Discussion
Evaluation of Aluminium and Copper Toxicity:

Toxicity can be defined as the inherent capacity of a toxicant to affect adversely any biological activity of an organism. The popular method of evaluating the toxicity of a toxicant is by the determination of \(LD_{50}\) or \(LC_{50}\). This value represents the amount of toxicant either in the form of dose (LD) or concentration (LC) which kills 50% population of test animals within a fixed period of time (Finney, 1971; D'Silva and Kureishy, 1978). The period of exposure is having considerable importance in evaluating the toxicity levels of toxicants in aquatic animals. Generally, depending on the nature of the toxicant, the toxicity levels will be assessed at 24, 48, 72 and 96 hours or even more (Spehar et al., 1982). However, most of the \(LC_{50}\) studies on metals in aquatic organisms have preferred 96 hour exposure period, keeping in view that their effects, if any, on these animals become consistent within this period (Eisler, 1977). In the present study also aluminium and copper \(LC_{50}\)s for the fingerlings of the fish \textit{C. carpio} are determined at 96 hour exposure only.

Most of the studies on the effects of metals on freshwater organisms commenced with the determination
of LC$_{50}$s (Spehar et al., 1978, 1982; Holocombe et al., 1979; Sastry and Agarwal, 1979; Andros and Garton, 1980; Gill and Pant, 1981; Kaviraj and Konar, 1982; Sanpera et al., 1983; Radhakrishnaiah and Busappa, 1984; Morgan et al., 1986; Mirenda, 1986; Victoriamma and Radhakrishnaiah, 1986; Datto and Sinha, 1987; Krishnakumar et al., 1987; Suresh et al., 1991; Sivaramakrishna et al., 1991). LC$_{50}$ studies are also made in the freshwater fishes on exposure to aluminium and copper concentrations (Andros and Garton, 1980; Miller and Mackey, 1980; Dixon and Sprague, 1981; Dixon and Hilton, 1981; Spehar et al., 1982; Staurnes et al., 1984; Olsson et al., 1987). These studies reported that the level of tolerance varies from species to species. Resistance to high amounts resulted in certain species by immobilizing greater amounts than their more sensitive counterparts (Majori and Pentronio, 1973). Further, the test conditions could also account for such variability. Many factors such as pH, water hardness, size, sex, temperature etc., modify the toxicity of aluminium and copper (Chakoumakos et al., 1979; Smith and Heath, 1979). In the present study, the 96 hour LC$_{50}$s obtained for aluminium and copper to the fingerlings of C. carpio, 44.61
mg/l and 0.133 mg/l respectively, are within the concentration ranges of earlier reports.

Generally, as per the literature available, copper is more toxic to animals as it is readily absorbed by the membranes and form complexes with number of proteins, including enzymes. Hence, in the present study, the LC$_{50}$ of copper to the fingerlings is very very less i.e., 0.133 mg/l, than aluminium, which is 44.61 mg/l. As aluminium is poorly absorbed due to insoluble colloidal aluminium oxide, its toxicity to the fish is less. But in high doses aluminium could accumulate in tissues and can cause lethality. Further, the fingerlings of C. carpio might have very less resistance capacity to copper, as evident by the high mortality in very low concentration, than to aluminium in which it showed greater resistance (Lucky et al., 1975).

LC$_{50}$ studies are highly useful in determining the sublethal concentrations of metals. Most of the information on the effects of metals in aquatic animals concerns short-term experiments carried out in lethal concentration. This information is not sufficient to determine the real damage to individuals and populations. Hence, there is a need for sublethal toxicity
studies, which prove to be of most practical value in evaluating the sequence of events involving in response of the test animal to sublethal concentrations (Nobbs and Pearu, 1976; Hoppenheit, 1977; Perkins, 1979). So, to derive such sublethal concentrations and to compare the responses of the animal in sublethal concentrations with those in lethal, $LC_{50}$ are highly required.

Various symptoms of poisoning can also be observed from studies involving the determination of $LC_{50}$s. In the present study, control fingerlings behaved normally in the sense that they were very active and movements were well coordinated. They were alert and at any slight possible disturbance they swam faster. But, in lethal and near to lethal concentrations of aluminium and copper, they became irritable and hyper-excitible; jumping and restless movements were observed particularly in aluminium concentrations. Further, a number of black patches were observed on the fish exposed to copper for 3 to 4 days; slowly they became sluggish and settled down on the bottom and died. In the case of aluminium exposed fish, the body became very smooth with mucus secretion; mucus threads were observed at the gill region particularly at the time of its death. Moreover, examination of gills of the dead fish revealed
that the gill lamellae colour changed from red to brown. This was more prevalent in the fingerlings exposed to copper than to aluminium. Some of these observations were also reported earlier in fishes exposed to heavy metals (Kaviraj and Konar, 1982; Bengeri and Patil, 1982; Ansari, 1984). Suffocation caused by the mucus film over the gills may be one of the reasons for the death of the fingerlings in lethal concentrations of aluminium and copper. In lower concentrations no significant behavioural changes were observed, except some hyper-excitability movements and a little mucus film over the gill region. On the whole, with the knowledge of toxicity studies it could be possible to establish limits and levels of susceptibility to aluminium and copper by the biotic components in the freshwater.

Effects of Aluminium and Copper on Carbohydrates:

Carbohydrates serve as a reservoir of chemical energy to be increased or decreased according to the animal's need. The major function of carbohydrates is to provide the required energy for all metabolic processes of animals. Changes in carbohydrates to meet the changing energy demands can be expected in animals exposed to stress. Most of the studies on carbohydrate metabolism in freshwater fishes exposed to heavy metals repo-
rtered an enhancement in the blood glucose level followed by the depletion of liver glycogen content leading to the development of hyperglycemia (Christensen et al., 1977; Gill and Pant, 1981; Singh and Srivastava, 1982). Glycogen is considered to be the major source of energy in animal tissues, and maintenance of glycogen reserves is an essential feature of normal organismal metabolism (Turner and Manchester, 1972). Further, it is well known that glycogen phosphorylase is the enzyme concerned with the breakdown of glycogen in liver and muscle tissues. Phosphorylase occupied a strategic position in the glycolytic sequence, since it is the initial catalytic force in the chain of chemical events that lead to phosphorylative degradation and utilization of glycogen (Stetten and Stetten, 1960).

In the present study, decrease in glucose level and the activity of glycogen phosphorylase along with an increase in the level of glycogen in the fingerlings of *C. carpio* on exposure to aluminium and copper clearly indicate the stimulation of glycogenesis in the fish exposed to metal stress. The glycogen synthesis might also be occurred through gluconeogenesis. Further, the decreased utilization of glucose for energy yielding purposes might have stimulated the synthesis glycogen under metal stress; which gives an opinion of metabolic
imbalance. The decrease in glucose utilization could be due to the decreased rate of oxygen consumption and suppression in the activities of glycolytic and TCA cycle enzymes. The glycogenesis in the fish could also be due to the stimulation of a variety of chemical released from the neuroendocrine system. There are reports in animals exposed to heavy metals on the shifts in glucose, glycogen and glycogen phosphorylase, indicating hyper- and hypo-glycemic conditions stimulated by the respective hormones (Nagarajan, 1982; Dhavale and Masurekar, 1986). In addition, the anaerobic stress on the fish could induce hepatic glycogenesis by the suppression of glycogen phosphorylase and glucose-6-phosphatase activities (Eckner, 1971).

It appears that higher is the concentration of metal, more is the stimulation of glycogenesis, mostly in liver and muscle. Hence, more decrease was observed in the level of glucose of the fingerlings of the fish exposed to lethal concentrations of aluminium and copper; and it increased over time of exposure. Probably, the increased hypoglycemia could be due to increased anaerobic stress; and the nonavailability of required energy to the fish for metabolic compensation might have lead to its death under high toxic conditions. In sublethal concentrations, though there is an initial decrease in
glucose level of the fingerlings, a drastic recovery was observed at 7 days of exposure in both aluminium and copper. Thus indicates the efforts taken by the fish to bring its metabolic rate to normal level on long and continuous exposure to sublethal toxic stress. However, on further exposure, the animal could successfully achieve the metabolic compensation and could regain normal metabolic rate in aluminium, but in copper it could not do so, may be due to the cumulative effect of this toxic metal on the phosphorylase systems of the cell. Thus, in *C. carpio* fingerlings, the metabolic compensation or adaptation to sublethal concentrations is metal-dependent.

Aluminium being a low toxic metal, the fish could eliminate or excrete or bind it by low molecular weight metal binding proteins. This could not be possible in lethal concentration because the bound metal may have some finite limit with regard to intracellular storage (Engel and Fowler, 1979). And, at high concentrations, aluminium ions can cause phosphate depletion (Luckey et al., 1975), hence the decreased phosphorylase activity could enhance the hypoglycemic condition. The toxic effect of copper on phosphorylase enzymatic machinery seems to be significantly more than that of aluminium, as evident not only from the higher degree of its suppr-
ession in the fish exposed to lethal concentration, but also from the failure of regaining normalcy on prolonged exposure to sublethal concentration. Probably, the excessive concentrations of copper could cause tissue damage and impairment of oxido-reductase activities in the organs of the fish (Deung et al., 1978). Hence, in sublethal concentration of copper, though the fish tried to regain the normalcy in its glucose and glycogen levels, but it failed to do so on prolonged exposure due to its sensitivity to the cumulative concentrations of the metal. The detoxification and metal elimination processes probably became less effective over the increased accumulation of metal.

**Effects of Aluminium and Copper on Proteins:**

Teleostean fishes are very suitable experimental animals for the study of protein metabolism, as these animals derive relatively large part of energy from the catabolism of proteins to carbon dioxide, water and ammonia (Goldstein and Foster, 1970; Vander Thillart, 1977). As these proteins are the important organic constituents of organs, their role in the compensatory mechanisms of an animal can be expected during toxic stress. The stress may invoke compensatory metabolic changes through modification and modulation of the
quantity and quality of proteins. Studies on the impact of toxicants, particularly heavy metals, on proteins of fishes are very limited (Helmy et al., 1979; Sharma and Davis, 1980; Dubale and Shah, 1981; Dixon and Sprague, 1981; Kito et al., 1982; Joseph et al., 1987; Suresh et al., 1991). These studies reported both the increase and decrease in tissue proteins of fishes exposed to lethal and sublethal concentrations of different heavy metals. Even in the present study, the decrease and increase in soluble, structural and total proteins are observed in the fingerlings of C. carpio on exposure to lethal and sublethal concentrations of aluminium and copper. Increase in proteins may indicate their synthesis to develop resistance to the imposed toxic stress and decrease in proteins may be due to the breakdown of proteins for metabolic utilization and energy production.

The breakdown of proteins can dominate over synthesis under enhanced proteolytic activity whereas, the synthesis of proteins can dominate over breakdown under enhanced anabolic processes (Harper et al., 1979). In the present study, the elevation and suppression in the protease activity and free amino acid levels in the fingerlings exposed to lethal and sublethal concentrations of aluminium and copper could indicate
the domination of proteolytic activity over synthesis where there is a decrease in protein content and vice versa where there is an increase in protein content. The maintenance of proteins in a highly organized state requires an active and continuous supply of energy. If this is impaired, the organ structures breakdown and the protein denature partially.

Proteases hydrolyse proteins and peptide bonds resulting in the production of amino acids as end products. The significant increase in protease activity and free amino acid levels at 1 day, with an insignificant decrease at 2 days and again a significant increase at 3 days on exposure of the fish to lethal concentration of aluminium; and a gradual increase over time of exposure in the fish exposed to lethal concentration of copper, with the corresponding decrease in soluble, structural and total proteins, clearly indicate the intensive proteolytic activity in the fingerlings exposed to acute metal stress. Though there is a little resistance at 2 days in the fish exposed to lethal concentration of aluminium, but it could not do so on further exposure, may be due to high accumulation of metal. Increase in protease activity, a lysosomal enzyme, could be due to the damage caused by high concentrations of metals to lysosomes resulting in the
release of enzymes present in them into the cytosol. Young (1982) reported that once the metal sequestering capacity of lysosomes exceeds, cell death occurs by the action of unsequestering metal ions in other parts of the cell. Further, it is also reported that the metal may cause destabilization of lysosomal membranes leading to the release of hydrolytic enzymes that in turn can cause autolysis. Sternlib and Goldfischer (1976) stated that the high concentration of metals can cause the release of enzymes from lysosomes by altering the structure, permeability and integrity of their membranes. Moore and Stebbing (1976) ascribed copper induced tissue degeneration in hydroids to decreased ability of lysosomal membranes. In addition, an increase in proteolytic activity could be due to the destruction of organ systems, and thereby disturbing the biochemical functioning of cellular activities (Karel and Saxena, 1975).

In the present study the severe proteolytic activity, whether due to the lysosomal instability or due to cellular destruction, might be the reason for the drastic decrease in soluble, structural and total proteins in the fingerlings exposed to lethal concentrations of aluminium and copper. The effect of copper on proteins seems to be more severe than aluminium as is evident by their drastic decrease over time of
exposure, as against to a slight rise at 2 days with a fall at 3 days in the case of aluminium. Whatsoever, the high proteolytic activity in the fingerlings could be due to the increased rate of accumulation of metals in the tissues of the animal. There are also reports indicating that high concentrations of metal decrease protein content by enhanced proteolytic activity (Sharma and Davis, 1980; Varaengo et al., 1980; Ramalingam and Ramalingam, 1982; Venkataramana and Radhakrishnaiah, 1987). The enhancement of acid phosphatases, due to which also protein synthesis impair resulting in the depletion of protein content (Benerjee et al., 1978; Dubale and Shah, 1981).

It appears that protein synthesis dominated over breakdown in the fingerlings of the fish exposed to sublethal concentrations of aluminium and copper. But, this change is more clear and increased over time of exposure in the fish exposed to aluminium whereas, on prolonged exposure, the proteolysis dominated over synthesis in those exposed to copper with a noticeable shift from 1 to 7 to 15 days; increase in proteins with a decrease in protease activity and free amino acid levels at 1 day of exposure to increase in all these three at 7 days, and decrease in proteins with a significant increase in free amino acid levels and protease activity at
15 days. The gradual increase in structural proteins in the fish exposed to aluminium could be helpful to fortify its organs for developing resistance to the imposed sublethal toxic stress. The increase in soluble proteins could indicate the synthesis of enzymes necessary for detoxification and also for metal binding (Dixon and Sprague, 1981; Stagg and Shuttleworth, 1982). This helps as a device to remove the fraction of aluminium from the intracellular environment so as to adapt to the imposed toxic stress. In copper exposed fish, the trend is different that it could not resist even the sublethal concentration. Though there is an initial resistance, the domination of proteolysis on prolonged exposure indicates the inability of the animal to adapt to sublethal stress; probably due to the increased rate of accumulation of copper than its elimination and detoxification. However, the increase in free amino acids can act as an osmotic and ionic effector (Jurss, 1980) to bring electrostatic equilibrium between the external medium and ions of the blood, and regulate ionic and osmotic balance (Schmidt-Nielson, 1975). In addition, the increase in amino acid level may partly helpful for the production of energy during stress conditions (Solinska et al., 1983) or for the structural reorganization of proteins.
by incorporating them into TCA cycle to favour gluconeogenesis. This is also evident by the increased glycogen levels during sublethal copper stress. The overall changes in proteins of the fingerlings indicated that copper, either lethal or sublethal, causes structural deformities and severe pathological conditions whereas the effect of aluminium is relatively less and the destruction of proteins could occur only at high concentrations.

Effects of Aluminium and Copper on Lipids:

Involvement of lipids in fishes exposed to toxic stress can reasonably expected essentially on two grounds. Firstly, to provide the necessary energy to meet the additional energy demands of various active processes involved during toxic stress, and secondly, to provide at least a part of the extra amount of water needed for the regulation of osmoconcentration of body fluids. Studies on the involvement of lipids in animals exposed to pesticides are many (Chefurka et al., 1980; Chang Jong et al., 1981; Kaphalia et al., 1981), but such studies in relation to heavy metal toxicity are limited (Rana and Ajaya Kumar, 1980; Urmila Devi and Radhakrishnaiah, 1989). However, these studies indicated both an increase and decrease in lipids of the
animals exposed to various toxicants. Interestingly, in the present study the total lipids increased in the fingerlings of *C. carpio* exposed to both lethal and sublethal concentrations of aluminium and copper, except at 1 day of exposure to lethal concentration of aluminium where a little decrease was observed.

Lipase activity is known to be present in the organs of fishes (Patton et al., 1975), however, there is no much information on its role during toxic stress. Generally, the activity of it is said to indicate the breakdown of lipids leading to the release of fatty acids and glycerol (Bilinski, 1969) and also the synthesis of neutral fats by its reverse reaction (George and Taleswara, 1962). In the present study, decrease in lipase activity in the fingerlings exposed to lethal and sublethal concentrations of aluminium and copper, except the increase observed at 1 and 3 days of exposure to lethal concentration of aluminium, indicated the suppression of this enzyme activity by metal concentrations, probably to favour the lipogenesis as evident by the increase in total lipids and decrease in free fatty acids. Swami et al., (1983) observed a metabolic shift from carbohydrate to lipid metabolism through acetyl-CoA barrier leading to an increment in lipids in the organs of the freshwater mussel, *Lamellidens margi-
nails, under pesticide toxicity. Perhaps, the same reason holds for the increased lipogenesis in the fingerlings exposed to metal stress. The probable reason for the induction of lipogenesis can be attributed to the attempts of the animal to safeguard the cellular integrity under metal stress. Further, in the fish exposed to lethal concentration of aluminium, the increase in free fatty acids, by the increased activity of lipase, could be useful for the liberation of energy required to sustain under toxic stress. But, here also, the domination of lypolysis seen at 1 day rapidly shifted to the domination of lypogenesis at 3 days of exposure. In lethal concentration of copper, the lypogenesis significantly increased over time of exposure. It is quite interesting and paradoxical to observe the glycogenesis and lypogenesis in the fish exposed to lethal concentrations of aluminium and copper as against to proteolysis. The former two are synthetic and energy conserving pathways whereas the latter one is catabolic and energy liberating pathway. The reasons for the stimulation of these pathways are not clear; however, the free amino acids liberated by the degradation of proteins could be utilized by the animal for glycogen and lipid syntheses. This may cause an imbalance in the homeostatic mechanism of the fish under acute toxicity.
Lipids also increased in the fingerlings over time of exposure to sublethal concentration of aluminium. The synthesis of lipids, along with proteins, probably, serve to mitigate the sublethal toxic stress. The lipoproteins and phospholipids may constitute the structural rigidity to prevent the entry of toxic ions at sublethal level. In copper exposed fish, the increase in lipids gradually decreased over time of exposure, may be due to decrease in protein and glycogen reserves which might have caused destabilization in glycolipids, lipoproteins and phospholipids. Thus, the results indicated that not only lethal concentrations but the sublethal concentrations of copper are also highly toxic to _C. carpio_ fingerlings on prolonged exposure. The toxicity of aluminium is on concentration-dependent, the fish exhibited a recovery under sublethal stress.

**Effects of Aluminium and Copper on Acetylcholine content and Acetylcholinesterase activity:**

Acetylcholinesterase (AchE) is an enzyme that modulates the amount of the neurotransmitter substance acetylcholine at the nerve cell junctions (Wustner and Fukuto, 1974; Rainsford, 1978). In addition to nervous tissue this enzyme also occurs in non-nervous tissues, and its level of activity in nervous as well as in non-
nervous tissues varies in response to environmental stresses, including heavy metals (Olson and Christensen, 1980; Sastry and Sharma, 1980; Sivprasad Rao et al., 1982; Moorthy et al., 1984).

Several different functions have been associated with AchE activity in the organs of fishes. Lundin (1962) suggested that cholinesterases are concerned with the maintenance of salt balance, and this coincides with the earlier suggestion of Van der Kloot (1958) that AchE activity is concerned with ionic fluxes in fish tissues. There are also a few evidences to indicate that ionic fluxes are in turn concerned with the regulation of cellular metabolic enzyme activity, nerve activity and permeable properties of the cell membrane (Apter and Koketsu, 1960; Saroja and Pampapathi Rao, 1965). Hence it may be that such AchE induced ionic fluxes may ultimately lead to the changes in membrane permeability, metabolism and nerve activity in different organs of the fish at different environmental conditions.

Inhibition of AchE activity is regarded as a significant parameter in assessing complex toxicogenic effects of various toxicants, including heavy metals (Olson and Christensen, 1980). In normal conditions, the enzyme AchE initially forms a complex with the
substrate Ach, which then acetylates the enzyme with the release of choline. Deacetylation occurs by the reaction of water with the acetylated enzyme, and form acetic acid and the original free enzyme. In the presence of pollutants the enzyme may react with them in a way precisely analogous to that of the normal substrate and form a pollutant-enzyme complex, instead of acetylated enzyme (Corbett et al., 1984).

In the present study, AchE activity suppressed at 1 day of exposure of the fingerlings to lethal and sublethal concentrations of aluminium, with the corresponding elevation in Ach content. This could indicate the metal-enzyme complex formation leading to the accumulation of Ach. However, on further exposure of the fish to either to lethal or sublethal concentration of aluminium, the AchE activity increased with the corresponding decrease in Ach content; thus indicate high membrane permeability and nerve activity of the fish under aluminium stress. The increase of this situation over time of exposure in lethal concentration reflects hyper-membrane potentials and hyper-excitability of the fish which might have lead to the death of the fish. In sublethal concentration, this situation slowly retreated on prolonged exposure indicating the adjustability or adaptability of the fish to chronic aluminium stress.
In the copper exposed fish, the drastic decrease in AchE activity, along with an increase in Ach content, could indicate the binding of copper to active sites of enzyme. This may prolong the inward Na\(^+\) current by suppressing the increase in K\(^+\) permeability, and lead to altered permeability properties of membranes (Van der Kloot, 1958). The reports of Coppage et al. (1975) revealed that death occurs in fishes when AchE activity fails to the level of 70 to 80 percent. This situation is seen in the present study at 3 days of exposure to lethal concentration. The steady decrease in AchE activity from 1 to 3 days of exposures could lead to the disruption of nervous activity and cause uncontrolled hormonal release leading to the degeneration of vital organs, inhibition of many biochemical functions and water loss (Corbett et al., 1984). Inhibition of acetylcholinesterase activity was reported in the fathead minnow, *Pimphales promelas*, treated with various groups of chemicals, including copper (Olson and Christensen, 1980). In sublethal concentration of copper also though AchE activity decreased, a recovery was observed over time of exposure. This could be due to the activation of detoxification mechanisms or reduced availability of copper ions to compete with acetylcholine.
On the whole, the effects of aluminium and copper on Ach and AchE are different, the former one more or less elevated the nervous activity and membrane permeability whereas the latter one inhibited the nervous activity and membrane permeability potentials. The hyper-excitability could be one of the reasons for the death of the fingerlings in lethal concentration of aluminium and numbing in lethal concentration of copper.