BIOASSAY

1.1. INTRODUCTION

The aquatic ecosystem has become the nodal point of not only serving as the receptacle for industrial wastes but also an area of intensive research for evaluating short and long term deleterious changes in the ecological milieu affecting other flora and fauna (Gautam and Lall, 1989).

Fish are employed as sensitive indicators of toxic pollution. Fish in nature may respond to several environmental factors and laboratory experiments may identify and clarify these responses (Peterson et al., 1989). Doudoroff (1957) observed that since the degree of toxicity to fish of complex industrial wastes and other pollutants in various receiving waters is unpredictable, experimental determination by an appropriate biological assay method is frequently necessary. Many bioassay methods have been described and used by investigators in the past.

Kelso et al. (1990) reported that the scientific basis for managing pollutants in aquatic ecosystems has been derived from the application of several approaches; these include bioassays (laboratory and in situ), ‘environmental impact’ studies (including surveys) and construction of models. The above authors further pointed out that intensity of toxicity can be easily measured by means of bioassay studies, which are of short duration with less cost. The purpose of a bioassay program is to determine the effects of a discharge on the aquatic life of the receiving body of water (Patrick, 1985). Barghigiani et al. (1983) have pointed out that experimental responses may be divided
into two groups: (1) acute effects (short-term effects occurring over hours or a few days) and (2) chronic effects (long-term effects occurring over more than a week).

McCarty *et al.* (1978) and Stratton (1986) stated that accurate assessment of acute toxicity is an essential step in the development of realistic water quality. The objective of an acute toxicity test is to determine the concentration of a test material (*e.g.*, chemical or effluent) or the level of an agent (*e.g.*, temperature or pH) that produces a deleterious effect on a group of test organisms during a short term exposure under controlled conditions (Parrish, 1985). The primary purpose of an acute test is to estimate the concentration of the test material that is lethal to 50% of animals of a given species within a specific length of time (usually 24, 48, 72 and 96 h), which is referred to as the median lethal concentration (LC 50) (Gelber *et al.*, 1985). According to Rand and Petrocelli (1985), mortality and survival over a specific period of time are typical effect criteria in short term (acute) exposure tests.

Barghigiani *et al.* (1983) are of the opinion that toxicant concentrations used must be rather high in order for one to observe significant effects in a short time. The authors further observed that the simpler experiments on toxicity tests employ mortality criteria (percentage mortality) in a group of tested organisms after a given exposure time (such as 24, 48, 72 or 96 h) to a single toxicant or a mixture of toxicants. Different indices are adopted to estimate the influence of a given pollutant on the mortality of a given species, *e.g.*, the TL (tolerance limit), the LD (lethal dose), the LC (lethal concentration) and the ST (survival time) (Sivakumari, 1997). The traditional use of death as an end point in toxicity testing has limited our ability to assess subtle effects of toxicants on fish populations (Hill, 1989). Wuhrmann and Woker (1948, 1950, 1953, 1955) found that time of survival or time required for development of lethal poisoning is the most objective criterion for evaluation of toxicity of different substances.
Description of sublethal effects of pollutants will probably be a more profitable application of fitted multivariable response surfaces (Sprague, 1970). Forlin et al. (1986) have stated that the effects of pollutants on a population can be better understood and predicted by studying the sublethal effects on the individual or by focusing on processes at lower levels of biological organization. The authors further added that use of sublethal physiological responses to detect pollutant-caused disturbances at a very early stage is possible, since individual responses always precede population responses. As a result of chronic poisoning, fish are slightly weakened, readily affected by unfavourable conditions in the external environment and more prone to diseases; the general resistance of the fish declines, growth and development are retarded and as a result it dies (Metelev et al., 1983).

Hobe et al. (1983) have stated that the occurrence of acid rain and the resultant acidification of many freshwater lakes and rivers have been recognized globally as an anthropogenic environmental problem. Acidification produces profound changes in the biota of aquatic ecosystems and has far-reaching consequences on all trophic levels of aquatic life (Muniz, 1981, Bauer and Fischer-Scherl, 1987).

Atmospheric inputs of strong acids to acid sensitive regions may contribute to surface water acidification (Johansson-Sjoback and Larsson, 1979, Driscoll and Newton, 1985). A variety of water quality problems are associated with this phenomenon, including elevated trace metal concentrations (Baker, 1982), increase in lake transparency and reduction in thermal stability (Yan, 1983; Effler et al., 1985) as well as effects on aquatic organisms (Fordham and Driscoll, 1989).

Field and laboratory investigations have demonstrated toxicity of acid waters to a number of fish species (Brown and Sadler, 1989), though few field data encompass a range of life stages (Lacroix et al., 1985; Johnson et al., 1987). In addition, several
laboratory studies have elucidated some of the physiological mechanisms involved mainly for adult fish (Wood, 1989). However, there are many uncertainties in extrapolating laboratory results to natural systems.

Haines and Schofield (1980) reported that sudden mortalities of fish which may result from acidification are rarely observed under field conditions and mortalities during episodic inputs of hydrogen ions may be more common than has been usually observed. The above authors have also stated that a sudden reduction in pH can cause more mortality than chronic exposure where pH decline is gradual.

Increased acidification of aquatic ecosystems as a result of atmospheric deposition of strong acid has been identified as the source of declines in fish populations (Beamins and Harvey, 1972; Trojnar, 1977a). Effects of acidification have been attributed to impaired fertilization, lowered survival of embryos and fry and decreased hatching success (Leivestad et al., 1976; Runn et al., 1977; Peterson et al., 1980; Rask, 1983; Cleveland et al., 1986; Vuorinen et al., 1990a). The decreased survival of early life stages has been proposed as the main reason for the loss of fish populations in acidified waters (Schofield, 1976; Vuorinen et al., 1990a).

Trojnar (1977a) observed that White sucker, *Catostomus commersoni* exposed to pH 4.53 died within 24 hours. According to Jagoe and Haines (1983), sunapee trout, *Salvelinus alpinus oquassa* treated at pH 3.0 died within 4 hours, whereas in pH 3.5, the survival time ranged from 16.5 to 19 hours and in pH 4.5 and 5.0 no mortality occurred after 19 days. *Coho salmon*, *Oncorhynchus kisutch* exposed to pH 4.36 survived for 21 days (Powell and McKeown, 1986b) and rainbow trout, *Salmo gairdneri* lived for 44 hours in pH 4.0 - 4.1 (McDonald et al., 1983).

Reduction of survival time of fish in acid waters was reported by Dunson and Martin (1973) and Menendez (1976) in brook trout, *Salvelinus fontinalis*, Morgan and

Booth *et al.* (1982, 1988) suggested that there is a compelling need for a complete understanding of the ability of aquatic organisms to tolerate acid stress resulting from both progressive reductions of the environmental pH and from short periods of severe stress. The above authors have pointed out that two important environmental factors that influence the survival of fish in acidified water are water hardness and elevated concentrations of trace elements. Raitaniemi and Rask (1994) indicated that liming has proved to be a successful way to maintain or restore the populations of even the most sensitive fish species, when an adequate method for liming is used. From the fisheries point of view, liming should be a part of a general programme of action for acidic lakes.

Fish species living in waters affected by acidification have been reported to have survived better after lime treatment of water (Bengtsson *et al.*, 1980; Hasselrot and Hultberg, 1984; Nyberg *et al.*, 1986; Gloss *et al.*, 1989). It is further stated that liming has proved to be a successful method in keeping fish populations alive or restoring them to at least partially acidified lakes, when sufficient amount of limestone is used in the neutralization and the liming is repeated frequently enough (Hultberg and Andersson, 1982; Nyberg, 1984; Weatherly, 1988).

Experimental liming of lakes and running waters has enhanced fish reproduction earlier endangered or inhibited by acidification (Bengtsson *et al.*, 1980; Eriksson *et al.*, 1983; Nyberg *et al.*, 1986; Eriksson and Tengelin, 1987; Raitaniemi and Rask, 1990). Liming of water from an acidic river has been shown to increase survival of salmon fry.
Salmo salar (Farmer et al., 1980). A similar observation was made by Raitaniemi and Rask (1990) in perch, Perca fluviatilis and roach, Rutilus rutilus.

Increase in the concentration of external Ca\(^{2+}\) enhances the survival of fish in acidified waters as reported by Brown (1981, 1982a) in brown trout, Salmo trutta, McDonald et al. (1983) in rainbow trout, Salmo gairdneri, Brown et al. (1990b) and Jagoe and Haines (1990) in salmon, Salmo salar and Ingersoll et al. (1990) in brook trout, Salvelinus fontinalis. Calcium is vital for the survival of sunshine bass, Morone chrysops and Morone saxatilis under stressful conditions (Grizzle et al., 1985; Weirich et al., 1992, 1993). Laboratory studies of Brown and Brocksen (1982) showed that calcium levels should be sufficient to ameliorate the direct effects of hydrogen ion toxicity at pH 4.5 or below.

The foregoing review of literature reveals that there is not much work on the toxic effect of low pH on fish with reference to Indian context. Hence, an attempt was made to assess the toxic impact of low pH on fish, Cyprinus carpio var. communis and the ameliorative effect of external calcium on acid toxicity under tropical conditions.

1.2. MATERIAL AND METHODS

Impact of pollution on aquatic communities can be evaluated by many methods. Frequently, it concentrates on structural features of one or more groups of organisms such as fish, macroinvertebrates, periphyton or microbial communities (Pandy, 1997).

Fishes can be employed for ecological assessments at all levels of biological organization (Harris, 1995). Fishes are used as indicators of pollution over a temporal range varying from minutes to decades and spatially from a local scale of 1 m to the entire river basin; it is possible to use fish in assessment and monitoring programmes that are both rapid and relatively inexpensive as pointed out by Arunachalam et al. (1997).
1.2.1. MATERIAL

The exotic scale carp, *Cyprinus carpio* var. *communis* (Linnaeus), is extensively cultivated in India and in the Far-East; it is omnivorous and a non-predatory form, efficiently converting the food ingested into flesh and grows very fast when fed with artificial feeds; hence it is called 'the swimming pig' (Jhingran, 1975).

**Reason for selecting fish**

The carp, *Cyprinus carpio* var. *communis* was chosen for the present investigation based on the following reasons:

- The family Cyprinidae is well represented amongst the piscine inhabitants of freshwater and estuaries of India.
- It is widely distributed throughout the world.
- It has economic, recreational, commercial and ecological importance both locally and nationally.
- It is readily available in good numbers throughout the year.
- It rapidly multiplies without much effort.
- It is easily adaptable to laboratory conditions.
- It is sensitive to material or environmental factors under consideration.
- It is an ideal animal for toxicity studies in aquatic biology.
- Less risk involved in handling and transporting.
- Feeding flexibility.

**Procurement**

Specimens of *Cyprinus carpio* var. *communis* were procured from Tamil Nadu Fisheries Development Corporation Limited, Aliyar Fish Farm, Aliyar, Tamil Nadu, India, and were transported to the laboratory in polythene bags containing oxygenated
water. Fish of approximately the same age and size which hatched from the same lot of eggs were collected; the age of the fish being 3 to 4 months old.

**Acclimatization**

Fish were stocked in a large, rectangular cement tank (4'x6'x3'), previously washed with soap water, disinfected with potassium permanganate and thoroughly washed with water. They were acclimatized to laboratory conditions for 20 days, before being used in experiments. During acclimatization and also during subsequent periods no symptoms of disease were apparent.

**Diet and feeding**

During the acclimation period, fish were fed *ad libitum* once daily with groundnut oil cake and rice bran (in the ratio of 1:2). Feeding was stopped two days prior to the commencement of experiments, so as to keep the experimental animals in same metabolic condition and to minimize possible effects on parameters measured.

**Maintenance**

Fish were maintained on a natural photoperiod and ambient water temperatures. Water was changed every 24 h and well aerated in order to reduce any accumulation of excretory products and to ensure sufficient oxygen supply to the fish respectively.

**Recruitment of fish for experiments**

After acclimation, fish with a length of 7-8 cm and weighing 5-6 g were selected and transferred into clean rectangular glass aquaria (75 x 35 x 37 cm) of 150-L water capacity. Fish of both sexes were used. These fish served as the ready stock for experimental schedules.
1.2.2. METHODS

Water characteristics

Many environmental factors cause stress in fish. In some cases, even the minutest environmental change is reflected by a measurable physiological alteration that may influence the results of the experiments (Klontz and Smith, 1968). Wuhrman and Woker (1955) reported that the physico-chemical features of water have a significant influence on the biodegradability, availability and toxicity of pollutants. Hence, in the present study, water characteristics such as temperature, pH, dissolved oxygen, total alkalinity, salinity, total hardness, calcium and magnesium were estimated.

Temperature of the water was monitored by a thermometer. pH value was determined by a pen type pH meter (pH Scan 1, Eutech Cybernetics PTE Ltd., Singapore). Dissolved oxygen content was estimated by Winkler's method using starch indicator. Total alkalinity was determined using methyl orange indicator. Salinity was measured by Mohr's method using potassium chromate indicator. Total hardness was estimated using eriochrome black-T indicator and calcium levels were determined using murexide indicator, while magnesium content was calculated by subtracting calcium hardness from total hardness. The physico-chemical analyses of water in the present experiments were carried out using the methods as per APHA et al. (1976). Temperature, pH, total hardness, calcium and magnesium were monitored daily and the rest of the parameters were determined weekly. The analytical data for one set of experiments for the above parameters are presented in Table 1, since the difference in the hydrobiological values of water used for different sets of experiments was negligible.

Acid toxicity

pH is considered to be a measure of environmental quality. Acid precipitation can affect fish populations of lakes and rivers resulting in decreased fish densities (Almer et al., 1974). Schofield (1976) is of the opinion that sulphuric acid is a common...
mineral acid pollutant in wild. So in the present study, sulphuric acid was chosen to investigate the impact of acid stress on fish. Sulphuric acid (analytical grade) was obtained from S.D. fine chem., Boisar, India.

Formulae : \( \text{H}_2\text{SO}_4 \)

Purity : 98%

Specific gravity : 1.835

Molecular weight : 98.07

In order to find out the survival time of fish at different pH levels (acidity), the following experiment was conducted. Eight circular plastic tubs, each of 12 L capacity with 10 L of water in each were taken. The pH of water in the experimental tubs was lowered to desired values (6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0) by adding appropriate quantity of 0.1 N sulphuric acid drop by drop. The eighth tub served as the control with a water pH of 7.4. 10 fish were introduced into each tub, which were not fed two days prior to start of experiment. For each concentration, four replicates were maintained. During experiment, fish were fed \textit{ad libitum} 1 h prior to water replacement. Water was changed every 24 h and pH was adjusted to the designated level. The survival time of fish in each pH was noted. As the fish survived for 7 days in pH 4.0, this pH was chosen for short term studies according to Sprague (1969,1973), Wood and McDonald (1982) and Parker \textit{et al.} (1985). Dead fish were removed immediately from the experimental tubs. Death was indicated by the fish when they lost their equilibrium, floated their belly up, became immobile and failed to respond when gently prodded with a glass rod.

\textbf{Influence of external calcium on acid stress}

To find out the influence of external calcium on acid stress, calcium carbonate was used as a source of external calcium. Calcium carbonate (Reagent grade) was
obtained from New India Chemical Enterprises, Kochi, India. The physical and chemical properties of calcium carbonate are as follows:

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>CaCO₃</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>100.059</td>
</tr>
<tr>
<td>Colour</td>
<td>White</td>
</tr>
<tr>
<td>Density</td>
<td>2.930</td>
</tr>
<tr>
<td>Boiling point</td>
<td>825 °C (d)</td>
</tr>
<tr>
<td>Solubility in cold water</td>
<td>0.00153g/100cc</td>
</tr>
</tbody>
</table>

Five tubs were taken with 10 L of water in each. One tub served as control, while the remaining four were acidified to pH 4.0. The calcium level in the control tub/water was noted and it was 6.3 mg/L. Then, different CaCO₃ concentrations (Broad range trials) ranging from 5 mg to 20 mg/L (5, 10, 15 and 20 mg/L) were added to each tub. The experiment was initiated by placing 10 fish in each tub and the survival/mortality was observed for 8 days. Only in tubs with acid plus 10 mg/L CaCO₃ there was 100 percent survival of fish for more than 7 days and after 7th day no mortality was observed. However, in other tubs fish showed mortality after second day onwards.

Following this, narrow range was selected ranging from 6 mg/L to 14 mg/L of CaCO₃ (6, 7, 8, 9, 10, 11, 12, 13 and 14 mg/L) in order to find out the optimum calcium level. Eleven tubs each of 12 L capacity with 10 L of water in each were taken and acidified to pH 4.0 and appropriate amount of calcium carbonate was added to each tub as mentioned above with one control tub. Then 10 fish were introduced in each tub. The minimum external calcium level at which 100 percent survival of fish was recorded for more than 7 days was taken as the optimum calcium level and it was 10 mg/L. In rest of the experimental tubs fish mortality was observed starting from fourth day of treatment.
1.3. RESULTS

According to Davey (1976) and Forstner and Wittmann (1983), the physicochemical characteristics of water may influence the availability and nature of toxicants in aquatic ecosystem.

Data depicting the hydrobiological features of the water used for toxicological studies are given in Table 1.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Hydrobiological features</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Temperature</td>
<td>26.00 ± 2.00 °C</td>
</tr>
<tr>
<td>2</td>
<td>pH</td>
<td>7.40 ± 0.10 Units</td>
</tr>
<tr>
<td>3</td>
<td>Dissolved Oxygen</td>
<td>6.92 ± 0.02 ml/L</td>
</tr>
<tr>
<td>4</td>
<td>Total alkalinity</td>
<td>19.18 ± 0.14 mg/L</td>
</tr>
<tr>
<td>5</td>
<td>Salinity</td>
<td>0.33 ± 0.01 ppt</td>
</tr>
<tr>
<td>6</td>
<td>Total hardness</td>
<td>9.21 ± 0.22 mg/L</td>
</tr>
<tr>
<td>7</td>
<td>Calcium</td>
<td>6.34 ± 0.02 mg/L</td>
</tr>
<tr>
<td>8</td>
<td>Magnesium</td>
<td>2.87 ± 0.20 mg/L</td>
</tr>
</tbody>
</table>

Values are mean of five individual observations

The survival time of *Cyprinus carpio* var. *communis* when exposed to different low pH ranges are given in Table 2 and Fig. 1. At pH 3.0, the fish survived only for 45 min, whereas when fish were exposed to pH ranges of 3.5, 4.0 and 4.5, they survived for 2 days, 8 days and 12 days, respectively. In the case of pH 5.0 and 5.5, the fish survived
for 40 and 52 days, respectively. On the other hand, in pH 6.0, the fish survived for more than 60 days. The data revealed that there exists a direct relationship between pH and survival time of fish i.e., when the pH of water was lowered, the survival time of fish was also reduced.

Table 3 and Fig.2 gives the percent survival of fish *Cyprinus carpio* var. *communis* at pH 4.0 for eight days. At the end of 4th and 5th days of treatment there was 84% and 56% survival, respectively. On subsequent days, the per cent survival of fish decreased gradually with 100% mortality after 8th day of exposure. Acid toxicity at low ranges caused severe behavioural changes in the fish. The fish exhibited fast jerky and erratic movements, restlessness, increased opercular movements, surfacing, gulping of air, turning upside down, and loss of balance. Excess mucous secretion at the gills was also noted in them. Exposure of fish to broad range trials of acid plus calcium treatments resulted in 100% survival up to 2nd day (Table 4 and Fig 3) in all levels of CaCO₃. But as the exposure period was extended, the per cent
survival of fish was reduced to 96% in acid plus 20 mg/L of CaCO₃ group after 3rd day. There was 89% survival after 4th day exposure in acid plus 5 mg/L CaCO₃.

On the other hand, acid plus 10 mg/L of calcium resulted in 100% survival even after the end of 7th day. In acid plus 15 mg/L treatment fish mortality started after 5th day. Close range trials also indicated 100% survival of fish only up to 3 days from 6 mg/L to 14 mg/L (Table 5 and Fig. 3B). However, except in 10 mg/L, in all the other ranges the percent survival of fish decreased as the exposure period was extended. But in acid plus 10 mg/L of external calcium, the fish survived throughout the experimental period of 7 days. The data showed that an external calcium level of 10 mg/L in acidified water (pH 4.0) prolonged the survival of fish and this was considered as the optimum calcium level for pH 4.0.

Fig. 3B
Per cent survival of fish *Cyprinus carpio* var. *communis* when exposed to acid and acid plus different calcium level (Narrow range trials)
1.4. DISCUSSION

The purpose of most biological assessments of freshwater habitats is to characterize the status of the aquatic resources and to monitor trends in the condition of biological communities occurring there; these assessments are often associated with evaluations of anthropogenic perturbation (Resh et al., 1995; Sivaramakrishnan et al., 1996). Knowledge about the fate of environmental chemicals in aquatic environments is essential for the understanding and prediction of possible ecotoxicological effects (Fent and Looser, 1995).

Kelso et al. (1990) argues that bioassay programmes have been generally effective at screening sensitivity to toxicants; laboratory and *in situ* bioassays have been used to evaluate biological responses to acidification. Ramade (1987) indicated that the aim of using bioassays in the monitoring of environmental pollution is to establish a relationship between the toxicity and the concentration of a pollutant being studied in the biotopes concerned. The author further stated that this sequential testing procedure provides a gradually more precise estimate of the minimum concentration of the pollutant likely to induce harmful ecotoxicological effects. Toxicity tests must accurately assess the toxicity of chemicals in aquatic environments, if they are to yield meaningful information for evaluating chemical hazards to aquatic biota (Howe et al., 1994).

Adverse or toxic effects can be produced in the laboratory or in the natural environment by acute (short term) or chronic (long-term) exposure to chemicals or other potentially toxic agents (Rand and Petrocelli, 1985). Douglas et al. (1986) pointed out that acute toxicity test of fish has been selected as a primary test for the prediction of environmental effects of chemicals. Acute (short term) toxicity studies offer substantial help in detection, evaluation and abatement of pollution by providing reliable estimates of concentrations from which water quality criteria can be derived (Selvakumar et al.,...
Chronic toxicity tests permit evaluation of possible adverse effects of the chemical under conditions of long term exposure at sublethal concentrations (Rand and Petrocelli, 1985).

Acute mortalities of fish which may result from acidification and/or metal toxicity are rarely observed under field conditions and mortalities during episodic inputs of hydrogen ion may be more common than has been commonly observed as reported by Jensen and Snekvik (1972), Leivestad and Muniz (1976) and Leivestad et al. (1976) in salmon, Salmo salar and brown trout, Salmo trutta. The pH measured under such situations ranged from 3.9 to 4.6.

The decreased survival of early life stages has been proposed as the main reason for the losses of fish populations in acidified waters (Schofield, 1976; Vuorinen et al., 1990a). Falk and Dunson (1977) found that the survival time of brook trout, Salvelinus fontinalis was 24 h at pH 5.0-5.8, whereas in pH 3.15-3.5, fish survived for 1 h. Under similar conditions at pH 3.5 and 4.0, the brown trout, Salmo trutta survived for 18 days (Brown, 1981). Palawski et al. (1989) reported that in pH 5.0 and 5.6, Chironomus riparius survived for more than 30 days. Ingersall et al. (1990) observed that the brook trout, Salvelinus fontinalis survived for 21 days in pH 4.0 - 6.5.

In the present study, the survival time of Cyprinus carpio var. communis and water pH showed direct relation supporting the observations of previous workers (Graham and Wood, 1981; Lacroix et al., 1985; Hutchinson and Sprague, 1989; Fiss and Carline, 1993; Gagen et al., 1994; Sivakumari, 1997; Keinanen et al., 1998).

One of the first and now extensively documented responses of fish to acid exposure is the increased release of mucus from cells in the skin and gill surfaces (McDonald, 1983a). The rapid appearance of mucus at low pH is probably due to both
enhanced release and to the precipitation of the protein components in the secretion (Westfall, 1945). A number of authors (Daye and Gairsie, 1976; Dively et al., 1977; Haines and Schofield, 1980) have suggested that a thick mucus layer upon the gills would impede oxygen transfer and thus would contribute to a severe tissue hypoxia. Daye and Garside (1976) pointed out that extensive damage to the gill lamellae, with separation of the outer epithelial layer also occurs.

Dively et al. (1977) and Ultsch and Gros (1979) reported that elevated hydrogen ion concentrations may cause excessive secretion of mucus from the gills, thereby reducing the rate of oxygen diffusion across the gill surface. Due to massive acidosis, a pronounced accumulation of mucus on the gills (Plonka and Neff, 1969) and a sloughing of the gill epithelial tissue (Daye and Garside, 1976) may severely impair branchial oxygen diffusion and reduction in blood oxygen carrying capacity.

Ultsch et al. (1981) and Booth et al. (1982) found that low water pH (3.5) resulted in reduced O₂ exchange at the gills and inadequate delivery of O₂ to tissues of carp. The above authors concluded that at pH 3.5, tissue hypoxia was the main factor contributing to death. Spry et al. (1981) reviewed that mechanism of acid toxicity in fish, and suggested that the primary cause of death at very low pH is anoxia.

Booth et al. (1982) reported that significant respiratory problems appear to arise at lethal levels of water pH. Likewise, brook trout, Salvelinus fontinalis exposed to pH values ranging from 2.0 - 3.5 (lethal in 30 min - 3h) showed significant reduction in O₂ uptake prior to death (Packer and Dunson 1972; Packer 1979). The increased influx of hydrogen ion reduces blood pH, which in turn reduces the oxygen-carrying capacity of hemoglobin (Haines and Schofield, 1980).
Haines (1981) and Wood and McDonald (1982) observed that iono-regulatory disturbance is a key component of the toxic syndrome during low pH exposure leading to death. The authors have also stated that the massive disruptions of cardiovascular and fluid volume homeostasis caused death in low pH exposed fishes. Gonzalez et al. (1976) proposed that calcium was liberated from the epithelium by low pH titration, thus opening of junctions to ionic efflux. McDonald (1982a) stated that H+ ion increase the permeability of the gills by displacing Ca$^{2+}$ from the paracellular diffusion pathway.

Transfer from pH 7.0 to 4.0 caused a three fold increase in sodium loss in brown trout, *Salmo trutta* sufficient to cause death in 24 to 48 hours (McWilliams and Potts, 1978, Potts, 1979). Death will also occur at higher pH in water with very low ionic content than in water with moderate or high ionic content, because of increased osmotic stress (Haines and Schofield, 1980).

McDonald (1983a) reported seven major effects of pH upon the gills of fish which are as follows (ranked in approximate order of their occurrence with declining pH): inhibition of Na$^+$ and Cl$^-$ uptake mechanisms, increased ion-permeability and diffusional ion efflux, increased hydrogen ion permeation of gills, enhanced mucus production and release, mucus coagulation and precipitation, inhibition of gas transfer across the gills, and damage and separation of gill epithelial layers.

It is not possible currently to determine whether osmotic or respiratory effects or both are responsible for death of fish at low pH or whether some other factors may be involved (Haines and Schofield, 1980). In the present study, death of fish during low pH treatment may be due to excess mucus in the gills, failure of oxygen delivery to the tissues and iono-regulatory failure; tissue hypoxia or anoxia may be possible reasons for the death of fish during low pH treatments recalling the observation of McDonald (1983a).
Toxicity of H⁺ ions cannot be considered in isolation. Even a small change in calcium concentration can significantly influence survival of fish as suggested by Haves et al. (1984). Increasing calcium concentrations was beneficial to all life stages, although the magnitude of this benefit depends on life stage and on the specific pH levels (Ingersoll, 1990). According to Wood et al. (1990), water calcium levels will have a marked effect under neutral conditions and even greater influence under low pH conditions. Addition of salts to acid waters has been shown to lead to improved survival (Brown, 1982a). Laboratory and field data indicate that water Ca²⁺ is clearly protective against acid toxicity (Lloyd and Jordan, 1964; Leivestad et al., 1976; Wright and Snekvik, 1978; Brown, 1981; Wood and McDonald, 1982; Howells et al., 1983).

Brown (1981) observed that calcium prolonged survival of yearling brown trout, *Salmo trutta* exposed to pH 3.5-4.0. Environmental calcium was also shown to promote the survival of cyprinodont, *Fundulus kansee* in deionized water (Pickford et al., 1966), in striped bass, *Morone saxatilis* and white bass, *Morone chryxops* (Grizzle et al., 1985; Weirich et al., 1992) in soft water. A similar observation was made by Farmer et al. (1980) in salmon, *Salmo salar* exposed to acid toxicity. Hutchinson et al. (1989) reported that 4mg/ CaCO₃ prolonged survival of lake trout, *Salvelinus namaycush* exposed to pH 4.8.

Survival of fish is enhanced in acidified water of increased Ca²⁺ levels (Brown 1981, 1983a) possibly by ameliorating the effects of acid toxicity on ion losses across the gills. At concentrations less than 2 mg/L, calcium can significantly influence survival time of fish exposed to acute acid stress in the laboratory (Brown, 1982a; Rago and Wiener, 1986). McWilliams (1983) indicated that gills from brown trout, *Salmo trutta* taken from acidic and low Ca waters, lost Ca less rapidly than their counterparts from high Ca waters. Tight binding of Ca may be a significant physiological adaptation for survival in acidic and low Ca waters.
McDonald (1983a) discussed the influence of external calcium and concluded that when both external calcium and pH are low, the displacement of membrane-bound calcium leads to increased ion losses. He suggested that calcium plays a crucial role in modifying the toxic syndrome exhibited by fish exposed to low pH. Thus, as the calcium concentration in water increases, the loss of ions is reduced and lethal pH also decreased (Fromm, 1980).

Ca$^{2+}$ is thought to regulate gill membrane permeability and therefore, prevent the loss of ions in rainbow trout, *Oncorhynchus mykiss* (Gundersen and Curtis, 1995). Calcium also determines the relative efflux rates of sodium and chloride by changing the cation/anion selectivity of the leakage pathways in gill epithelia (McWilliams and Potts, 1978; Wood, 1989). Sodium loss may be a proximate cause of mortality in acid-exposed fishes (Pedder and Maly, 1987, Hill, 1989).

Presence of calcium in the external medium has been shown to reduce branchial sodium fluxes in the gold fish, *Carassius auratus* (Cuthbert and Maetz, 1972). Potts and McWilliams (1989) stated that any increase in the external calcium concentration in acid water is therefore beneficial not only because it directly reduces sodium permeability, but it also decreases hydrogen ion permeability. Calcium binds to the surface of cells and the main binding groups are oxyanions; this binding to sites on organic molecules facilitates the control of the steady state of structures of the cell membrane, cell-cell conglomerates, and structural units inside the cell (Williams, 1974).

Experiments with *Daphnia magna* showed that both influx and outflux of Na$^+$ were significantly altered at pH 4.5 in soft water but not in hard water as opined by Haves et al. (1984). The authors further added that in addition to the protective effect of calcium on membrane permeability and consequently on rate of Na$^+$ loss, calcium
may also indirectly protect the enzyme, ATPase, which is located within the membrane and is responsible for the active uptake of sodium.

In the present study, increased survival of fish in high calcium level may be due to neutralization of acid toxicity, protection of membrane permeability, reduced ion loss across the gill or preferential ion regulation or any combination.