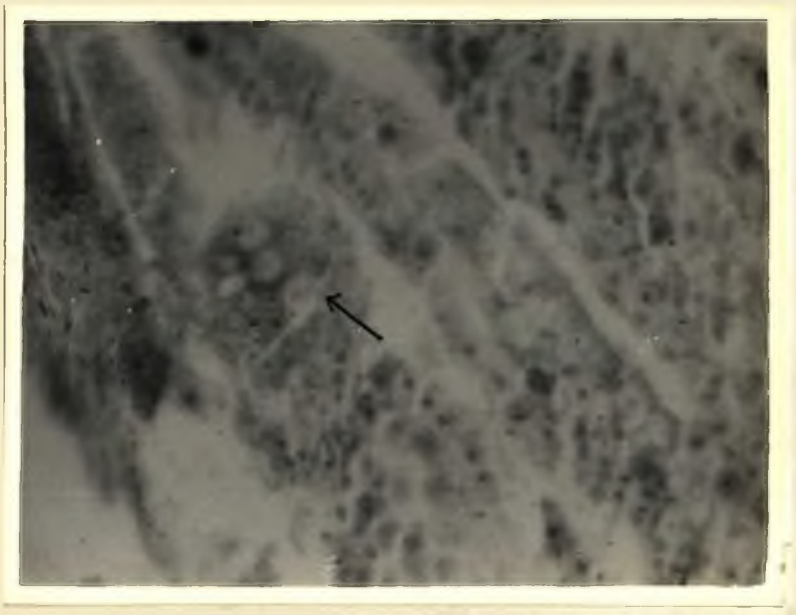

Fig. 9. L.S. of hepatopancreatic tubules of P. indicus exposed to 100 ppb of zinc for 20 days; arrow indicates clump of epithelial cells in the midregion of the tubule. **200X**

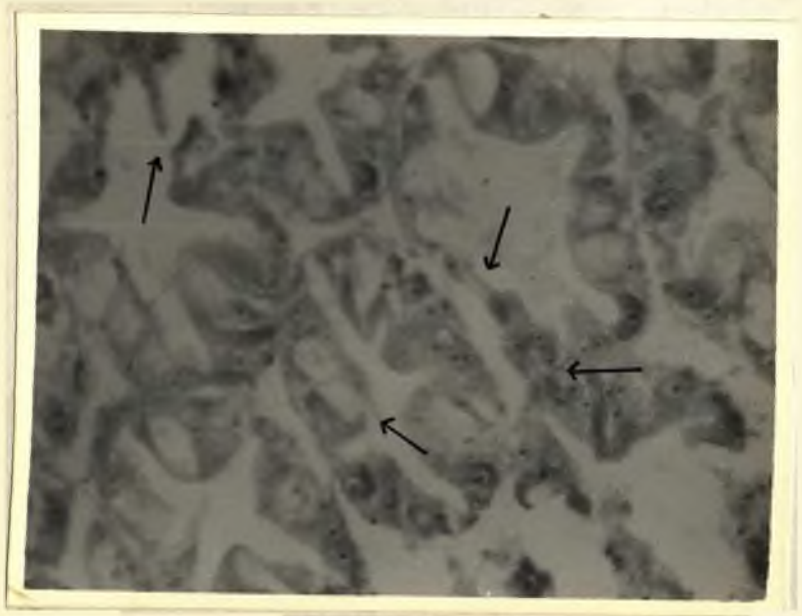

Fig.10. T.S. of hepatopancreatic tubules of P. indicus exposed to 300 ppb of zinc for 20 days; arrow indicates tubular rupture, increased vacuolation, delamination and pycnotic nuclei. **200X**

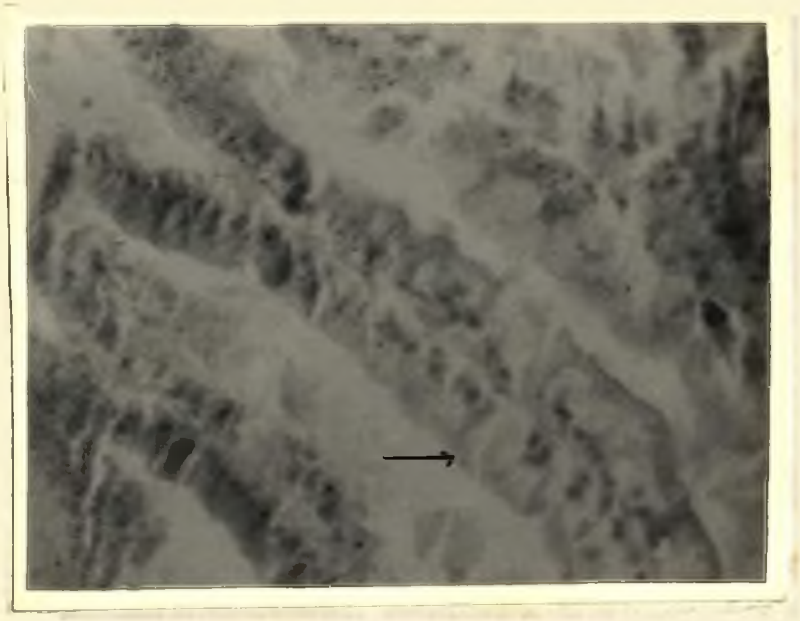

Fig.11. L.S. of hepatopancreatic tubules of P. indicus exposed to 300 ppb of zinc for 20 days; arrow indicates increased vacuolation. **200X**

hepatocytes of all treated animals in general, showed considerably reduced cellular inclusions. Occurrence of pycnotic nuclei was a common feature observed in the hepatocytes of animals exposed to the toxicant (Fig.10).

3.2.2 HISTOPATHOLOGY OF GILLS

The decapod gills are well-developed and enclosed in branchial chambers formed between the thoracic body wall and the inner surface of the carapace. The central axis of each gill is attached to the cephalothoracic wall by a tubular structure joining the ventral end of the central axis. Primary filaments branching out from the central axis divide further into secondary filaments which are generally non-branching. Each secondary filament contains a basally located afferent and efferent vessel separated by a thin septum. These lamellae are characterized by epithelial pillar cells between opposite cuticular walls, interspersed with interconnecting lacunae, through which the haemolymph moves. The distal tips of the filament often contain enlarged lacunae. The gill processes normally contain circulating haemocytes.

Gills of P. indicus exposed to 300 ppb of zinc often developed blackened gills within 3 to 4 days of exposure (Figs. 5a and b), while no such incidence was noticed in any of the animals exposed to 100 ppb of the metal, even after 20 days exposure. Histological examination of sections prepared from the gills revealed infiltration by haemocytes (Fig. 18), following zinc treatment. The extent of haemocyte infiltration increased with the increase of zinc concentrations and exposure time. Distension of secondary lamellae, as a result of

Fig.12. L.S. of gills of P. indicus maintained as control. **100X**

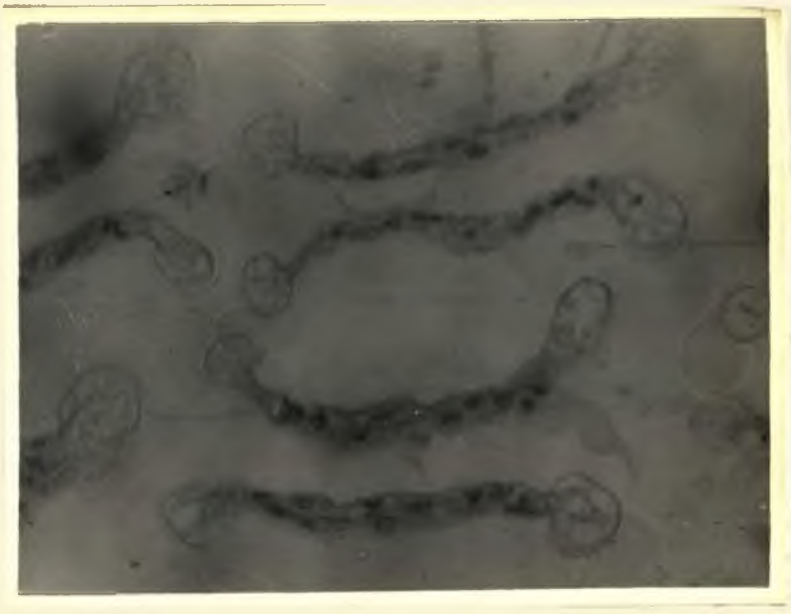

Fig.13. L.S. of gills of P. indicus maintained as control. **400X**

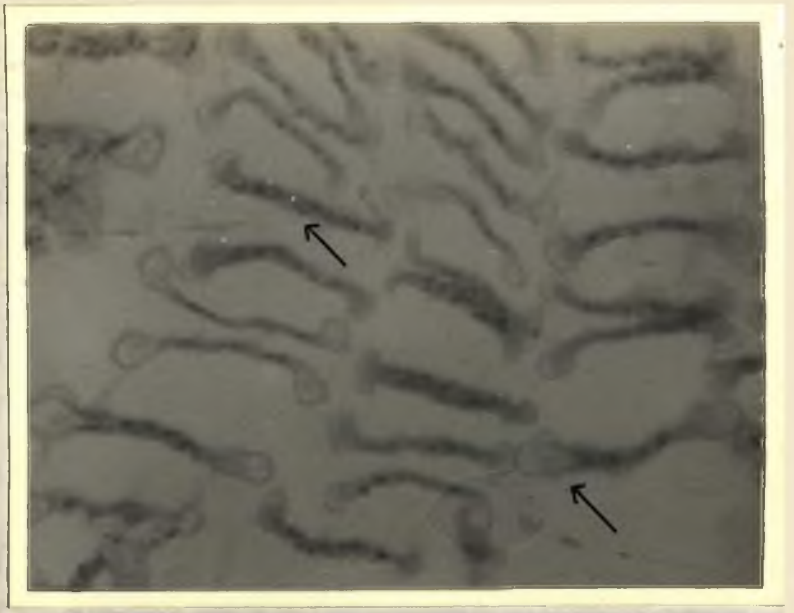

Fig.14. L.S. of gills of P. indicus exposed to 100 ppb of zinc for 10 days; arrows indicate initiation of distension and distortion of secondary lamellae. **200X**

Fig.15. L.S. of gills of P. indicus exposed to 100 ppb of zinc for 10 days; arrows indicate empty distal tips of secondary lamellae. **200X**

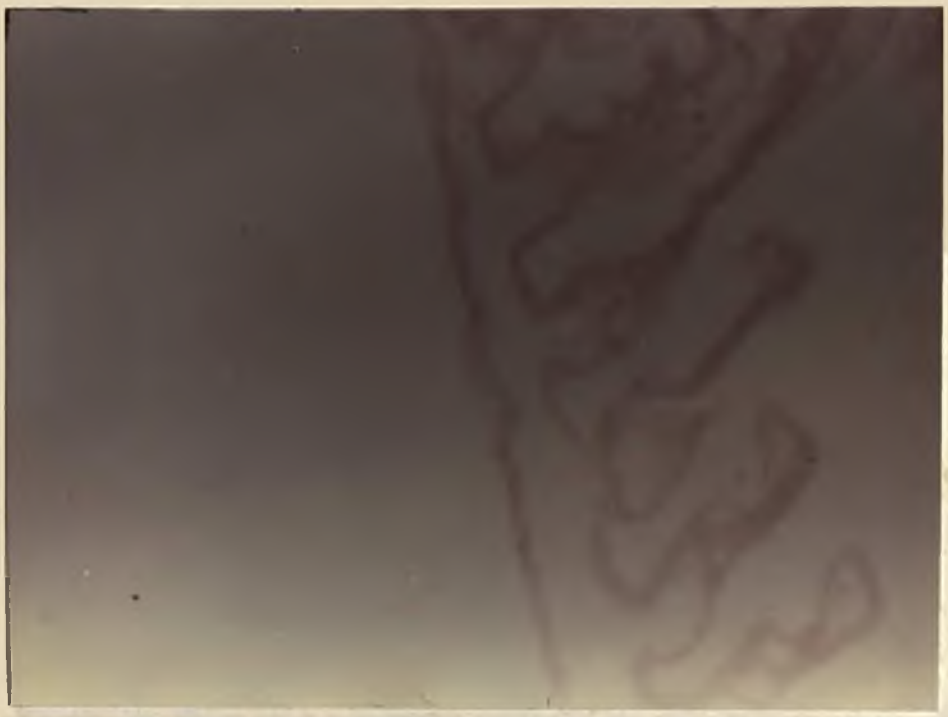

Fig.16. L.S. of gills of P. indicus exposed to 300 ppb of zinc for 10 days showing sloughing off of primary and secondary gill processes. **200X**

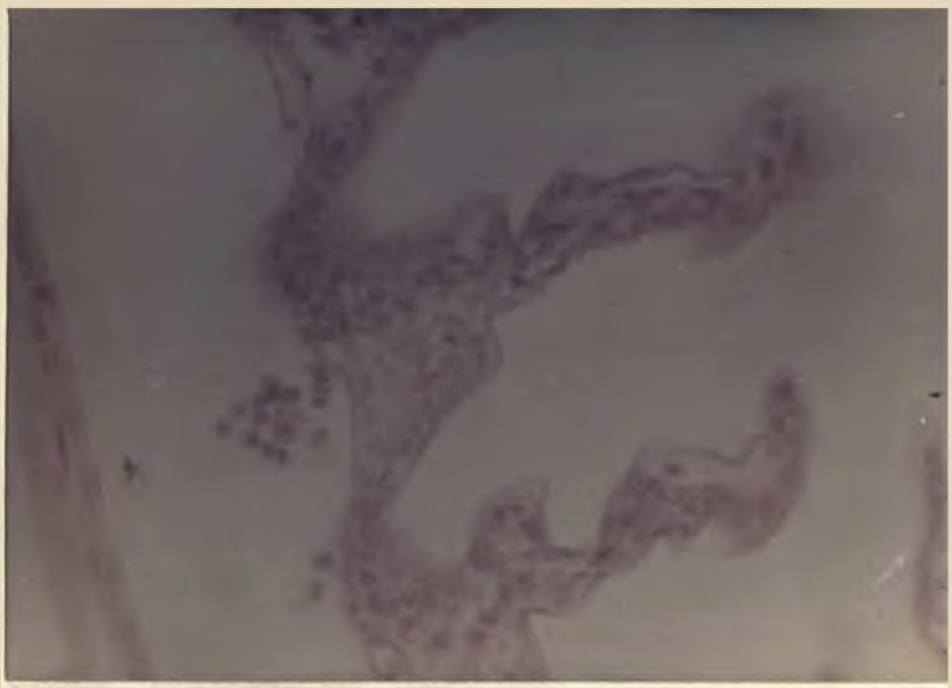

Fig.17. L.S. of gills of P. indicus exposed to 300 ppb of zinc for 10 days showing haemocyte infiltration in the primary processes. **400X**

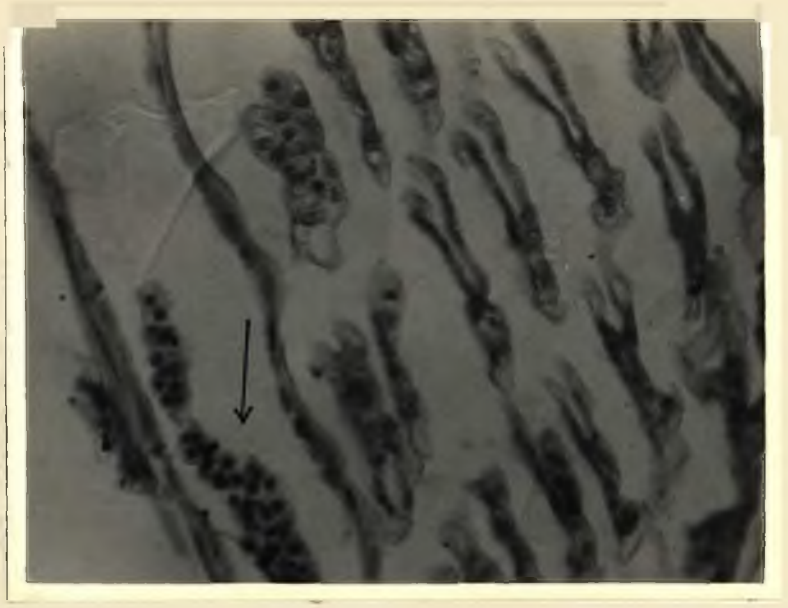

Fig.18. L.S. of gills of P. indicus exposed to 300 ppb of zinc for 10 days; arrow indicates haemocyte infiltration in the central axis. **200X**

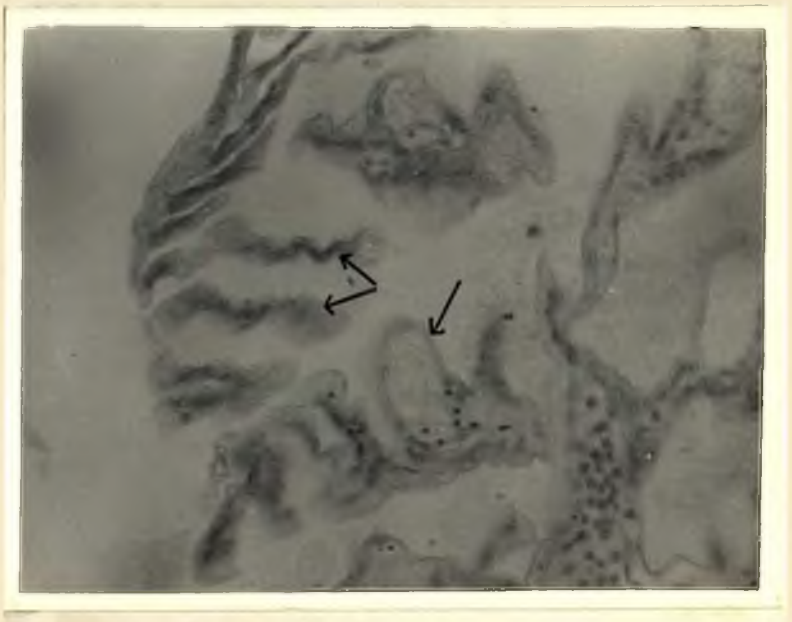

Fig.19. L.S. of gills of P. indicus exposed to 300 ppb of zinc for 20 days; arrows indicate highly distended and empty and highly distorted secondary lamellae. **200X**

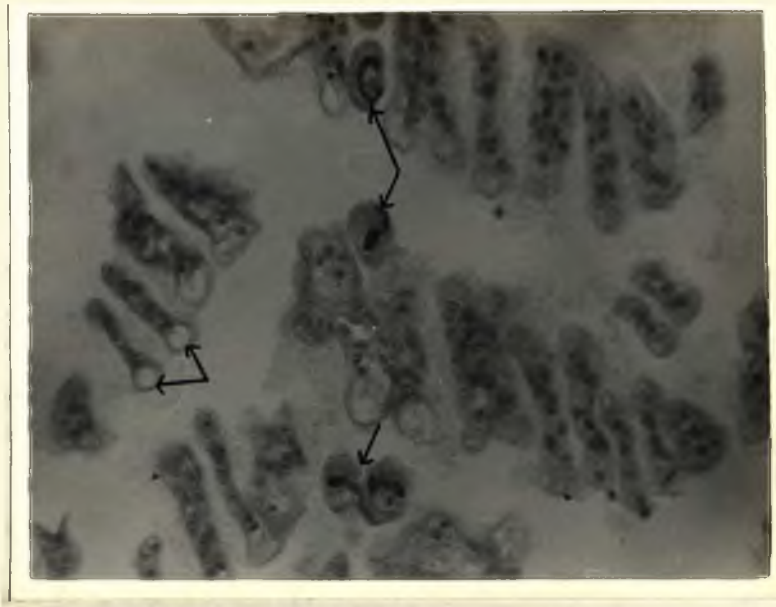

Fig.20. L.S. of gills of P. indicus exposed to 300 ppb of zinc for 20 days; arrows indicate empty tips and lesions in secondary lamellae. **200X**

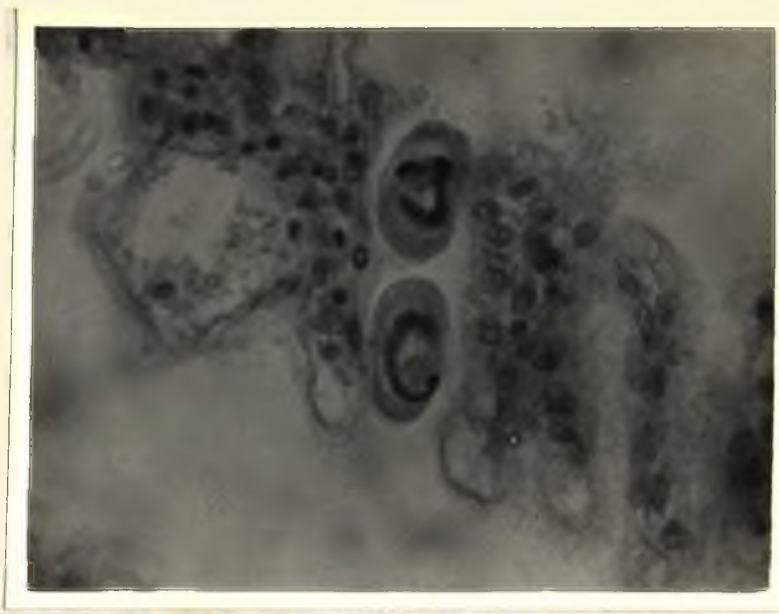

Fig.21. L.S. of gills of P. indicus exposed to 300 ppb of zinc for 20 days showing lesions, pycnotic nuclei, haemocyte infiltration and distension of secondary lamellae. **400X**

haemocyte accumulation, was noticed in gills of prawns exposed to both, 100 and 300 ppb of zinc (Figs. 14 and 21). The distal tips of the lamellar filaments, which often contain enlarged lacunae, seemed to be devoid of haemolymph and circulating haemocytes in animals exposed to both the concentrations of the toxicant (Figs. 15 and 20). Sloughing of primary and secondary gill filaments was noticed in portions of the gills, following treatment with 300 ppb of zinc (Fig. 16). Degeneration of primary processes was accompanied by haemocyte accumulation, pycnotic nuclei and sloughing off of secondary lamellae (Fig. 17). Secondary lamellar degeneration was also of common occurence, especially in animals exposed to 300 ppb of zinc for 20 days. Necrotic black lesions (Figs. 20 and 21) were seen in portions of secondary lamellae and distorted and empty lamellae (Fig. 19) were also seen in many areas in the case of animals exposed to 300 ppb of the metal. In most cases, the entire gill filament was seldom affected; often normal and affected gill processes and lamella were found to occur in the same areas.

3.3 STUDIES ON STARVATION

Subjecting juvenile P. indicus to starvation for 10 days resulted in considerable degenerative changes in the epithelial cells of the hepatopancreas. Morphologically, the hepatopancreas of the starved animals appeared considerably shrunken. Histological examination revealed autolytic changes in many parts of the tissues. Most of the cells were more or less obliterated and differentiation of the cell types was difficult (Fig. 27). There was a marked reduction in the vacuolar contents, with most of the vacuoles being empty (Fig. 23).

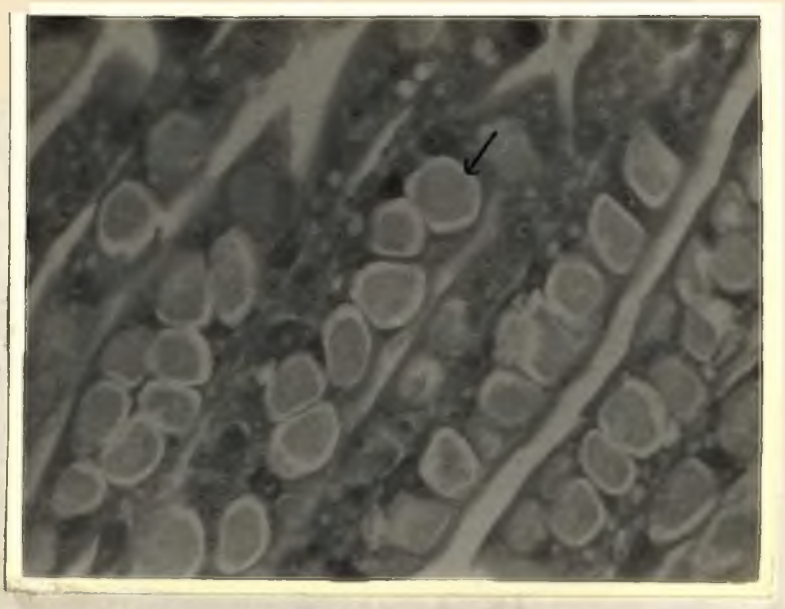

Fig.22. L.S. of hepatopancreatic tubules of P. indicus maintained as control; arrow indicates homogenous vacuolar contents. **200X**

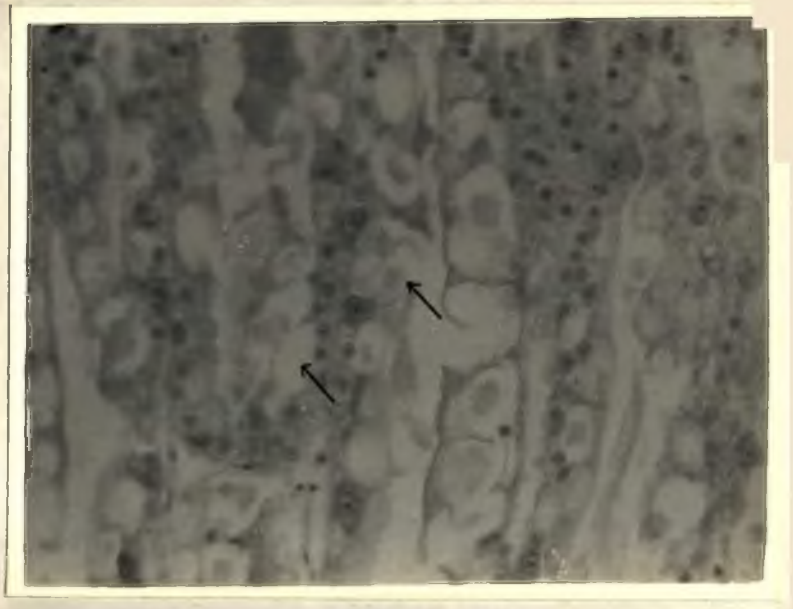

Fig.23. L.S. of hepatopancreatic tubules of P. indicus starved for 10 days; arrows indicate loss of homogeneity in vacuolar contents and empty vacuoles. **200X**

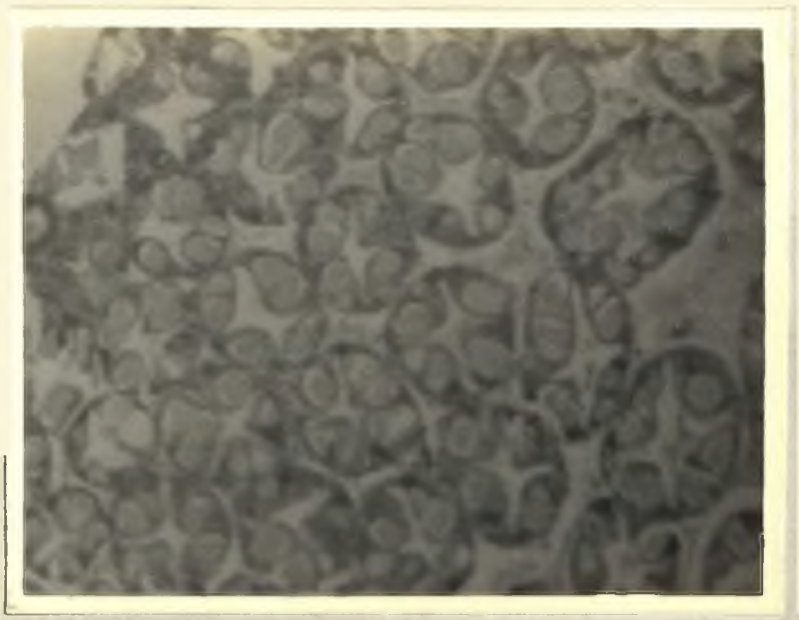

Fig.24. T.S. of hepatopancreatic tubules of P. indicus maintained as control. **100X**

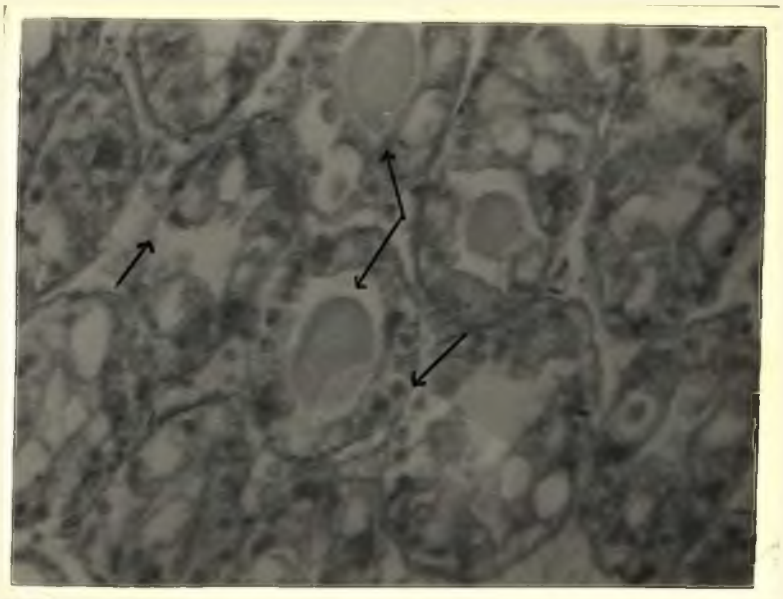

Fig.25. T.S. of hepatopancreatic tubules of P. indicus starved for 10 days; arrows indicate tubular rupture, accumulation of residual contents in the lumen and pycnotic nuclei. **200X**

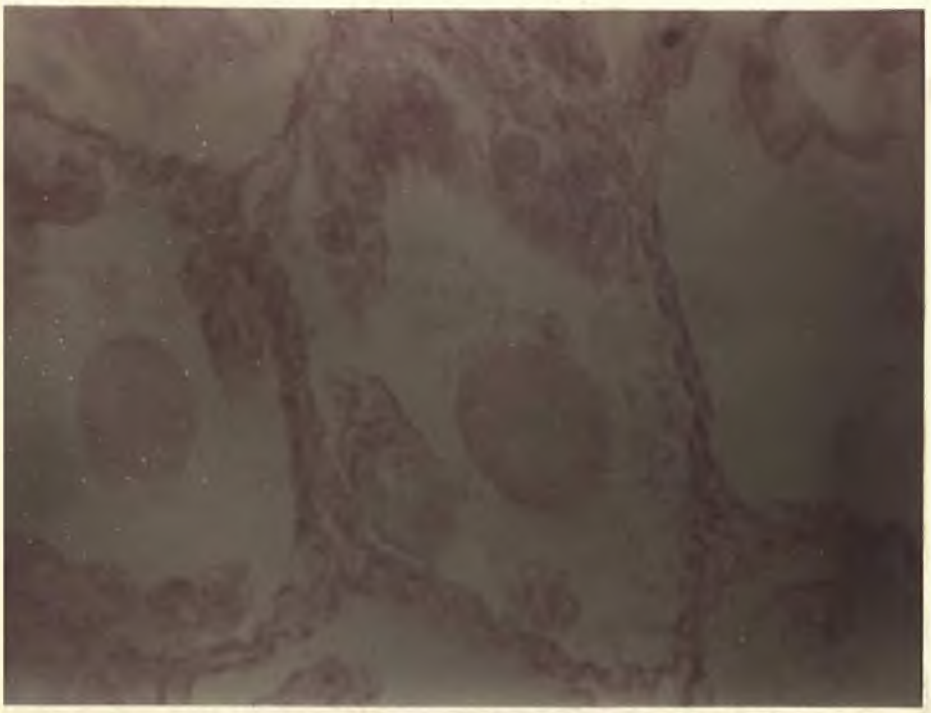

Fig.26. T.S. of hepatopancreatic tubules of P. indicus starved for 10 days showing shrunken basal lamina of disintegrated epithelial cells and pycnotic nuclei. **400X**

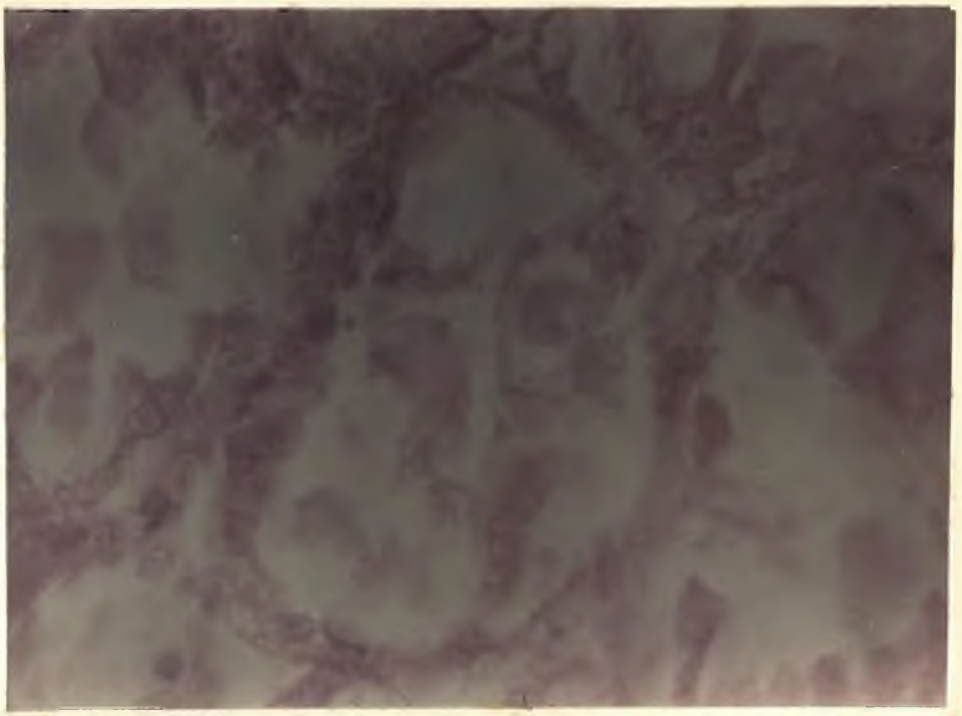

Fig.27. T.S. of hepatopancreatic tubules of P. indicus starved for 10 days showing autophagy and loss of cellular architecture. **400X**

Vacuolar contents, when present, lacked the homogeneity seen in the contents of vacuoles (Fig.22) in the hepatocytes of animals maintained under normal conditions. There was a general shrinkage of the tubular structures, with the shrinkage of the basal lamina being marked, especially in areas where autophagy had progressed to some extent (Fig. 26). Unlike what was observed in control animals, residual contents were commonly seen in the lumen of many tubules of the starved animals (Fig. 25). In general, cellular contents were considerably less in the latter. The occurence of pycnotic nuclei was also marked (Fig. 25), especially in the highly degenerated areas of the hepatopancreas of animals subjected to starvation.

Chapter IV

IV - DISCUSSION

4.1 LETHAL TOXICITY STUDIES

The capacity of a toxicant to effect death in an organism, with respect to concentration and time, is best assessed by means of lethal toxicity tests conducted under laboratory conditions. The 96 hr LC_{50} values determined by these studies are normally used to express the lethal toxicity of the xenobiotic to the organism concerned and to fix sublethal concentrations for subsequent chronic toxicological investigations. Thus, lethal toxicity tests often become an integral part of laboratory based sublethal chronic exposure studies.

The accumulation of zinc in crustaceans has been shown to occur by absorption from food and the surrounding medium. The hepatopancreas, gills and the entire body surface have been reported to play an active role in zinc accumulation in decapod crustaceans. The ability of these animals to regulate zinc levels upto certain operational limits before regulation breaks down, is indicated by the studies conducted by White and Rainbow (1982, 1984.) Rainbow (1985) and Nugegoda and Rainbow (1987), on the prawn, Palaemon elegans. Similar inferences have been drawn from studies conducted on the lobster, Homarus vulgaris and the freshwater crayfish (Bryan, 1964, 1967). In view of these findings it is likely that these animals are capable of tolerating comparatively high levels of zinc.

The present study reveals that juvenile P. indicus can tolerate zinc concentrations to some extent; the 96 hr LC_{50} was found to be as high as

1668.16 ppb. Sivadasan (1987), studying the effects of zinc on the prawn, Metapenaeus dobsoni (30-50 mm size), reported a 96 hr LC_{50} value of 0.76 mg/l in water of 25 ppt salinity. Ahsanullah (1976) showed the 96 hr LC_{50} of zinc to be 9.5 mg/l for Palaemon sp. and 11.0 mg/l for Paragrapsus quadridentatus. Thorp and Lake (1974) reported a 96 hr LC_{50} value of 1.21 mg/l of zinc for the crustacean Paratya tasmaniensis.

The legal limit for zinc in fish and fishery products (India) and the maximum permissible level of zinc in water (India), as fixed by Marine Products Export Development Authority (MPEDA), are 50 ppm and 0.25 ppm respectively. Studies by Sankaranarayanan and Stephen (1978) revealed that zinc occurs in the range of 2.3 to 113.2 mg/l in the Cochin backwaters. Subhash Chander (1986), Joshi (1990) and Ouseph (1992) obtained zinc concentrations ranging from 5 to 50 ppb in the perennial fields and canal systems along Cochin backwaters, with the values rising during the monsoons (Ouseph, 1992). The authors have reported the increased concentrations to be caused by the influx of zinc-contaminated fresh water from nearby industrial areas. Taking into account these relatively low zinc levels and the higher legal limits for the metal in Indian waters and fish and fishery products and considering the high values of LC_{50} obtained in the present study, it is easy to conclude that P. indicus in these waters does not, at present, face any threat due to zinc.

However, one of the important aspects which lethal toxicity tests often overlook, is the fact that though animals succumb to toxic substances after

exposure to specific concentrations for specific periods of time, the actual damage to their physiology is initiated at a much earlier stage, by even meagre concentrations of the substance. In order to evaluate the actual potency of a substance in causing deleterious effects in an organism, it is necessary to conduct sublethal chronic exposure studies and demarcate the stage when the animal is first affected by the pollutant stress, from the stage in which the animal succumbs to the fatal effect of the toxicant.

One of the objectives of lethal toxicity tests is to enable prediction of the concentration of a pollutant, as accurately as possible, that will not harm the ecosystem and the biota under study. The use of application factors (AF) applied to acute toxicity tests in water quality criteria has been recognized as a temporary solution to the problem of pollution by toxicants (NAS/NAE, 1973). These factors vary from 0.9 to 0.0001. 'Safe concentrations,' which presumably have no sublethal chronic effects are derived as a product of the LC_{50} and the application factor. EPA (1979) recommends safe concentrations derived by applying an AF of 0.01 to the 96 hr LC_{50} value of the toxicant.

4.2 CHRONIC EXPOSURE STUDIES

The assessment of sub-lethal toxic effects of hazardous substances on organisms has been mostly centered around investigations into changes in the physiological functions of the animals. Numerous cytological, histochemical and histological approaches have also been employed to detect

pathological disturbances in aquatic animals. It is a known fact that particular organ systems, rather than the whole organism, are easily affected by external pollutants. However, toxicological investigations regarding physiological malfunctioning and morphological aberrations seldom throw light on such aspects. Standard histopathological approaches are more useful in providing an overall picture of the degree of disturbance within the organ systems concerned (Moore, 1988). There is no doubt as to the fact that the structural damage caused to the tissues, which can be detected through standard histological studies also get reflected on the physiology of the animal also (Mathew, 1990), and histological responses to environmental conditions, as observed from animal tissue sections, form an important link between effects at the cytochemical level and those measured in the whole organism (Moore, 1988).

4.2.1 HISTOPATHOLOGY OF HEPATOPANCREAS

The hepatopancreas is one of the most dynamic organs which is highly sensitive to heavy metals and other xenobiotics. In decapod crustaceans, digestive gland functions as the main site for digestion and absorption of nutrients, storage of reserve food and excretion (Al-Mohanna and Nott, 1989). The cytology of the decapod hepatopancreas has been studied extensively by light and electron microscopy (Gibson and Barker, 1979). In general, 4 types of cells, namely, E-, R-, F- and B- cells, have been recognised in most decapods, based on the classification put forth by Hirsch and Jacobs (1928). Later reports by Hopkin and Nott (1979, 1980) have revealed further cytological

characters confirming the earlier observations that the tubule epithelium contains 4 cell types (Mohanna et al., 1985). The individual tubules, which are well-defined in decapods, are loosely held together by basophilic connective tissue strands (Barker and Gibson, 1978). Hirsch and Jacobs (1928, 1930) first described the process by which new cells are formed at the apex of each diverticulum, with differentiation progressing down the tubule, towards the proximal end.

Crustaceans in general and decapod crustaceans in particular, have been shown to possess the ability to regulate internal concentrations of essential but potentially toxic metals to presumably safe constant body levels. Bryan (1968), discussing the concentrations of zinc and copper in the tissues of decapod crustaceans, came to the conclusion that the chief regulatory methods employed by these animals include temporary absorption and storage by hepatopancreas, loss across body surface and excretion through urine and faeces. Studies on zinc regulation in the lobster, Homarus vulgaris (Bryan, 1964) revealed the possibility of involvement of the hepatopancreas in zinc regulation, there being a nominal increase in hepatopancreatic zinc concentrations in zinc-exposed animals as compared to normal ones. The author came to the conclusion that, at its simplest, the role of the hepatopancreas appears to be that of a sponge which mops up excess zinc from the blood and, with the excretory organs, helps to keep the blood zinc level fairly normal. In the absence of feeding, it was understood that the uptake was effected by absorption over the body surface.

Dall and Moriarty (1983) have reported the role of the crustacean midgut gland in the inactivation of a number of potentially toxic metals. Al-Mohanna and Nott (1989), in a study on the functional cytology of the hepatopancreas of the penaeid prawn, Penaeus semisulcatus, which can contain high levels of zinc (Al-Mohanna and Nott, 1985), showed, through X-ray micro-analyses of dense absorbed materials, that the B-cell residues in the proximal region of the tubules contain the maximum amount of zinc in association with sulphur, calcium and potassium. This probably indicates the ability of the B-cell in retaining zinc for discharge into the lumen. The potentially toxic zinc is rendered inactive through binding as the phosphate in separate in the cellular granules. In the present study, the nominal increase in cellular vacuolation and the number of B-cells, in the hepatopancreas of P. indicus exposed to zinc may be an indication of greater zinc retention as a means of detoxification. Along with number, the size of the vacuoles too showed a slight increase at higher concentrations and longer exposure time.

Bryan (1968) has shown that decapod hepatopancreas are capable of reabsorbing zinc from the blood and can store an excess of zinc. Al-Mohanna and Nott (1989) have reported the presence of zinc in R-cell inclusions in P. semisulcatus. They also found that zinc is present in abundant, small, spherical lysosomal structures present in the R-cell. Lysosomal responses to environmental stress factors, including many pollutants, have been described by Hawkins (1980) to manifest as changes, either infusion/events or in membrane permeability of lysosomal complements. Cytoplasmic vacuolation has been reported to occur in the hepatic cells of fishes exposed to zinc (Kumar and

Pant, 1981; Kothari and Suneeta, 1990). Increased vacuolation results, as is evident in the present study in obliteration of the tubular lumen. Delamination of the hepatocytes from the basement membrane of the tubular epithelium, is yet another phenomenon noticed in zinc-abused animals. Such delamination was found to cause "clumps" of cells in the lumen of the tubules, especially in animals exposed the lower concentration of zinc. Dislodgement of B-cells into the tubular lumen has been reported to occur normally in decapods. However, clumping of the other cell types as noticed in the present study, has been acknowledged as a sensitive histopathological reaction of stress from xenobiotics in general. Similar instances of clumps in the tubular lumen have been reported in the prawn, Metapenaeus dobsoni, exposed to the heavy metals, copper and mercury (Manisseri, 1993). Another feature observed in the present study was the increase in F-cells in animals exposed to the higher concentration of zinc. This could possibly be an indication of either increased secretory activity and protein production, or the ability of the pollutant to induce variability in cell proportion in the hepatopancreas. Reduction in the cellular inclusions was of common occurrence even in animals exposed to the lower dose of zinc and could be indicative of excessive energy utilisation as a result of heavy metal stress. There was a marked occurrence of pycnotic nuclei in animals exposed to both high and low concentrations of zinc. Occurrence of pycnosis has been reported to be a common phenomenon in aquatic organisms subjected to heavy metal stress. Nuclear hypertrophy of hepatocytes has been reported in fishes exposed to heavy metals. Dinesan (1988) has reported pycnosis in the tissues of the milkfish, Chanos chanos exposed to sublethal doses of zinc.

An overall atrophy in tubular structures was noted especially in the case of animals exposed to higher concentrations of zinc. Moore (1985) has identified the appearance of severe degenerative changes in the epithelia of the digestive gland of bivalves as an indication of autolytic processes resulting from lysosomal destabilization. Crespo and Sala (1986) reported that two organelles of chloride cells that were mainly altered, following short-term exposure of the dogfish, Scyliorhinus canicula, to lethal does of zinc, were lysosomes and mitochondria. Hiltibran (1971) and Brown et al. (1974) have stressed on the effects of toxic metals on the cellular respiratory metabolism of hepatic cells. Similar effects can possibly be the cause for destructive changes noted in the present study.

4.2.2 HISTOPATHOLOGY OF GILLS

The decapod gills follows the basic gill pattern wherein a series of 4 gills is attached to each thoracic somite. However, the presence of this full complement has not been reported in any modern decapod. A single gill essentially consists of an elongated central axis and a series of lateral branches. The central axis is provided with afferent and efferent blood channels. The decapod gill may be dendrobranchiate, trichobranchiate or phyllobranchiate Penaeoids and Sergestoids are the only decapods with dendrobrachiate gills.

The dendrobranchiate gill consists of a long central axis that carries a series of paired branches (the primary filaments), at right angles along its

length. Each branch gives rise to numerous perpendicularly oriented secondary filaments. A longtitudinal septum divides the lumen of each axis, branch and filament into afferent and efferent chambers. The secondary lamellae are transversed by epithelial pillar cells between opposite cuticular walls. The haemolymph moves through interconnecting lacunae which are interspersed among the pillar cells. The base of the secondary lamella houses an afferent and an efferent vessel, separated by a thin septum and the distal end is generally swollen, containing enlarged lacunae. Circulating haemocytes are seen in the gill processes.

Electron microscopic studies on the structure of the gill lamellae of Crangon crangon (Papathanassiou, 1985) reveals an outer cuticle beneath which are the epithelial cells and central haemocoel. The epithelial cells have numerous mitochondria. Each cell has a nucleus containing dispersed chromatin near the periphery and is connected to the next one by septate desmosomes. The apical region of the cell has prominent invaginations which produce small vesicles by micropinocytosis. Golgi complexes rough endoplasmic reticulum and abundant free ribosomes are also present in the cells.

Being the most permeable region of the body constantly in contact with the surrounding water, the role of the gills in the essential life-sustaining functions of respiration and osmoregulation cannot be doubted. Baker (1969), Eisler and Gardner (1975) and Jones (1975) have shown through physiological, histological and ultrastructural studies that heavy metal ions interfere with respiration and osmoregulation by disrupting the structure of the gill cells

in fish and crustaceans. The gills have been reported to be the main sites of absorption of heavy metals present in the medium (Bryan, 1964, 1968).

Studying the effect of zinc on the hermit crab, Dardanus arrosar, Cuadros et al. (1981) reported a high uptake of the metal through the gills, leading to a wide degree of variations in the gill structure. Gills of P. indicus exposed to zinc, in the present study, developed several pathological alterations. The most significant effect noted was the occurrence of blackened lamellae which often developed even within 3 to 4 days of exposure to 300 ppb of zinc. Similar effects were noticed by Nimmo et al. (1977) in the gills of P. duorarum and Palaemonetes vulgaris exposed to cadmium. However, no such incidence was noticed in the case of animals subjected to the lower concentration of zinc. This could possibly suggest a better ability of the animal, to regulate, to an extent, the body zinc level upto a threshold value, beyond which accumulation occurs, as suggested by Rainbow (1988).

Critical examination of the sections of the gills of zinc exposed animals revealed several marked variations from the normal structure. The heavy haemocyte infiltration noticed, especially in the case of animals exposed to higher concentration of zinc suggests an inflammatory reaction to the heavy metal stress. Distension of secondary lamellae was also more in this case. Pycnotic nuclei, commonly seen in animals exposed to stress, were abundant. The inhibition of gas exchange through distension of gill epithelium has been noted by Patil and Kalimal (1989) in zinc exposed prawns, Macrobrachium

hendersodyanum. They reported wide spread occurrence of gill lesions, pycnotic nuclei, necrotic areas, oedema and loss of cuticular lining of the lamellae. Degeneration of primary processes and secondary lamellae and sloughing off of secondary lamellae were also met with, in the present study. The secondary lamellae of animals exposed to 300 ppb of zinc exhibited maximum distortion in shape. Instances of lamellar shrinkage were also noted. Necrotic black lesions were commonly found in portions of gill processes from animals subjected to 300 ppb of zinc. Nimmo et al. (1977) had observed multiple lessons of this sort on gill processes, following cadmium treatment in P. duorarum.

Occurrence of secondary lamellae with empty tips was noticed in the case of animals exposed to both the concentrations of zinc. However, while only portions of the lamellar tips were empty in animals exposed to 100 ppb solution, the entire tips seemd to be empty in some lamellae of animals treated with 300 ppb of zinc. The occurrence of totally empty distended and degenerated secondary lamellae were also noticed in the case of animals exposed to the higher concentration. Wobeser (1975) observed cytoplasmic vacuolation and pycnosis, followed by distension and desquanation of the hyperplastic epithelium, which resulted in bare central pillars surrounded by a mat of epithelial cell remnants in the gill lamellae of rainbow trout exposed to methyl mercury chloride. Similar instances have been noticed in the present study in the gills of P. indicus treated to high zinc concentration.

The congestion of gill lamellae by haemocytes has been widely reported fro heavy metal poisoning; such gill lamellae after assume enormous proportions,

almost twice the size of normal lamellae, as noticed by Nimmo et al. (1977) in cadmium treated P. duorarum. A similar observation has been made in the present study on P. indicus exposed to zinc. Heavy haemocyte infiltration can lead to blockage of haemolymph channels and thus render the gills physiologically non-functional. The death of zinc treated fish due to insufficiency of the gas exchange process at the gills to satisfy the oxygen requirements of the animal has been reported by Skidmore (1970).

The malfunctioning of damaged gills can probably be related to the effect of the heavy metal on the cell organelles, especially the mitochondria, several studies in the past have revealed the immense sensitivity of mitochondria to metal ions. Crespo and Sala (1986) have shown great alterations in mitochondrial structure and function in the chloride cells of zinc treated dogfish, Scyliorhinus canicula. These alterations, followed by digestion of altered mitochondria by cytolysosomes, can lead to impairment of oxidative phosphorylation, causing a blockade of the sodium-potassium pump and the inhibition of several enzymatic activities involved in respiration and osmoregulation. Structural deformity suffered by the gills, which amounts to such magnitude as has been noticed in the present study, as a result of zinc toxicity, would invariably affect the efficiency of functions such as respiration and osmoregulation, performed by this vital organ. This in turn, would imply perturbations in the physiological functioning of the animal as a whole.

4.3 STUDIES ON STARVATION

The crustacean hepatopancreas has the function of both, the liver and the pancreas of vertebrates (Vonk, 1960), and is therefore involved in the

secretion of digestive enzymes and the absorption and storage of lipid material (Bunt, 1968; Smith et al., 1975). Starvation, which is often encountered by animals in both natural and culture ecosystems, lays a great stress on the morphology, histology and functional physiology of the hepatopancreas.

The hepatopancreas of P. indicus starved for 10 days exhibited a considerable shrinkage in size. Starvation resulted in the general atrophy of the tubule cells, with most of cells having undergone autolysis, making it difficult to differentiate the different cell-types. Lawrence et al. (1965) showed that the size of the digestive gland in the chiton, Katharina tunicata is influenced by the amount of food available and shrinks during starvation. Similar findings were reported in the mussel, Mytilus edulis by Thompson et al. (1974).

In a study on the effects of starvation on juvenile lobsters, Rosemark et al. (1980) noticed a general atrophy, especially of the absorptive and secretory cells in the hepatopancreas after just 4 days of starvation. They also reported a reduction in the size and number of B-cells and in the vacuolation of R-cells, accompanied by a progressive appearance of connective tissue and haemocyte infiltration. A regular feature noticed in the present study was the occurrence of empty vacuoles. Vacuolar contents, if present, were meagre and lacked the homogenous nature of the contents seen in control animals. On the other hand, there was a considerable accumulation of homogenous inclusions within the tubular lumen which could indicate increased atrophy of the epithelial cells. Cellular inclusions were, considerably less in almost all the cell types.

According to Storch et al. (1981), in starved decapod crustaceans, it is the R-cell in the hepatopancreas that reacts more than the F-cell or the B-cell. In a study on starvation in juvenile P. monodon, Storch et al. (1984) noticed a decrease in the size of the R-cell, depletion of stored lipid inclusions, pronounced swelling of mitochondria and increase in the thickness of the basal lamina. Overall tubular degeneration has been reported in the hepatopancreas of the starved lobsters, Homarus americanus (Stewart et al., 1967). According to Thompson et al. (1974) digestive gland material is utilised during periods of starvation and this process is associated with the degeneration of hepatic tubules. Fawcett (1959) reported rapid and drastic changes in the form of the endoplasmic reticulum of the digestive gland cells on starvation. Protein secretory cells have been found to be especially susceptible during starvation. Starvation induced changes in the histology of hepatopancreas of Metapenaeus dobsoni include considerable shrinkage of the tissue, general atrophy of the epithelial cells, reduction in cellular inclusions, obliteration of tubular lumen, slight swelling of the cells lack of homogenous contents in the vacuoles of the B-cells and occurrence of pycnotic nuclei (Manisseri, 1993). The histopathological alterations noticed in the hepatopancreatic tissue of starved P. indicus during the present study are in general conformity with the observations made in the different species of decapod crustaceans by several authors.

Mayzaud (1973) reported that starvation leads to protein metabolism, but owing to limited reserves, the animals would not be able to maintain a basal level of metabolism and would therefore, have to resort to degradation

of body proteins at an increasing rate to ensure a viable level of oxidative metabolism. Dall and Smith (1986) found protein to be the only energy substrate, under starvation, in Penaeus esculentes, supporting the conclusion of Barclay et al. (1983) which attributed proteis to be the main body reserve metabolised during starvation. Clifford and Brick (1983) however, concluded that in starved Macrobrachium rosenbergii, energy metabolism is dominated by the oxidation of carbohydrate while lipid and protein were only secondary and tertiary substrates respectively.

According to Thompson et al. (1974), there is no doubt about the rapid utilisation of digestive gland material during starvation. The authors state that recovery of the digestive gland with respect to both, structure and function, during resumption of feeding, is essential for survival of the animal since all metabolic processes are ultimately dependent upon the functional integrity of the digestive gland.

Chapter V

V - SUMMARY

Pollution is one of the major limiting factors that poses a potential threat to the sustenance of aquaculture systems. Keeping in view the importance of heavy metals as pollutants, a study was undertaken to assess the extent of harm done by the heavy metal zinc, which finds its way to natural water bodies mainly through industrial effluents, on the Indian White Prawn, Penaeus indicus, one if the important cultivable penaeid prawns in the country. The study was aimed at estimating the toxicity of the metal to the species at lethal and sub-lethal concentration levels. The sub-lethal toxicity was assessed through studies on the histopathological manifestations in vital organs such as hepatopancreas and gills. The histopathological effects of starvation, which is a stress these animals often encounter in both, natural and culture ecosystems, were also worked out.

Juvenile P. indicus were exposed to various lethal concentrations of zinc to estimate the 96 hr LC_{50}, following the method of Static Renewable Bioassay with 100% water exchange once every 24 hrs. The 96 hr LC_{50}, as analysed by Probit method, was worked out to be 1668.16 ppb.

Exposure of the animals to sublethal doses (100 and 300 ppb) of zinc was done for 20 days, with the animals being sacrificed after the 10th and the 20th days of exposure. The hepatopancreas and gills of these animals were subjected to critical histological examination, to identify the histopathological changes, if any, effected in them by the toxicant. The hepatopancreas

of zinc-exposed animals showed marked deviation from the normal structure. Vacuolation, especially of the B-cells, loss of homogeneity of vacuolar contents, reduction in cellular inclusions, occurrence of pycnotic nuclei, delamination of epithelial cells, clumping of cells in the tubular lumen, and rupture of tubules were among the histopathological alterations encountered. Higher concentrations seemed to result in more deteriorative changes.

The gills showed drastic changes under zinc exposure, often undergoing blackening in higher concentrations. Haemocyte infiltration, distension and distortion of the secondary lamellae, and sloughing off of gill processes were of wide occurrence in the gills of zinc abused animals. There a marked occurrence of pycnotic nuclei. Necrotic lesions and development of empty tips in secondary lamellae were also among the alterations induced by the heavy metal. All the histopathological changes noted did not, however, occur in the entire tissue, either in the hepatopancreas or in the gills; often damaged and intact structures occurredside by side.

Critical histological examination of the hepatopancreas of juvenile prawns starved for 10 days revealed a high degree of degeneration. A lesser homogeneity of vacuolar contents, empty vacuoles, presence of residual contents in the tubular lumen, reduction in cellular inclusions, pycnosis of nuclei, shrinkage of basal lamina and autolytic changes resulting in cellular atrophy were noticed widely. These observations indicate that starvation brings about drastic changes in the structure of the hepatopancreas, which can thus be used as

an indicator organ in the assessment of the nutritional status of the animal and any dietary stress it is subjected to.

The present study indicates that P. indicus is able to tolerate the stress induced by zinc upto a certain limit, beyond which, it succumbs to the toxicant. Thus, based on the lethal toxicity data obtained, 'safe levels' of zinc that can be encountered by the animals without being considerably harmed, can be predicted. However, from the histopathological observations, it is clear that even a concentration, which is more than 10 times less than the 96 hr LC_{50} obtained, can cause drastic changes in different organ systems of the animal, inflicting structural deformity of the organs, which, undoubtedly, reflects on the animal's functional integrity. The hepatopancreas, which, plays a major role in the digestive physiology of the animal, is no doubt easily affected by the metal. The damage caused to the gills is also a matter of concern since the vital functions of respiration and osmoregulation, which are effected chiefly through the gills, are affected adversely.

Based on the present findings, it would be desirable to limit the 'safe level' of the metal to a very narrow range, which will ensure the availability of the metal in trace amounts for the functioning of various enzymatic and physiological activities of the animals, without effecting any potential harm in them. The prediction of such a limit should undoubtedly be of help in effecting proper regulatory measures in the management of pollution problems caused by zinc in culture systems and in the natural ecosystem.

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