THE EFFECTS OF ACID AND WATER HARDNESS ON BLUEGILL EMBRYO-LARVAE DETERMINED BY LABORATORY AND ON-SITE TOXICITY TESTS.

by

Kathleen M. Moynan

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Department of Fisheries and Wildlife Sciences

APPROVED:

Louis A. Helfrich, Chair

Donald J. Orth

Larry A. Nielsen

November 1989

Blacksburg, Virginia

THE EFFECTS OF ACID AND WATER HARDNESS ON BLUEGILL EMBRYO-LARVAE DETERMINED BY LABORATORY AND ON-SITE TOXICITY TESTS.

by

Kathleen M. Moynan

Louis A. Helfrich,Chair

Department of Fisheries and Wildlife Sciences

(ABSTRACT)

The sensitivity of bluegill (*Lepomis macrochirus*) to low pH in soft (12 or 18 mg/L $CaCO_3$) and hard (165 or 197 mg/L $CaCO_3$) water was compared in five day laboratory toxicity tests. Embryo-larval bluegill were exposed to pH levels ranging from 3.8 to 7.0 in soft water and from 3.8 to 8.0 in hard water. An on-site toxicity test, using lake water (3.4 mg/L $CaCO_3$) adjusted to pH levels ranging from 3.5 to 7.3, was conducted to compare laboratory and field results. At low pH, hatching was reduced, the hatching period prolonged, and the incidence of partial hatching increased. Increased water hardness mitigated acid toxicity, enhanced larval survival, and promoted hatchability. Hatching rates were decreased over those of the controls by 76 percent in soft water and by 23 percent in hard water at pH 4.0, and hatching was negligible at pH 3.8. The length of the hatching period was prolonged by 24 to 48 hours at pH levels $\leq$4.6. Partial hatching averaged 43 percent in pH 4.4. Increasing acidity resulted in increased embryo-larval mortality, averaging >62 percent at pH levels <4.6; mortality was 100 percent at pH levels <4.4 in soft water and 3.8 in hard water. Bluegill larvae were more sensitive than eggs. The embryo-larval LC50's were pH 4.67 in soft water and pH 4.06 in hard water; LC1's were pH 5.66 in soft water and 5.04 in hard water. In the on-site field experiment, approximately 50 percent mortality

occurred at pH 4.6 which was nearly identical to the LC50 of pH 4.67 in laboratory (soft water).

Growth of larvae was not affected by low pH. As acid levels increased, yolk-sac volumes decreased, physical deformities (fin, eye, and spinal abnormalities) increased and behavioral abnormalities were evident. Yolk-sac volume was decreased at pH levels <5.5 in soft water and <5.1 in hard water, perhaps reflecting higher energy expenditures by larvae held at sublethal pH levels. Spinal curvature and fin erosion of larvae were apparent at pH levels <5.25 in soft water. Larvae were lethargic and swimming ability was impaired at pH levels ≤5.5 in soft water and ≤5.1 in hard water. Exposure of embryo-larval bluegill to low pH levels (<5.7) in soft water may compromise their ability to survive, forage efficiently, and escape predation under natural conditions.

Acknowledgements

I would like to thank The International Science and Technology Corporation (IS&T), the Department of Fisheries and Wildlife Sciences, and the Flat Top Lakes Owners Association for providing the financial support that made this educational opportunity possible. I sincerely thank Dr. L.A. Helfrich for providing a context for my research that included freedom and professionalism, and for providing guidance, assistance and editorial advice. I would also like to thank the other members of committee Dr. D.J. Orth and L.A. Nielsen for their council and their commitment to excellence in fisheries education.

I thank and , for providing excellent technical assistance. , , , and , are graduate students who assisted in the completion of this project and they have my sincerest thanks. I would especially like to thank and for the technical fisheries training, physical labor, and friendship they unselfishly provided.

Intellectual stimulation, enlightenment, emotional support, and my "happiest hours" were provided by the International Club students and the VPI graduate students; thank you.

I am grateful to Dr. G.A. Atchison and for their friendship, encouragement, and confidence in me. My parents , my sister , my partners and , and my friends , , and are the source of my life and the work that I do; thank you.

Table of Contents

List of Illustrations

List of Tables

Introduction

Acid deposition is having a major impact on the aquatic organisms in eastern North America and Europe and is an emerging problem in Asia, South America, and the Soviet Union (WRI 1986, Schindler 1988). A large portion of the North American and European continents now receive precipitation with a pH averaging between 4.1 and 4.5 (WRI 1986). In eastern North America, the area receiving acid precipitation has expanded north westward and southward, and the intensity of acidification has increased in New York and New England (Cogbill and Likens 1974). The pH of the surface waters has decreased significantly in impacted regions, where the watershed soils have only a limited acid neutralizing capacity (Arnold et al. 1985). Areas of New York, North Carolina, Maine, New Hampshire, New Jersey, Nova Scotia, Quebec and Ontario have exhibited alkalinity and pH declines in surface waters due to acidic precipitation (Haines 1981, Haines and Baker 1986). Virginia and West Virginia are included in the area that receives the highest sulfate deposition (>35 kg/ha/yr) in eastern North America (Zeba et al. 1988), and acidification of high elevation, low order streams is occurring in these states. (Webb et al. 1989, Haines and Baker 1986, Jordahl and Benson 1987). In West Virginia, a survey of 82 streams revealed that 10

percent had pH levels less than 5.0 and 24 percent had alkalinities of less than 20 meq/L (Haines and Baker 1986).

The long-term solution to the problem of acid deposition is the reduction of emissions of sulfur and nitrogen oxides (Schindler 1988). However, enactment of laws and implementation of acid pollution control strategies has been substantially delayed by industrial and political influences. As a result, many lacustrine and riverine populations have been adversely impacted by acidification (Haines 1981, Pearce 1987).

The extensive research conducted to assess the effects of acid deposition on fish populations, has identified several factors that influence acid toxicity including, species (Johnsson et al. 1977 Carrick 1979, Baker and Schofield 1982, Rombough 1983, Palmer et al. 1988), stock and strain (Robinson et al. 1976, Swarts et al. 1978), life stage (Daye and Garside 1977, Lee and Gerking 1980, Baker and Schofield 1982, Kwain and Rose 1985, Vinogradov and Komov 1985), water hardness (Lloyd and Jordan 1964, Muniz and Leivestad 1980, Rosseland 1980, Seip 1980, Wood and McDonald 1982, Frenette and Richard 1986), condition of the fish (Kwain et al. 1984), and temperature (Kwain 1975). Extremes in acid tolerance are exhibited by fish species such as desert pupfish (*Cyprinodon nevadensis*) and European perch (*Perca fluviatilis*). The desert pupfish inhabits waters of pH 8.3 and exhibit reproductive impairment at pH 7.0 (Lee and Gerking 1980). Conversely, European perch inhabit oligotrophic lakes, low in ionic strength and exhibit reduced egg hatching between pH's 4.0 and 4.5 (Vinogradov and Komov 1985). Research concerning the effects of acid deposition on fishes have focused mainly on salmonid species because of their economic and recreational importance and the fact that they inhabit waters

susceptible to acidification. However, widespread geographic expansion of acid deposition has resulted in significant increasing risk to other fish species and aquatic ecosystems. Information on the acid tolerances of widely distributed, non-salmonid species that inhabit acid sensitive waters is needed. The focus of my research is the exposure of bluegill (*Lepomis macrochirus*) to reduced pH in laboratory and on-site toxicity tests.

The effects of acidification on a fish population are not usually sudden or spectacular, rather the population becomes increasingly composed of larger, older individuals due to recruitment failure (Haines 1981, Harvy 1982, Hulsman et al. 1983). The acid tolerance of adults generally is higher than earlier life stages of most fishes (Daye and Garside 1977, Lee and Gerking 1980, Baker and Schofield 1982, Kwain and Rose 1985, Vinogradov and Komov 1985). For example, pH levels as low as 4.2 are tolerated by adult white sucker (*Catostomus commersoni*) (Beamish 1976), whereas pH's 4.9, 5.3 and 5.6 are the limits of tolerance for post-larvae, larvae, and eggs, respectively for this species (Baker and Schofield 1982). Documentation of the acid tolerance of early life stages is essential to predict the response of fish populations to acidification.

Acidification causes high mortality of developing embryos and newly hatched larvae (Muniz and Leivestad 1980, Haines 1981, Vinogradov and Komov 1985). High mortality in embryos results from reduced activity of the choriolytic enzymes in low pH (Johansson et al. 1977, Daye and Garside 1980, Peterson et al. 1980, Vinogradov and Komov 1985, von Westernhagen 1988), whereas high mortality in larvae is probably attributable to erosion of the epidermal layer of the integument and gills, brain, kidney and spleen injury, lysis of erythrocytes (Daye and Garside 1980, Hill et

al. 1988), and failure of the ionoregulatory system leading to circulatory collapse (Packer and Dunson 1972, McWilliams and Potts 1978, Peterson and Martin-Robichaud 1986). The highly efficient ion transportation systems of juvenile and adult freshwater fishes allow them to maintain a more effective salt balance than early life stages, particularly when exposed to acid conditions. Incomplete development of the ion regulation system in newly hatched larvae may underlie their reduced acid tolerance (Daye and Garside 1980). Because of the acknowledged sensitivity of early life stages to acidification, embryo-larval stages of bluegill were used in this experiment.

Aluminum is present in many soils and becomes mobilized under acidic conditions (Schindler et al. 1980, Thomsen et al. 1988). Relatively low levels of aluminum and low pH conditions are lethal to many aquatic organisms (Baker and Schofield 1982, Eriksson et al. 1983, Hasselrot and Hultberg 1984). Smallmouth bass (*Micropterus dolomieui*) exhibited 100 percent mortality in five days when exposed to pH 5.1 and 180 ug/L of aluminum, and approximately 75 percent mortality occurred in 30 days in pH 5.1 without aluminum (Kane and Rabeni 1987). In acidified waters, the toxicological response of fishes probably is mediated mainly though the effects of acid and not aluminum (Holtze and Hutchison 1989). Holtze and Hutchison (1989) found that aluminum was most toxic within 0.3 pH units of the pH which was lethal in the absence of aluminum. They recommended safe pH levels of greater than 5.9 for common shiner (*Notropis cornutus*), greater than 5.4 for lake whitefish (*Coregonus clupeaformis*), white sucker and walleye (*Stizostedion vitreum*), and greater than 5.1 for smallmouth and largemouth (*Micropterus salmoides*) bass.

Many laboratory and field studies have demonstrated the mitigation effects of water hardness and particularly calcium ions (McDonald et al. 1980), in low pH environments (Lloyd and Jordan 1964, Muniz and Leivestad 1980, Rosseland 1980, Seip 1980, Wood and McDonald 1980, Frenette and Richard 1986). Mortality increased in brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) in pH 4.0 when water hardness was decreased by 50 percent (Carrick 1979). Rainbow trout (*Oncorhychus mykiss*) also exhibited increased mortality in soft water; a decrease in hardness, from 320 mg/L to 12 mg/L $CaCO_3$ resulted in an increase in the pH causing 50 percent mortality from 4.12 to 4.25 (LLoyd and Jordan 1964).

Increasing water hardness decreases the toxicity of acid to fish embryos and larvae, although the reduction in toxic effect is mediated by different mechanisms. The activity of the hatching enzyme is reduced by both low pH and low calcium ion concentration, thus reducing hatching and survival in soft, acidic conditions (Bell et al. 1969). In larval fishes, acidic conditions cause severe ion disturbance, leading to internal salt depletion of the entire body (Wood and McDonald 1982). The development of ionoregulatory failure is a function of effects of acid on fish gills, and include: 1) passive entry of acid across a concentration gradient, 2) inhibition of active Na^+/H^+ or NH_4^+ exchange (Packer and Dunson 1970, McWilliams and Potts 1978), and 3) increasing passive permeability to $Na,^+$ $K,^+$ Cl^- exiting across a concentration gradient (Eddy 1975, Wood and McDonald 1982). Exchange of H^+ through the gills is initially buffered by blood proteins and bicarbonate ions followed by intracellular and skeletal buffering by K^+ (Wood and McDonald 1982) and bone $CaCO_3$ and $Ca_3(PO_4)_2$ (Nelson 1982). Ionic disturbance in larval and adult fishes exposed to acidified water was increased in soft water, probably through reduced gill membrane stability and decreased ion up-take (McDonald et al. 1983). Because water hardness can have

significant effects on acid toxicity, bluegill embryo-larvae in this study were exposed to low pH levels at two levels of water hardness.

Fish population declines due to acid toxicity do not occur at a single, well defined pH level, rather they are mediated by several biotic and abiotic factors. A single experiment can not account for all of the possible variables, therefore, estimates of acid toxicity derived in the laboratory often do not accurately predict toxicity levels in field situations (Muniz and Leivestad 1980, Kennedy 1980). The purpose of my research was to estimate acid toxicity and tolerances of embryo-larval bluegill and to improve the predictive ability and realism of such estimates by investigating the influence of water hardness on acid toxicity in the laboratory and by conducting an on-site verification experiment.

Specific objectives of this research were as follows:

1. To assess the toxicity of sulfuric acid to embryo-larvae bluegill through short-term (120 or 168 hour), static-renewal toxicity tests conducted in the laboratory and in an on-site experiment.

2. To determine the tolerance range of embryo-larvae bluegill exposed to reduced pH conditions by characterizing eggs hatching rates, embryo-larval LC50 and LC1 (threshold toxicity levels) values, yolk-sac absorption rates, and larval behavior.

3. To examine the influence of two water hardness levels (hard and soft) on acid toxicity in embryo-larval bluegill.

Methods

Test Species Selection

Bluegills (*Lepomis macrochirus*) are abundant inhabitants of widely distributed, acid sensitive lakes. Because acid precipitation has spread southward from northeastern North America (Cogbill and Likens 1974) more warmwater lakes supporting bluegills are susceptible to acidification. Bluegills are important forage for large predators such as largemouth bass, northern pike (*Esox lucius*), and walleye (*Stizostedion vitreum*) and their decline would significantly alter sportfish growth and community structure. Except for the black bass, bluegill sunfish are the primary freshwater sportfish species targeted by U.S. anglers. In 1985, of the 40 million freshwater anglers in the United States, 14 million or 37 percent spent 264 million angling days in pursuit of panfish (U.S. Dept of the Interior 1988). Freshwater fishing expenditures totaled 17.8 billion dollars, a significant proportion of which can be attributed to the popularity of bluegill fishing, in 1985 (U.S. Dept. of the Interior 1988).

Bluegill sunfish are among the eight freshwater fish species recommended by the American Society for Testing and Materials (ASTM 1980) for use in toxicity tests based on availability, and commercial, recreational, and ecological importance, past use, and ease of laboratory handling. Little information is available about bluegill acid tolerance; existing data consists mainly of observations of the occurrence of adults in acidified lakes (Harvey 1982) and limited 96-hour toxicity tests on juveniles and adults (Trama 1954, Ultsch 1978, Ellgaard and Gilmore 1985, and Palmer et al. 1988). The early life stages, those that are probably the least acid tolerant, have not been addressed by previous studies of bluegill acid toxicity.

Test Procedure Selection

A short-term embryo-larval test protocol proposed by Birge et al. (1985) was chosen to estimate acid toxicity in bluegill in this study because it focuses on the life stages known to be most sensitive to acidification. The test is initiated shortly after fertilization and continues through four days post-hatch. A modification of this test, used in the on-site experiment, extended the exposure period to complete yolk-sac absorption (seven days) in order to maximize exposure time, but avoid exogenous feeding. Complete yolk-sac absorption was not a a well defined end-point, differential rates of yolk depletion occurred at various pH levels. Therefore, a standard exposure period of four days post-hatch was used in all laboratory tests. Standard procedural guidelines Horning and Weber (1985), and ASTM (1988) also were followed.

In this study, a short-term embryo-larval test was used to determine the acid level lethal to 50 percent of the population, or the median lethal concentration (LC50), and the threshold concentration (LC1), the lethal concentration to one percent of the population. LC1 values may be used to estimate chronic life-cycle values. An estimate of chronic toxicity is the maximum acceptable toxicant concentration (MATC). Data derived from full life cycle toxicity tests (chronic test) expose all life stages to the toxicant, are used to determine the MATC (Mount and Stephan 1976). A MATC is defined as the hypothetical toxic threshold concentration between the highest concentration tested having no observed effects and the next higher toxicant concentration having significant toxic effects (Mount and Stephan 1976). The experiment-wide MATC range is often taken as the lowest concentrations that demonstrates an effect on any of the specified response end points, such as reduced egg production or hatching (Rand and Petrocelli 1985). Birge et al. (1985) reviewed the published metal toxicity data and found that all LC1 values were within the MATC range.

Only complete life-cycle tests can be used to definitively determine acceptable toxicant concentration, but the cost and time requirements of chronic procedures have limited their use. Development of shorter term tests has evolved from the need to rapidly estimate toxicity, yet maintain reliability. In theory, because embryonic development is the most dynamic life stage, and because of the variety and number of receptor sites available for exposure during the sequential development of the embryo, it probably is also the most susceptible to toxic action (Birge et al. 1985). Results from full life cycle test have indicated that early life stages generally are more sensitive than other life stages (McKim 1977, Macek and Slight 1977). Evaluation of 56 full life-cycle tests revealed that 82 percent of the embryo-larval or early juvenile

MATC's were essentially identical to those estimated by chronic testing (McKim 1977).

The estimation of threshold toxicity from embryo-larval data requires three assumptions, 1) that the chemical does not exhibit significant cumulative toxicity, 2) that the effects of the chemical on fish is mediated through a single mechanism which manifests itself in a relatively short period of time, and 3) the effect is most severe on early life stage. These assumption seem to hold true for acid toxicity. Acid does not concentrate within the organism. One mechanism, failure of the ionoregulation, is the mode of mortality at environmentally important levels. Moreover, in acid toxicity tests, early life stages have been shown to be more sensitive than others, especially when exposure includes the embryonic period (Daye and Garside 1979, Fritz 1980, Kwain and Rose 1985, Johnson et al. 1987). MATC's derived from an embryo-larval testing are considered estimates, because not all developmental stages were exposed, and another life stage may be less tolerant (Benoit et al. 1982).

The short-term static-renewal procedure was most practical for use in this study, because it eliminates the need for exogenous feeding which can increase procedural difficulties, especially in field settings (Birge et al. 1985). In a static-renewal test, the treatment solutions are replaced periodically, usually once every 24 hours (ASTM 1980). Flow-through systems are preferred for toxicity testing, because they avoid the problems associated with the build up of metabolic wastes. However, Birge et al. (1985) demonstrated that LC50's and threshold levels (LC1's) could be determined with a high degree of precision and acceptable repeatability using static-renewal procedures. Flow-through systems use large volumes of water compared to static

renewal procedures. Reconstituted water was used in this study to minimize the volume of the test solution needed.

Study Sites

On-site and laboratory tests were incorporated in this research effort. Laboratory test were conducted in an environmental chamber in Cheatham Hall on the campus of Virginia Polytechnic Institute and State University. The on-site test was conducted at Flat Top Lake in Raleigh County, West Virginia. Flat Top Lake is a 94 hectar, mesoeutrophic, V-shaped impoundment (Figure 1). The riparian community consists of 370 lots, most of which have been developed into either permanent or seasonal homes. The lake is a source of drinking water and recreation (swimming, fishing, and boating) for the lake property owners. A summary of the fishes collected in July 1986 using a combination of gear types including trap nets, gill nets, and electroshocking is provided in Table 1 (unpublished data, Helfrich 1986). The dominant fish were bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), yellow perch (*Perca flavescens*). The adults of these species were reported to be tolerant of soft, acid waters (Harvey 1982). Acidifying conditions, increasing competition, a depressed forage base, and accelerating siltation were plausible explanations for the relative abundance of these species in Flat Top Lake (Helfrich and Orth 1988).

Water quality data collected at Flat Top Lake from 1983 to 1985 indicated that alkalinity was declining and iron and manganese were increasing (aluminum was not measured) (Sheehan and Leonard 1986). These observations lead the investigators

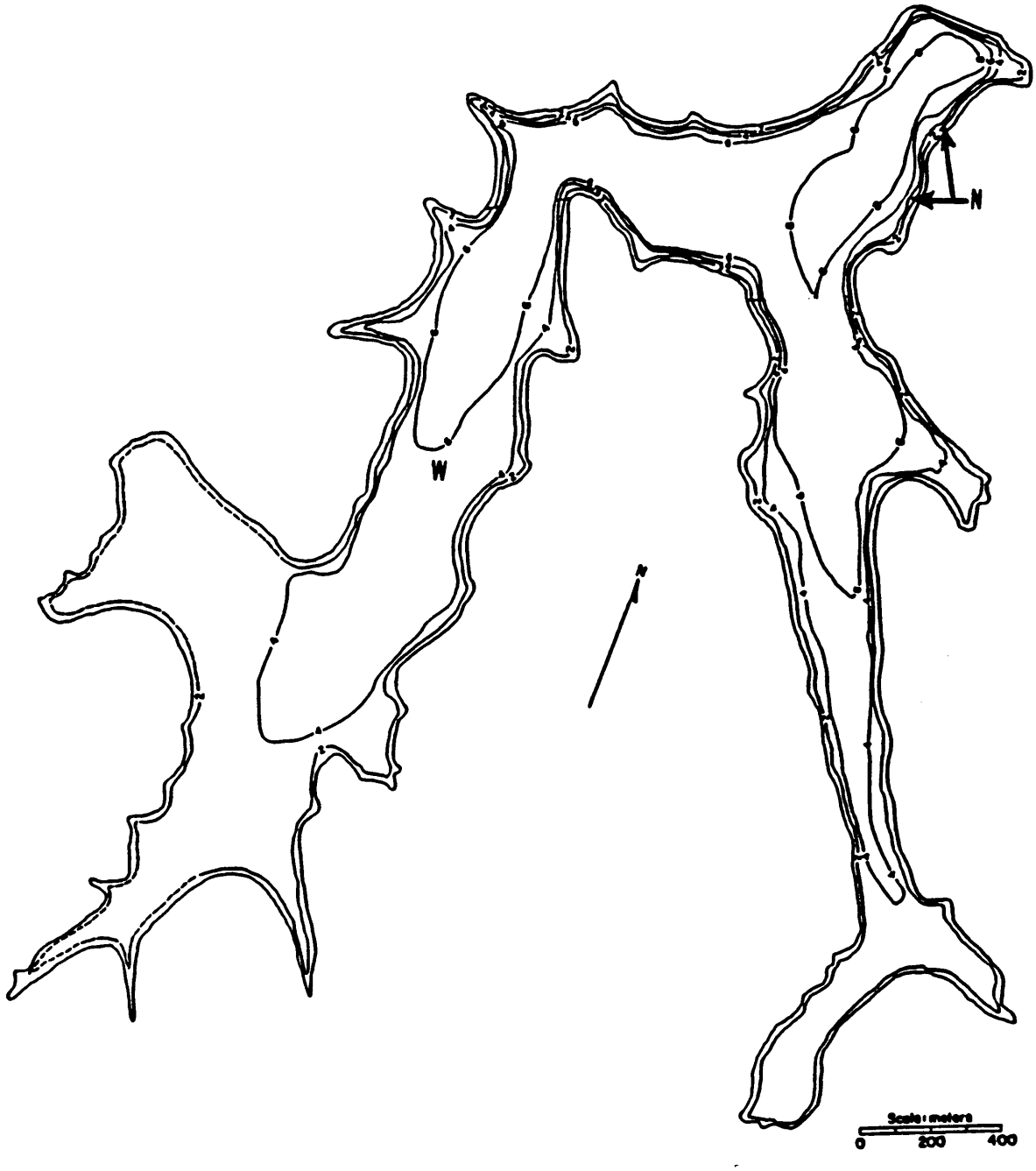

Figure 1. Location of the nests (N) for on-site and laboratory experiments, and the source of dilution water (W) for the on-site test at Flat Top Lake.

Table 1. Species composition of fishes in Flat Top Lake in July 1986. Total catch represents a composite of all gear types (trap nets, gill nets, and electroshocking) used in collections.

Species	Number	% of Total Catch
Bluegill	347	40.9
Largemouth bass	276	32.5
Yellow perch	121	14.3
Smallmouth bass	4	0.5
Black crappie	73	8.6
Green sunfish	12	1.4
Rainbow trout	1	0.1
Channel catfish	3	0.4
Black bullhead	2	0.2
Brown bullhead	4	0.5
White sucker	4	0.5
Redhorse	1	0.1
TOTAL	848	100.0

to suspect that the lake was acidifying. To avert further declines in water quality, 228,000 kg of calcium carbonate was added to the lake in July, 1987 (Helfrich and Orth 1988). The liming of Flat Top Lake was sponsored by 1) Living Lakes Corporation (a non-profit lake restoration organization), 2) the International Science and Technology Corporation (IS&T) and, 3) the Flat Top Lake owners association. The Flat Top Lake Association and the Living Lakes Corporation provided the funding for the liming and part of this research project. IS&T conducted water chemistry analysis, and supplied the calcium carbonate, and helped distribute the lime. Lime was added to the lake in the form of a slurry from a flat-bottomed boat.

In August 1987, post-treatment measurements indicated that water quality had improved. Elevated metal concentrations were no longer evident, although alkalinity and hardness were still low, 21 mg/L and 14 mg/L $CaCO_3$, respectively. Water quality characteristics in Flat Top Lake during the on-site experiment, August 12-19, 1987 are summarized in Table 2. Heavy metals were within EPA standard limits (Thurston et al. 1979). Sodium and chloride levels were elevated compared to other ions in Flat Top Lake, probably the result of runoff from a Highway Department salt storage site located upstream of the reservoir. These ions, however, did not appear to interfere with the results of the experiments. Physiological evidence suggests that calcium ions are solely responsible for fish responses to acidification (Graham and Wood 1981).

To increase the predictive ability of laboratory derived data, Leivestad (1982) recommended that acid toxicity experiments be conducted with fish that are adapted to water quality condition that typify acid sensitive environments. For this reason adult bluegill from Flat Top Lake were selected as the source of bluegill eggs.

Table 2. Water quality parameters (mg/l) measured mid-channel in the West arm of Flattop Lake during the on-site toxicity test 8-12 to 8-19-87. A less than symbol (<) indicates the parameter is below detection limits.

Parameter	Mean mg/L	Standard Deviation	Range mg/L	N
pH	7.60	...	7.67 - 7.46	8
Temp.(°C)	21.4	0.84	21.0 - 22.9	8
Alkalinity	14.25	0.00	14.25 - 14.25	3
Hardness	3.4	0.42	3.0 - 4.0	8
Calcium	7.49	0.14	7.40 - 7.65	3
Magnesium	2.26	0.05	2.22 - 2.32	3
Manganese	0.02	0.01	0.01 - 0.02	3
Phosphorus	<0.06	...	...	3
Potassium	0.92	0.04	0.88 - 0.95	3
Aluminum	<0.025	...	...	3
Boron	<0.006	...	...	3
Copper	<0.002	...	...	3
Iron	0.008	0.003	0.006 - 0.011	3
Sodium	28.95	0.244	28.67 - 29.12	3
Sulfur	2.08	0.035	2.04 - 2.10	3
Zinc	<0.004	...	...	3
chloride[a]	53.60	...	...	2
Acid neutralizing capacity (ueq/L)[a]	233.8	...	...	2
Dissolved inorganic carbon[a]	2.7	...	...	2
Dissolved organic carbon[a]	1.99	...	...	2
Conductivity (uS/cm)[a]	231.0	...	...	2

[a] International Science and Technology Corporation (unpublished data).

Experimental Treatments

Three types of dilution water were used in this study to establish water hardness treatments: 1) natural water (Flat Top Lake), 2) reconstituted soft water, 3) reconstituted hard water. Distilled water was reconstituted to either "very soft" or "hard" water by the addition of the appropriate quantities of ASC reagent-grade (Fisher Scientific Company) sodium, magnesium, calcium, and potassium salts as recommended by Marking and Dawson (1973) and ASTM (1980). These quantities are tabulated in Appendix A. The "very soft" water formula was representative of conditions in acid sensitive waters such as those found in Flat Top Lake. The term "soft" will be used to denote this treatment. The "hard" water formula was used to achieve significantly different treatment conditions for comparative purposes, and the term "hard" will be used to denote this treatment.

Two hardness levels were used in each laboratory experiment. Hardness levels averaged 197.8 mg/L and 164.7 mg/L $CaCO_3$ for the hard water treatment in experiment I and II, respectively; and 18.3 mg/L and 11.7 mg/L $CaCO_3$ for the soft water treatment in experiment I and II, respectively. Treatment solutions were held in 12, 100 liter plastic reservoirs. These reservoirs were filled to 80 liters with distilled water; six containers received reconstituted soft water and six received reconstituted hard water. Salts needed to establish hardness levels were weighed on a Mettler H45 analytical balance and then were added to reservoirs. To ensure uniform quality within each water hardness treatment, water for all six reservoirs was mixed by a siphon system until only "minimal detectable differences" in alkalinity and hardness occurred. Water quality characteristics were determined from samples

taken from each reservoir on the initial and final days of experiments I and II (Tables 3 and 4) Elemental concentrations were analyzed by the Virginia Tech Soil Sciences Laboratory using an inductively coupled plasma emission spectrophotometer (Jarrell ash model 9000). Alkalinity was determined by potentiometric titration with 0.02N H_2SO_4 for low alkalinity; and hardness was determined by titration with 0.02N EDTA using standard methods (APHA, 1975).

The hardness of Flat Top lake water during the on-site experiment averaged 3.4 mg/L $CaCO_3$ and was comparable to the soft water condition in laboratory experiments I and II. Surface water used in the on-site experiment was collected from mid-channel in the west arm of Flat Top Lake (Figure 1) because water quality was being monitored at this site by the IS&T in conjunction with base addition to the lake (Table 2). Water was collected in 15 liter plastic reservoirs 24 hours prior to use in the experiment. Water quality analysis was conducted on samples taken from the reservoirs on the initial and final day of the experiment. Alkalinity and hardness were determined in the field using standard methods as described for laboratory experiments (APHA 1975). Elemental analysis was determined from samples that were collected and placed on ice for transportation to the laboratory where they were frozen until processed by Virginia Tech Soil Sciences Laboratory.

In both field and laboratory experiments, concentrated sulfuric acid was used to achieve pH treatment levels. Many studies have indicated that atmospheric sulfur oxides and nitrogen oxides are the major causes of acid deposition, but most of precipitation acidity (60 percent to 70 percent) in northeastern United States originated from sulfate (Cogbill and Likens 1974, Canter 1986). Sulfuric acid was used

Table 3. Water quality parameters (mg/L) measured in dilution water for laboratory experiment I. A less than symbol (<) indicates that the parameter is below detection limits.

Parameter	Ca Level	Mean	Standard Deviation	Range	N
pH	High	7.90	...	7.80 - 8.1	12
	Low	7.00	...	6.9 - 7.0	12
Alkalinity	High	124.6	1.49	126.5 - 132.0	4
	Low	8.8	0.54	7.7 - 9.6	4
Hardness	High	197.8	6.7	186 - 210	11
	Low	18.3	2.0	15.0 - 20.0	12
Calcium	High	30.41	0.36	30.0 - 30.9	6
	Low	2.55	0.15	2.30 - 2.80	6
Sodium	High	58.60	0.99	57.0 - 60.0	6
	Low	3.71	0.09	3.60 - 3.80	6
Magnesium	High	26.75	0.29	26.30 - 27.20	6
	Low	1.66	0.02	1.60 - 1.70	6
Manganese	High	0.003	0.001	0.001 - 0.003	6
	Low	...	...	...	6
Phosphorus	High	<0.060	...	...	6
	Low	<0.060	...	...	6
Potassium	High	4.96	0.11	4.80 - 5.10	6
	Low	0.97	0.03	0.95 - 1.01	6
Aluminum	High	<0.025	...	...	6
	Low	<0.025	...	...	6
Boron	High	<0.006	...	...	6
	Low	<0.006	...	...	6
Copper	High	0.013	0.009	0.010 - 0.032	6
	Low	0.008	0.002	0.005 - 0.010	6
Iron	High	0.007	0.004	0.010 - 0.014	6
	Low	0.005	...	...	6
Sulfur	High	94.09[a]	0.83	93.3 - 95.2	5
	Low[a]	6.77	0.64	6.10 - 7.80	5
Zinc	High	0.015	0.008	0.011 - 0.030	6
	Low	0.012	0.004	0.010 - 0.020	6
Conductivity(uS/cm)	High	712	43.50	630 - 750	6
	Low	89.83	27.30	52 - 135	6

[a] Sulfer levels were 61.16 (mg/l) and 3.41 (mg/l) for control high and low, respectively.

Table 4. Water quality parameters (mg/l) measured in dilution water for laboratory experiment II. A less than symbol (<) indicates that the parameter is below detection limits.

Parameter	Ca Level	Mean	Standard Deviation	Range	N
pH	High	8.00	...	8.0 - 8.3	12
	Low	7.00	...	6.9 - 7.1	12
Alkalinity	High	110.9	4.0	109 - 120	7
	Low	7.5	0.6	7.0 - 8.4	7
Hardness	High	164.7	6.1	158 - 170	11
	Low	11.7	1.5	10.0 - 15.0	12
Calcium	High	28.02	0.52	27.2 - 28.6	6
	Low	1.78	0.05	1.70 - 1.80	6
Sodium	High	53.01	0.60	52.4 - 53.8	6
	Low	3.31	0.08	32.0 - 3.40	6
Magnesium	High	24.64	0.35	24.30 - 24.70	6
	Low	1.53	0.03	1.50 - 1.60	6
Manganese	High	<0.001	...	...	6
	Low	<0.001	...	...	6
Phosphorus	High	<0.060	...	...	3
	Low	<0.060	...	...	3
Potassium	High	4.71	0.18	4.50 - 5.00	6
	Low	0.54	0.07	0.46 - 0.58	6
Aluminum	High	<0.025	...	...	6
	Low	<0.025	...	...	6
Boron	High	<0.006	...	...	6
	Low	<0.006	...	...	6
Copper	High	0.004	0.001	0.0 - 0.007	6
	Low	0.005	0.002	0.003 - 0.008	6
Iron	High	0.027	0.026	0.0 - 0.090	6
	Low	<0.005	...	...	6
Sulfur	High[a]	92.00	3.08	87.9 - 97.9	5
	Low[a]	6.29	0.19	6.10 - 6.60	5
Zinc	High	0.011	0.003	0.009 - 0.020	6
	Low	0.007	0.002	0.005 - 0.010	6
Conductivity (uS/cm)	High	611	13.20	600 - 650	6
	Low	51.33	6.59	40 - 62	6

[a]Sulfur levels were 52.41 (mg/l) and 3.41 (mg/l) for control high and low calcium, respectively.

in these experiments because of its wide occurrence and dominant role in acidic deposition.

In laboratory experiment I, treatment pH levels for hard and soft water were fixed within the reservoirs at 4.6, 4.4, 4.2, 4.0, 3.8, the soft water control pH was 7.0 and the hard water control pH was 8.0. In experiment II, treatment pH levels for hard water were 8.0, 5.1, 4.9, 4.7, 4.5 and 4.3; soft water treatment levels were 7.0, 5.5, 5.25, 5.0, 4.75 and 4.5. Water was reconstituted, pH adjusted, and vigorously aerated for at least four days prior to the initiation of the experiments. This stabilization period allowed for the removal of acid-generated CO_2 and assisted in minimizing pH fluctuations during the test. Treatment solutions were renewed once every 24 hours and pH measurements were taken before and after each renewal. The addition of sulfuric acid affected the alkalinity and conductivity of the test solutions. The alkalinity (mg/L $CaCO_3$) of test solutions in laboratory I ranged from 0 to 9.6 in soft water and from 0 to 123 in hard water; conductivity (μS/cm) ranged from 52 to 135 in soft water and 630 to 750 in hard water. The alkalinity in laboratory test II ranged from 0.10 to 17.3 in soft water and 0 to 120 in hard water; conductivity ranged from 42 to 60 in soft water 600 to 650 in hard water.

In the on-site experiment, four pH levels (7.3, 5.5, 4.5 and 3.5) were established by acidifying Flat Top Lake water within the reservoirs 24 hours prior to use. The solutions were vigorously stirred to remove CO_2 produced as acid reacts with bicarbonate (Lloyd and Jordan 1964). The acid treatment levels fluctuated daily. A mean pH level for each treatment was calculated and used to discuss the results. The alkalinity (mg/L $CaCO_3$) of the field test treatment solutions ranged from 0 to 14.5.

Acid levels in all experiments were measured with a Fisher 825 MP pH meter using Fisher model 13-620-8 calomel reference electrode, and Fisher model 13-639-61 sleeve junction universal glass electrode. Calibration of the pH meter using a two-point calibration method was performed using Fisher certified buffer solutions pH 4.00 and 7.00. Meter calibration was checked frequently each day and recalibrated when necessary. The mean pH of the test solution in laboratory and field tests were based on the average of the 24 hour exposure levels, and calculated by converting all pH values to hydrogen ion normalities prior to averaging (Kinney 1973).

Toxicity Test Procedures

Bluegill eggs for the toxicity tests were collected from active nest using SCUBA in Flat Top Lake (Figure 1). Eggs for the field experiment were obtained on August 12, 1987 from two nests guarded by large (140 to 180 mm) male bluegill, approximately 20 meters off the north east shore in two meters of water. Though eggs from three spawns are considered ideal (ASTM 1980), only two nests with freshly fertilized eggs in an early developmental stage were located at Flat Top Lake on day one of the on-site field experiment. Eggs for laboratory experiments were collected on July 5, 1987 and July 16, 1987 for experiments I and II, (the initial day of the experiments), from three guarded nests. The nests were located three meters off the north east shore, near the dam in two meters of water (Figure 1).

The eggs were collected by extracting rocks with adhered eggs from nests and placing them in screened baskets (17 cm diameter PVC pipe with 0.05 mm nitex mesh fixed to one end). The screened baskets were placed in a cooler filled with aerated lake water for transportation to the field site, or to the laboratory at Virginia Polytechnic Institute and State University. The eggs were continually shielded from harmful ultra-violet radiation and kept submerged at all times. Initial attempts to transport eggs to the laboratory resulted in heavy fungal infections even though aeration was provided throughout the two hour trip. To reduce infections, the following fungicides were tested for their effects on egg survival, formalin (1 percent), malachite green (5 mg/L), and methylene blue (5 mg/L). The results indicated that methylene blue would control fungus without decreasing egg survival; and it is not a carcinogen as is malachite green. Therefore, 5 mg/L of methylene blue was added to the water prior to transportation of all eggs. Immediately upon arrival at the laboratory eggs were rinsed with fresh lake water.

In both laboratory and field experiments soft paint brushes (Prang #7) were used to gently separate eggs from rock nest substrate. Aeration was supplied by filtered compressed air during the removal process. Fertilized eggs from all spawns were pooled and mixed to ensure that eggs from different spawn were evenly dispersed. Eggs were microscopically examined to select only those fertilized ones that were developed to stage L, formation of the embryonic shield or less (Kim et al. 1987). Eggs used in experiment II were less developed than those used in Experiment I or the field experiment, most were in stage I, gastrulation (Kim et al. 1987), at the beginning of the test.

Laboratory tests were conducted using static-renewal procedures. The laboratory experimental design consisted of six pH treatments (each with two replicates) each at two levels of water hardness (hard and soft) for both experiment I and II. To facilitate monitoring egg and larval survival, 10 eggs each were held in 144, 2.5-liter plastic, cylindrical incubation chambers (7 cm in diameter x 7 cm in depth). Lids on the incubation chambers were fitted with nitex screening (mesh size 0.5 mm) to allow the test solution to circulate freely. Circulation was enhanced by aeration with filtered, compressed air supplied at nearly identical pressures to each of the treatment aquaria. Six incubation chambers (60 eggs) each were submerged in 24 rectangular, seven-liter, plastic aquaria filled to five liters with the treatment solution. The treatment solution in each aquaria was renewed at 24-hour intervals by draining all but 0.5 liters needed to keep the eggs and larvae wetted.

The 1,440 eggs collected from spawning fish in Flat Top Lake just prior to the initiation of the laboratory experiments were separated from the nesting substrate (as described above) and randomly partitioned, (two at a time), into petri dishes. In each petri dish, Flat Top Lake water diluted by 50 percent with either soft or hard reconstituted water was used to acclimate (four hours) the eggs to the ionic strength of the test solution prior to acid treatments. The experiment began with the introduction of the eggs to incubation chambers which were immersed in treatment solution in the test aquaria. Test aquaria were arranged in two blocks (laboratory benches) of twelve; treatment positions within blocks were randomly assigned.

Within 24 hours of the initiation of the test, all eggs with fungus were removed from the incubation chambers and excluded from population counts as recommended by Birge et al. (1985) and ASTM (1988). Dead eggs (discolored opaque and white ones)

and larvae (unresponsive to gentle prodding) were enumerated and removed every 24 hours. Hatching was determined by enumerating hatched live and dead larvae. Daily cumulative hatching rates were calculated as follows:

$$H\ \% = 100 \times \frac{((CDL + LL + CNTDL)}{(60 - FE))}$$

where (H) is the daily percent cumulative hatch, (CDL) is the cumulative number of dead larvae, (LL) is the cumulative number of live larvae, (CNTDL) is the number of larvae that died because they were caught in the lids of the incubation chambers, 60 is the original number of eggs per replicate and (FE) is the number of eggs that fungused.

The number of partially hatched larvae (those that do not completely escape the chorion) was determined visual every 24 hours. Cumulative mortality was considered to include all dead eggs, dead larvae and severely deformed larvae enumerated by the final day of the test. Physically damaged larvae were excluded from the population counts when mortality was calculated. Daily cumulative mortality was calculated as follows.

$$M\ \% = 100 \times \left[1 - \frac{((LL + NTDL)}{(60 - FE - CNTDL))} \right]$$

where (M) is the percent daily cumulative mortality, (NTDL) is the cumulative number of larvae physically damaged larvae up to but not including the examination day (larvae damaged by removing lids were considered live larvae until the next count).

Growth was determined only for experiment II because high mortalities in experiment I (as a function of the low pH treatments) resulted in a prohibitively small sample of live larvae at the termination of this test. Standard length was determined from a subsample of larvae ($n = 5$) on the first day of hatching and all live larvae at the termination of the experiment. Larvae used in the growth analysis were preserved in 10 percent formalin, and later (four months) measured. Measurements were obtained by projecting larvae images on to a 50.8 × 50.8 centimeter digitizing pad (Numonics Corp) with a Bauch and Lomb microprojector. The image of the anterior margin of the snout, the posterior margin of the notochord (standard length), and the posterior margin of the caudal fin (total length) (Snyder 1983) were digitized to the nearest millimeter (Revelation software program, Release G 5-13-85, Cosmos Inc., supplement developed by A.B. Jones III, personal communication). Image lengths were converted by stage micrometer to actual lengths. The projected image using this technique, had more clearly defined margins than did the microscopically magnified image used in the field experiment, allowing for more accurate measurements.

Larval yolk-sac volumes were examined for differential absorption rates as a function of pH. The maximum length and width of yolk-sacs of larvae alive at the termination of experiment II were measured using a microscope fitted with an occular micrometer. High mortality rates of larvae prevented yolk-sac volume analysis in experiment I. Yolk-sac volumes were calculated as follows:

$$V = \frac{4}{3}\pi AB^2$$

where (V) is the volume of a prolate spheriod, (A) is one half the length of the major axis, (B) is one half the length of the minor axis of the spheriod (Moriarity 1986).

Physical abnormalities were enumerated by examining preserved larvae that were alive at the termination of the test using a dissecting microscope 40X. Larvae from all treatments were examined on three separate occasions. The criteria used for identifying aberrant morphological features were determined in the first two examinations; the final examination was performed to enumerate physical aberrations. Fin erosion and eyes exhibiting exophthalmos (popeye) were difficult to determine on larval fish because they occurred as a gradation from "normal" (as determined by comparison with control larvae) to abnormal. Only clearly eroded fins and exophthalmos eyes were considered. Spinal deformities, mostly lordosis, also showed a graded response but were, in general, more easily observed.

Laboratory tests were conducted in an environmentally controlled room with a photoperiod of 16 hours of light, eight hours of dark. Room temperature was maintained at 26 C. Dissolved oxygen (YSI model 58 DO meter) and pH (as described above), were measured daily just prior to and after the daily renewal of test solution in each aquaria.

Field toxicity tests also were conducted using static-renewal procedures. The egg incubation chambers were similar to those designed by Runn et al. (1977), consisted of two cylinders of plastic (PVC) tubing (15 cm in diameter × 10 cm in depth), bound together with nylon straps. The cylindrical incubation chambers were fitted with nitex

screening (0.5 mm mesh) at each end to allow free flow of the test solution between the incubation chamber and the treatment aquaria. One incubation chamber each was suspended with nylon rope 15 cm below the waters surface in 12, 19-liter plastic aquaria filled to 15 liters with the treatment solutions. The treatment solution in each aquaria was renewed every 24 hours by draining all but 0.5 liters.

In the field test, 600 eggs (obtained as described above) were partitioned into twelve petri dishes (50 eggs/dish) filled with Flat Top Lake water. Each group of 50 eggs was then randomly assigned to one of the 12 treatment aquaria. The test began with the transfer of eggs and a minimal amount of Flat Top Lake water to the incubation chambers immersed within the test aquaria. Two sets of six aquaria were suspended (partially submerged) at the surface of Flat Top Lake by plywood and styrofoam floats. Aquaria positions within the floats were randomly assigned.

Dead eggs (discolored opaque and white ones) and larvae (unresponsive to prodding) were enumerated and removed every 24 hours until hatching was complete (two days), thereafter only one replicate per treatment was examined each day to reduce the time it took to renew the water within treatment aquaria. Hatching was determined by enumerating hatched larvae live or dead (unresponsive to prodding) and subtracting this number from the 50 original eggs. The number of partially hatched larvae (those that did not completely emerge from the chorion) were determined throughout the hatching period. Cumulative mortality included all dead eggs, and dead and severely deformed larvae enumerated by the final day of the test. Some bluegill larvae were physically injured due to their small size and the design of the egg baskets. These larvae were excluded from the total before mortality was calculated as recommended by Finney (1971). Growth was determined from a

subsample of 15 larvae (five from each treatment replicate) from each treatment on the first day of hatching and all live larvae at the termination of the test. Larvae used in the growth analysis were preserved in 70 percent alcohol, and later (1 month) measured with a dissecting microscope fitted with an occular micrometer.

Temperature and light conditions within the treatment aquaria fluctuated with the ambient conditions in the lake, although lids on the aquaria excluded rain and potentially harmful ultra-violet light. No aeration was provided to treatment aquaria, but openings located just below the lid provided air exchange. In each aquaria, temperature, dissolved oxygen (YSI model 58 DO meter), and pH (as described above) were measured daily prior to and after the renewal of the test solution.

Statistical Analysis

Statistical analyses of mortality, hatching, and partial hatching were conducted using a one-way analysis of variance (ANOVA) on arcsin-transformed data. If significant treatments effects were found cell means were compared using Dunnett's test (Miller 1981). All comparisons were made at $\alpha < 0.05$. Differences in hatching were between days were tested using a repeated measures two way (experiment I) or a one way (experiment II) analysis of variance on arcsine-transformed data (Milliken and Johnson 1985), followed by testing cell means using Duncan's test if significant treatment effects were found.

Probit analysis was used to calculate LC50 and LC1 values and the associated fiducial limits (Finney 1971). Parallel line analysis was used to to determine differences in the potency of acid in hard and soft water treatments (Finney 1971, Daum 1970). Differences in mortality between hard and soft water treatments were determined by examining 95 percent fiducial limits around the potency estimate, the non-inclusion of zero in the limits was indicative of a significant difference (Daum 1970). Differences in growth and yolk-sac absorption were determined using a one-way ANOVA for each water hardness treatment and significant treatment effects were compared using Tukey's test. Tukey's test is a conservative multiple comparison test appropriate for use when there are differences in the number of data points (n) constituting cell means. Mortality due to the treatments resulted in unequal sample sizes. All statistical analysis were conducted using the Stastical Analysis System (SAS 1985), except for Dunnett's tests which were made using a Hewlett and Packard 41CV calculator.

Results

Laboratory Experiment I

The mean hydrogen ion concentrations and ranges for the treatments in experiment I are shown in Table 5. The mean pH level was never greater than 0.06 pH units from the nominal value, and the average pH increase for a 24 hour period generally was low, about 0.024 pH units, although some were as high as 0.37 pH units. Levels of acid close to the limit of bicarbonate alkalinity (pH 5.0) showed greatest fluctuations because changes in CO_2 resulted in pH changes. The pH within the treatment aquaria usually increased during 24 hour periods between water renewal. The temperature for experiment I averaged 26.3 ± 2.1 C. Dissolved oxygen was 80 percent of saturation or higher in all treatment aquaria during the experiment.

Hatching Success

Cumulative hatching success of bluegill eggs was relatively high at most hardness and pH levels tested in experiment I (Table 5). Hatching began within the first 24 hours of the experiment and continued for approximately 96 hours; no hatching occurred after day four. In soft water, hatching success exceeded 87 percent for pH pH treatment levels at or above 4.2. At pH 4.0 hatching success declined ($P < 0.05$), averaging only 20 percent. At pH 3.8, hatching was minimal, averaging less than 1 percent. In hard water, cumulative hatching success was similar to that of soft water. Hatching success was greater than 82 percent in all treatments greater than or equal to pH 4.2. At pH 4.0, cumulative hatching success declined ($P < 0.05$), averaging 74.5 percent, but was greater ($P < 0.05$) than in soft water. Eggs incubated in pH 3.8 in hard water exhibited low hatching success, averaging 2.5 percent. In both hard and soft water, hatching was variable at pH levels near 4.0 and negligible below this pH. High water hardness appeared to promoted hatching at pH 4.0.

Hatching was affected substantially by low pH. A comparison of the cumulative hatching success on days one through four at each pH level and among pH levels on each of these days revealed that increasing acidity reduced and delayed hatching in both hard and soft water (Figure 2). The time to 80 percent egg hatching increased as pH decreased. An 80 percent cumulative hatching rate occurred on day one in pH's 7.0, 4.6, and 4.4, on day three in pH 4.2, and never occurred at pH's 4.0 and 3.8. In soft water, hatching success was was 76.6 percent at pH 7.0 and 67.4 percent at pH 4.6 on day one and increased ($P < 0.05$) to 96.4 percent and 97.0 percent, respectively on day two. All eggs either hatched or died by day two at these pH levels. Hatching success at pH 4.4 on day one was only 31.6 percent, but increased to 91.8 percent and

Table 5. Hatching success of bluegill eggs as a function of acid and water hardness levels in experiment I.

Soft Water					Hard Water				
Nominal pH	Mean pH	pH Range	Eggs Tested (n)	Eggs Hatching (%)	Nominal pH	Mean pH	pH Range	Eggs Tested (n)	Eggs Hatching (%)
7.00	6.99	6.87-7.14	55	96	8.00	7.90	7.80-8.06	55	98
7.00	7.01	6.87-7.19	56	97	8.00	7.90	7.80-8.10	54	98
4.60	4.66	4.57-4.97	52	96	4.60	4.67	4.57-5.13	51	100
4.60	4.64	4.58-4.77	47	98	4.60	4.67	4.59-4.82	52	96
4.40	4.43	4.38-4.51	52	92	4.40	4.43	4.37-4.58	57	95
4.40	4.42	4.38-4.51	46	100	4.40	4.41	4.37-4.48	50	98
4.20	4.22	4.19-4.32	46	97	4.20	4.22	4.18-4.33	49	88
4.20	4.21	4.19-4.26	51	88	4.20	4.21	4.18-4.26	53	83
4.00	4.03	3.99-4.17	41	36	4.00	4.01	3.89-4.09	54	80
4.00	4.02	3.99-4.06	49	6	4.00	4.01	3.99-4.06	42	69
3.80	3.81	3.80-3.86	55	0	3.80	3.86	3.78-3.87	42	5
3.80	3.79	3.80-3.78	56	2	3.80	3.82	3.79-3.85	38	0

95.9 percent on day two and three, respectively. Differences in hatching rates on days one, two, and three at this pH were significant ($P < 0.05$). All eggs either hatched or died by day four at pH 4.4. Hatching in pH 4.2 was a similar to that of 4.4. At pH 4.2 hatching success averaged 14.4 percent, 57.7 percent and 92.8 percent on days one, two and three, respectively. Differences in hatching success between all days were significant ($P < 0.05$). Hatching was complete by day three in pH 4.2. At pH 4.0, no hatching occurred on day one of the experiment, but hatching success averaged 7.8 percent, 17.8 percent and 20 percent on days two, three and four, respectively. Hatching success in pH 4.0 on day one and two was similar but was different ($P < 0.05$) from day three. Hatching success in pH 3.8 was 0.1 percent on the first day. No hatching occurred on subsequent days at this pH level.

In soft water, cumulative hatching rates varied among pH treatments within days (Figure 2). On day one, hatching rates at pH 7.0 and 4.6 were similar, but higher ($P < 0.05$) than those at all other pH levels. Cumulative hatching rates in pH 4.4 and pH 4.2 were similar, although those in pH 4.4 were higher ($P < 0.05$) than those at pH 3.8. On day two, pH 7.0, 4.6, and 4.4 all exhibited similar cumulative hatching rates, above 90 percent. Hatching rates at pH levels of 4.4 and greater was significantly higher than that of pH 4.2 and lower. Hatching rates in pH 4.0 and 3.8 were similar and significantly lower than all other pH levels. By day three, hatching in pH 7.0, 4.6, 4.4, and 4.2 was greater than 90 percent. Hatching rates at pH 4.0 and 3.8 remained lower ($P < 0.05$) than those at all other pH levels. Differences in hatching rates between pH levels on day four were indistinct from those of day three.

In hard water, patterns of cumulative hatching rates were similar to those in soft water (Figure 3). As in soft water, differences were apparent in hatching success

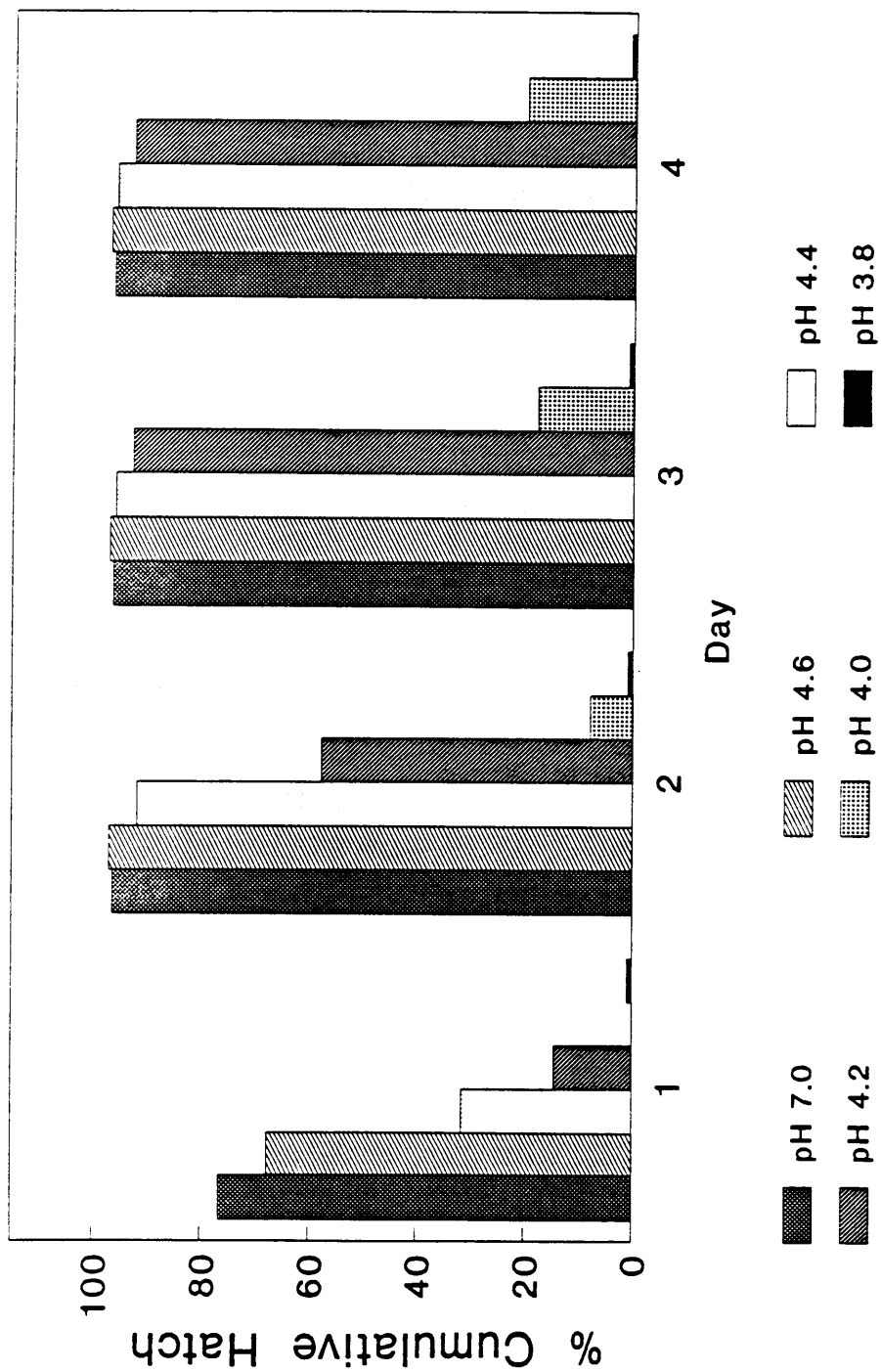

Figure 2. Cumulative hatching rates (%) of bluegill eggs in soft water for days one to four of laboratory experiment 1.

among days within pH levels and among pH levels within days. Delayed and reduced hatching success was apparent at low pH. The time to 80 percent egg hatching increased as pH decreased. An 80 percent cumulative hatching rate occurred on day one in pH's 8.0 and 4.6, on day two at 4.4, on day three in pH 4.2, and never occurred at pH's 4.0 and 3.8. Hatching success on day one was 93.6 percent and 87.4 percent for pH 8.0 and 4.6, respectively. On day two, the hatching rate was greater ($P < 0.05$) than on day one, averaging 98.2 percent and 98.1 percent for pH 8.0 and 4.6, respectively; no further hatching occurred after day two at these pH levels. Hatching success at pH 4.4 was 47.7 percent on day one and was greater ($P < 0.05$) than on day two, averaging 94.4 percent. No further hatching occurred at this pH. Hatching success in pH 4.2 on day one was 22.5 percent, 63.7 percent on day two, 83.3 percent on day three, and 85.3 percent on day four. Hatching success one, two, and three differed ($P < 0.05$). At pH 4.0, hatching success on days one and two were similar averaging 15.6 percent and 52.1 percent, respectively. On day three, hatching increased ($P < 0.05$) to 71.9 percent. On day four, hatching success was indistinct ($P > 0.05$) from that on day three, averaging 72.9 percent. No hatching occurred at pH 3.8 during the first two days, and only two eggs hatched by day four.

In hard water, differences ($P < 0.05$) in cumulative hatching success occurred among pH levels within days (Figure 3). On day one, hatching success in pH 8.0 was similar to that at 4.6, and these treatment levels were higher ($P < 0.05$) from all other pH levels. At pH 4.4, hatching was significantly higher than that of the others tested (pH 4.2, 4.0, and 3.8). Hatching success in pH 4.2, 4.0 and 3.8 was not different ($P > 0.05$). On day two, hatching success at 8.0, 4.6, and 4.4 were similar, and higher ($P < 0.05$) than those at all other pH levels. In pH 4.2, hatching success was higher ($P < 0.05$) than at pH's 4.0 and 3.8. On day three, hatching rates in pH 8.0, 4.6, and 4.4 were

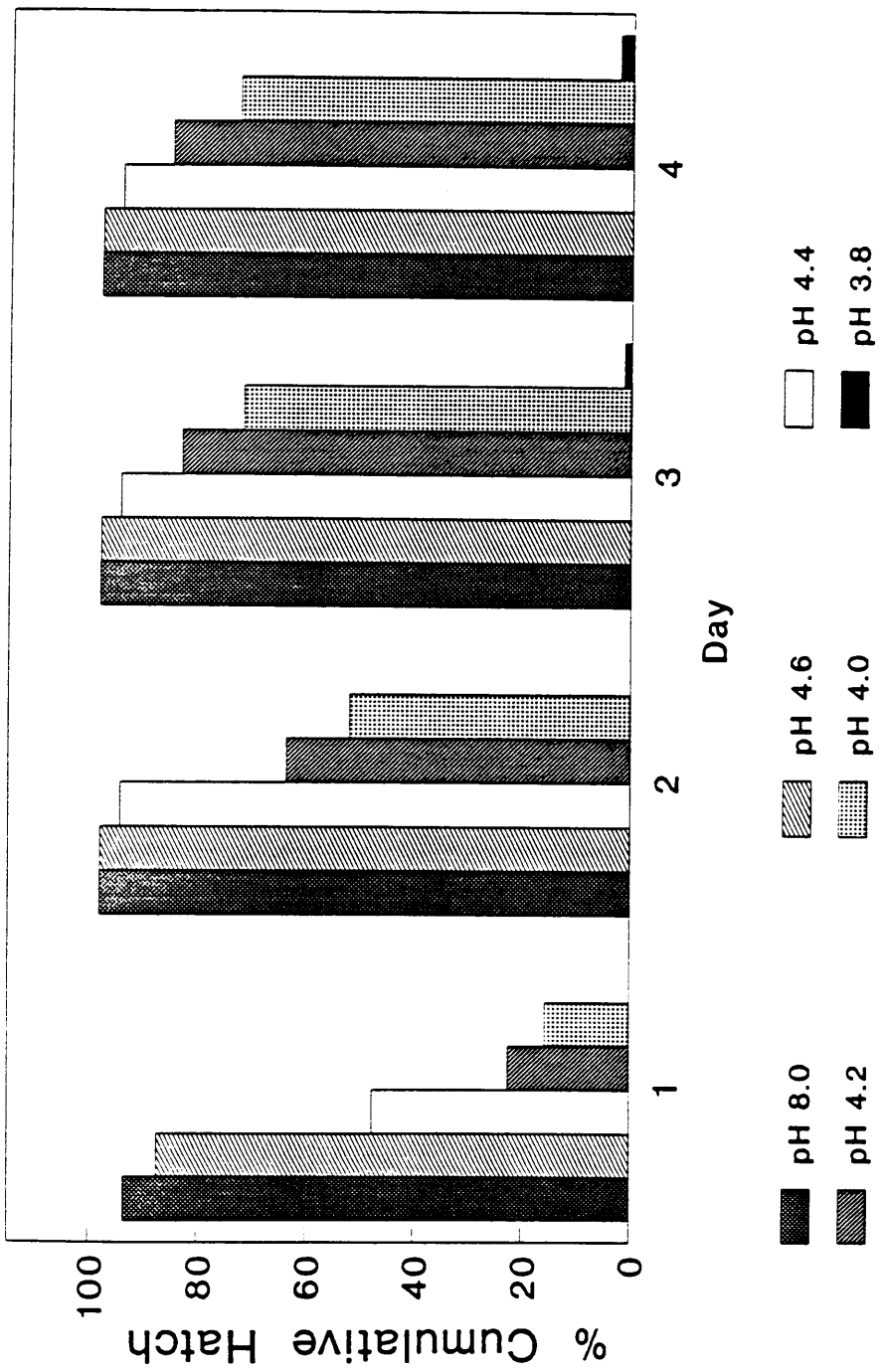

Figure 3. Cumulative hatching rates (%) of bluegill eggs in hard water on days one to four of laboratory experiment 1.

higher than all other treatment levels. At pH 4.2 and 4.0, hatching success was similar on day three, and had increased ($P < 0.05$) from day two. At pH 3.8 cumulative hatching success was lower ($P < 0.05$) than all other pH levels for all days.

Total cumulative hatching success in experiment I in hard and soft water within each day (one through four) is shown in Figure 4. On day one, hatching rates were higher ($P < 0.05$) in the hard water control (pH 8.0), than in the soft water control (pH 7.0). In pH 4.6 hatching success was higher ($P < 0.05$) in hard water than soft water. On day one, at treatment levels below pH 4.6 differences in hatching success between hard and soft water were indistinguishable. On day two, no differences ($P > 0.05$) in cumulative hatching success between hard and soft water at any pH level were observed. On days three and four, the percentage of cumulative hatched eggs in pH 4.0 was greater ($P < 0.05$) in hard water than in soft water.

Partial Hatching

Partial hatching occurred in both hard and soft water treatments at all pH levels except 3.8 during experiment I (Table 6). A large proportion of larvae (44 percent combining hard and soft water) attempting to hatch at low pH levels (≤ 4.4) were unable to exit the chorion completely. The head and yolk-sac of larvae remained encapsulated, only the tail was liberated. In soft water, partial hatching was minimal, averaging one and four percent at pH 7.0 and 4.6, respectively. A higher ($P < 0.05$) number of partially hatched larvae occurred at pH 4.4, 4.2 and 4.0 than at other pH treatments. The highest rates of partial hatching occurred in soft water at pH's 4.2 and 4.0 (52 and 32 percent, respectively). The trend in hard water was the same as

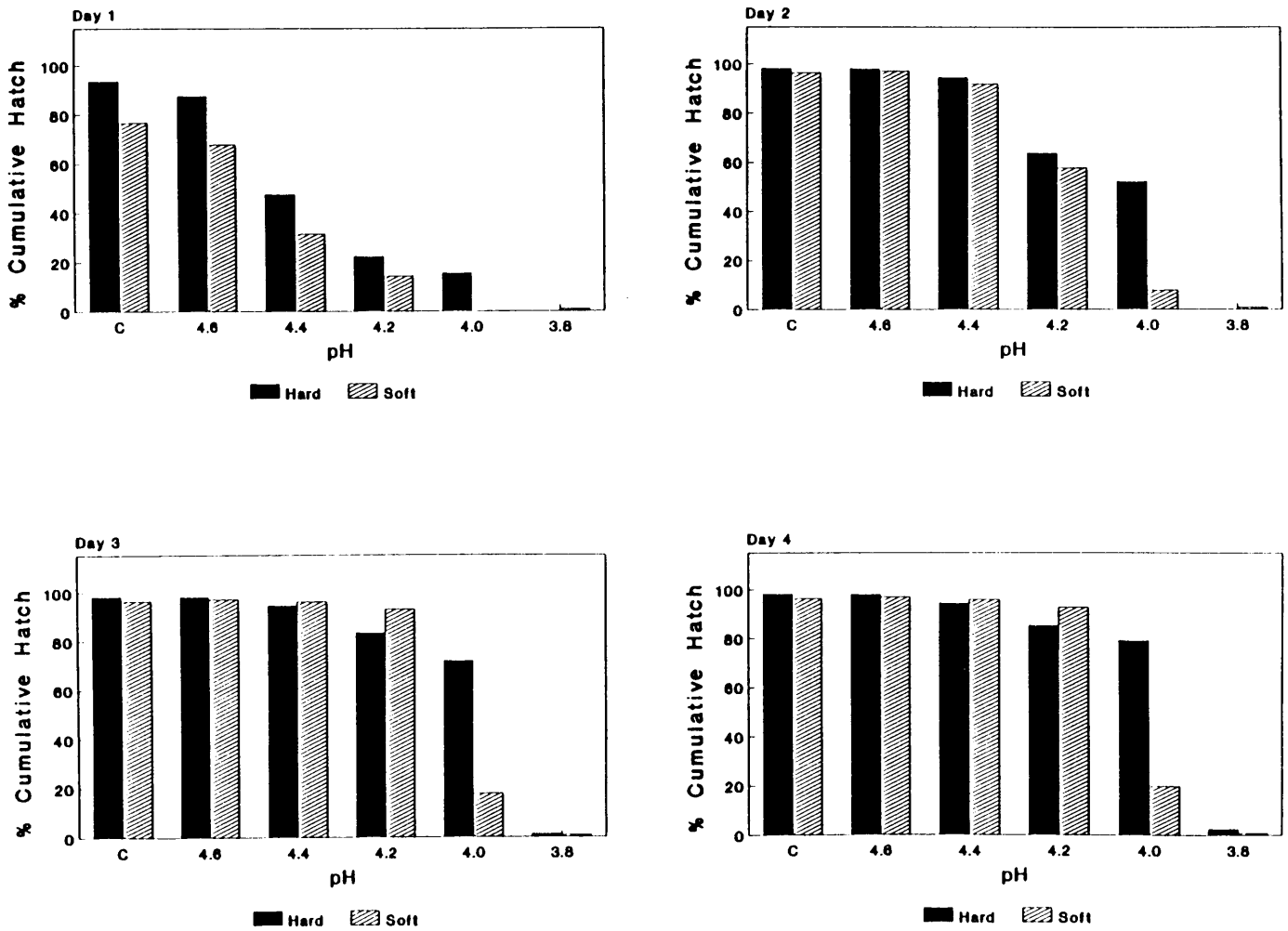

Figure 4. Cumulative hatching rates (%) of bluegill eggs in hard and soft water for days one to four in laboratory experiment I.

in soft water. Partial hatching was negatively correlated with pH except for pH 3.8. Low levels of partial hatching at pH 3.8 were the result of high egg mortalities at both hardness levels. In general, low pH levels tended to delay hatching (≤4.4), reduce hatching success (≤4.0), and increase the incidence of partial hatching (≤4.6).

Laboratory Experiment II

In experiment II, as in experiment I, pH levels within the treatment aquaria usually increased during the 24 hour periods between water renewal, but the increases were less pronounced than in experiment I. The maximum range and mean hydrogen ion concentrations in experiment II are presented in Table 7. The mean pH level was never greater than 0.04 pH units from the nominal value. The temperature for experiment II averaged 26.7 ± 1.7 C. Dissolved oxygen was always greater than 80 percent of saturation in the treatment chambers during the experiment.

Hatching success

Hatching in experiment II began within the first 24 hours and was completed in 96 hours in all treatments. No differences ($P > 0.05$) in hatching success among any of the pH treatment levels were detected (Table 7). In experiment II, the range of pH levels were chosen to obtain a greater number of treatments exhibiting partial embryo-larval mortality, which are essential for accurate LC50 estimates. Differences in cumulative hatching success in experiment II were not expected since all acid

Table 6. The percent of partially hatched bluegill larvae observed on day two of laboratory experiment I.

Soft Water			Hard Water		
Nominal pH	Eggs Tested (n)	Partially Hatched (%)	Nominal pH	Eggs Tested (n)	Partially Hatched (%)
7.00	111	0.9[a]	8.00	109	0.9[a]
4.60	99	4.1[b]	4.60	103	10.7[b]
4.40	98	19.4[c]	4.40	107	48.6[c]
4.20	97	52.6[d]	4.20	102	64.7[d]
4.00	90	32.2[e]	4.00	96	43.6[c]
3.80	111	0.0[a]	3.80	81	3.7[ab]

Treatment comparisons in columns with different superscripts are significantly different at ($p < 0.05$).

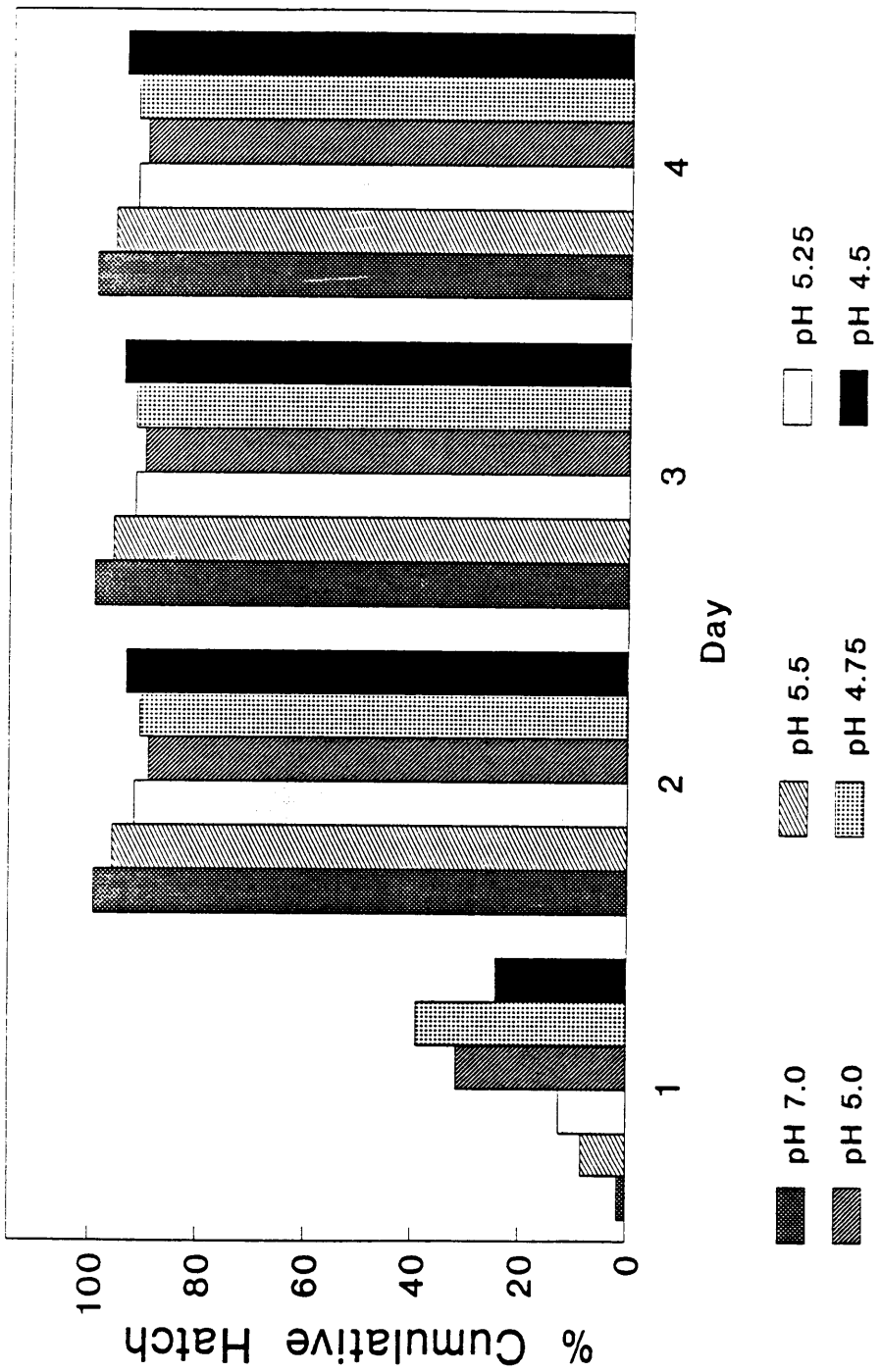

Figure 5. Cumulative hatching rates (%) of bluegill eggs in soft water for days one to four of laboratory experiment II.

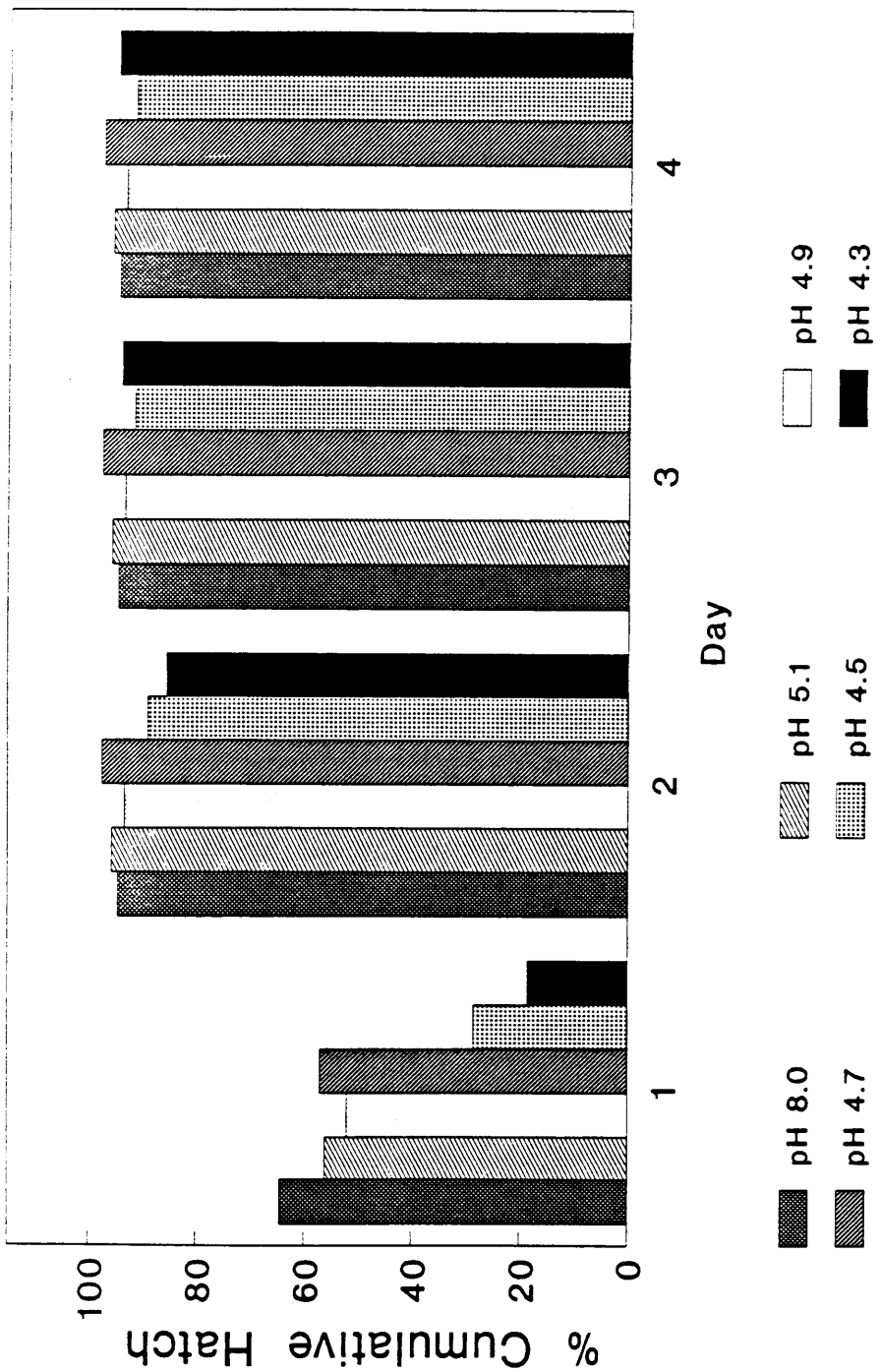

Figure 6. Cumulative hatching rates (%) of bluegill eggs in hard water for days one to four of laboratory experiment II.

treatment levels exceeded pH 4.0, the pH level found to decrease hatching success in experiment I.

Cumulative hatching success was similar ($P > 0.05$) at all pH and hardness levels (Figures 5 and 6). Hatching in all treatments was below 50 percent on day one, but increased to 80 percent, and then above 90 percent on days two and three, respectively. A comparison of cumulative hatching success among days within pH levels and among pH levels within days revealed that the treatment pH levels did not reduce hatching success or delay hatching in either hard and soft water (Figure 5 and 6).

Partial Hatching

Partial hatching rates were minimal at all pH and hardness levels (Table 8). In soft water, the lowest pH (4.5) tested exhibited the highest percentage of partially hatching larvae (1.7%). In experiment I, partial hatching increased from 4.1 percent to 19.4 percent at pH's 4.6 and 4.4, respectively, and corresponded to the results found in experiment II. The range of pH levels that caused high levels of partial hatching in experiment I did not cause them in experiment II. In experiment I at pH levels 4.2 and 4.4, partial hatching incidence was 64.7 percent and 48.6 percent, respectively. In contrast, at pH levels 4.3 and 4.5 in experiment II partial hatching only was 2.5 percent and 8.5 percent, respectively. The observed differences in partial hatching between experiment I and I in hard water was perhaps a function of the lengthy interval (24 hours) between observations. Greater partial hatching may have occurred, but was not observed.

Table 7. Hatching success of bluegill eggs as a function of acid and water hardness levels in experiment II.

Soft Water					Hard Water				
Nominal pH	Mean pH	pH Range	Eggs Tested (n)	Eggs Hatching (%)	Nominal pH	Mean pH	pH Range	Eggs Tested (n)	Eggs Hatching (%)
7.00	7.03	6.94-7.18	60	98	8.00	7.99	7.94-8.08	60	92
7.00	6.99	6.90-7.14	59	100	8.00	7.99	7.94-8.11	50	98
5.50	5.54	5.42-5.78	60	98	5.10	5.09	5.05-5.17	58	91
5.50	5.49	5.39-5.70	60	98	5.10	5.06	5.04-5.13	56	94
5.25	5.25	5.19-5.40	60	88	4.90	4.88	4.85-4.95	60	99
5.25	5.24	5.19-5.36	60	95	4.90	4.88	4.85-4.93	59	88
5.00	4.98	4.94-5.09	60	92	4.70	4.69	4.66-4.76	60	100
5.00	4.98	4.94-5.07	60	87	4.70	4.69	4.66-4.73	59	94
4.75	4.74	4.71-4.80	60	92	4.50	4.48	4.46-4.52	59	95
4.75	4.74	4.72-4.79	60	90	4.50	4.48	4.46-4.50	60	88
4.50	4.48	4.45-4.51	60	95	4.30	4.29	4.27-4.32	60	92
4.50	4.48	4.48-4.51	59	93	4.30	4.29	4.27 4.31	58	98

Table 8. The percent of partially hatched bluegill larvae observed on day two of laboratory experiment II.

Soft Water			Hard Water		
Nominal pH	Eggs Tested (n)	Partially Hatched (%)	Nominal pH	Eggs Tested (n)	Partially Hatched (%)
7.00	119	0.0	8.00	110	0.0
5.50	120	0.0	5.10	114	0.9
5.25	120	0.0	4.90	119	0.0
5.00	120	0.8	4.70	119	0.0
4.75	120	0.0	4.50	119	2.5
4.50	120	1.7	4.30	118	8.5

Embryo-larval Mortality

In experiment I, embryo-larval mortality was high (> 62 percent) in the soft water treatment all pH levels tested (≤4.6), excluding the control level, pH 7.0 (Table 9). At pH 4.6, mortality averaged 65.2 percent. At the next lower level, pH 4.4, mortality was 95.3 percent, at lower pH levels mortality was 100 percent. Embryo-larval mortality at all pH levels tested was greater ($P < 0.05$) than that found in controls. In contrast, embryo-mortality in the hard water treatment was low (averaging ≤ 31 percent) at pH levels greater than 4.0, and did not reach 100 percent until pH 3.8 (Table 9). At pH 4.0 the average embryo-larval mortality was 60 percent. Differences in mortality at treatment pH levels compared to the controls occurred only at pH 4.0 and 3.8 ($P < 0.05$). Complete (100 percent) mortality in hard water only occurred at the extreme pH of 3.8.

In experiment II lower hydrogen ion concentrations were used to increase the number of treatments exhibiting mortality rates less that 100 percent so that an LC50 could be estimated for soft water conditions. Mortality was considerably lower than in experiment I at all treatment pH and hardness levels and no pH level produced complete (100 percent) mortality (Table 10). Embryo-larval mortality in soft water was not different ($P < 0.05$) control levels until the acid level reached pH 4.5, where mortality averaged 77 percent. In hard water, embryo-larval mortality did not differ ($P > 0.05$) from that of the controls at any pH treatment level. In both hard and soft water, only small reductions in survival on days one to four, at pH levels greater than 4.2 were observed; the greatest declines in survival occurred on days five and six (Figures 7 and 8).

Table 9. Mortality of bluegill eggs and larvae as a function of acid and water hardness levels in experiment I.

Soft Water					Hard Water				
Nominal pH	Mean pH	pH Range	Egg + Larvae Tested (n)	Egg + Larvae Mortality (%)	Nominal pH	Mean pH	pH Range	Egg + Larvae Tested (n)	Egg + Larvae Mortality (%)
7.00	6.99	6.87-7.14	42	12	8.00	7.90	7.80-8.06	39	10
7.00	7.01	6.87-7.19	47	5	8.00	7.90	7.80-8.10	47	0
4.60	4.66	4.57-4.97	46	67	4.60	4.67	4.57-5.13	36	0
4.60	4.64	4.58-4.77	43	63	4.60	4.67	4.59-4.82	46	10
4.40	4.43	4.38-4.51	47	100	4.40	4.43	4.37-4.58	46	20
4.40	4.42	4.38-4.51	38	92	4.40	4.41	4.37-4.48	31	30
4.20	4.22	4.19-4.32	42	100	4.20	4.22	4.18-4.33	44	20
4.20	4.21	4.19-4.26	44	100	4.20	4.21	4.18-4.26	48	40
4.00	4.03	3.99-4.17	38	100	4.00	4.01	3.89-4.09	50	50
4.00	4.02	3.99-4.06	49	100	4.00	4.01	3.99-4.06	40	70
3.80	3.81	3.80-3.86	55	100	3.80	3.86	3.78-3.87	42	100
3.80	3.79	3.79-3.80	56	100	3.80	3.82	3.79-3.85	38	100

Table 10. Mortality of bluegill eggs and larvae as a function of acid and water hardness levels in experiment II.

Soft Water					Hard Water				
Nominal pH	Mean pH	pH Range	Egg + Larvae Tested (n)	Egg + Larvae Mortality (%)	Nominal pH	Mean pH	pH Range	Egg + Larvae Tested (n)	Egg + Larvae Mortality (%)
7.00	7.03	6.94-7.18	54	2	8.00	7.99	7.94-8.08	44	14
7.00	6.99	6.90-7.14	37	3	8.00	7.99	7.94-8.11	44	3
5.50	5.54	5.42-5.78	--[a]	--[a]	5.10	5.09	5.05-5.17	45	14
5.50	5.49	5.39-5.70	60	2	5.10	5.06	5.04-5.13	42	8
5.25	5.25	5.19-5.40	48	17	4.90	4.88	4.85-4.95	48	9
5.25	5.24	5.19-5.36	53	8	4.90	4.88	4.85-4.93	53	14
5.00	4.98	4.94-5.09	39	36	4.70	4.69	4.66-4.76	48	0
5.00	4.98	4.94-5.07	46	22	4.70	4.69	4.66-4.73	45	12
4.75	4.74	4.71-4.80	54	19	4.50	4.48	4.46-4.52	54	7
4.75	4.74	4.72-4.79	51	27	4.50	4.48	4.46-4.50	51	16
4.50	4.48	4.45-4.51	44	80	4.30	4.29	4.27-4.32	45	18
4.50	4.48	4.48-4.51	52	74	4.30	4.29	4.27 4.31	52	5

[a] pH was not maintained at the treatment level.

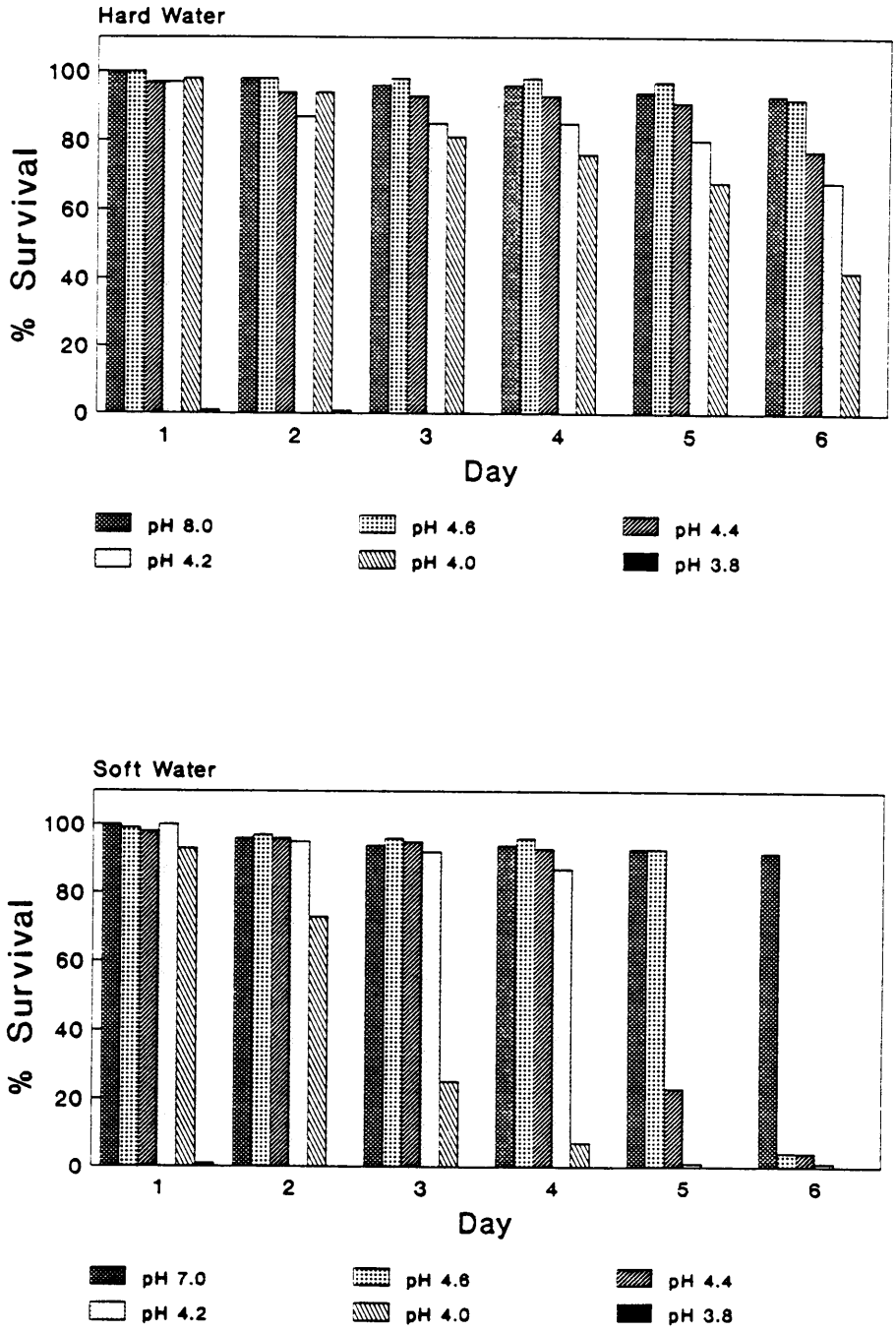

Figure 7. Cumulative survival in laboratory experiment I in hard and soft water conditions.

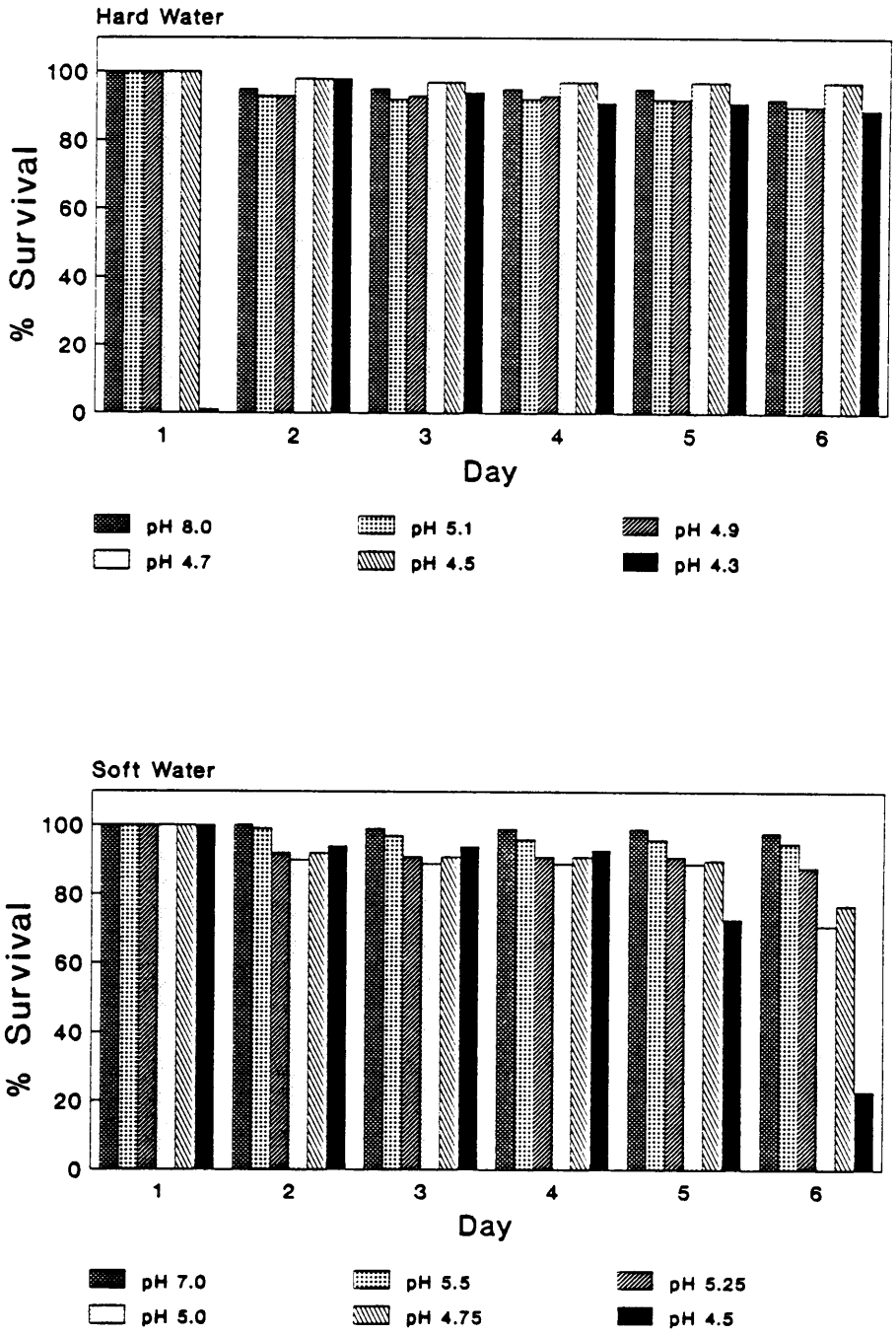

Figure 8. Cumulative survival in laboratory experiment II in hard and soft water conditions.

The LC50 for hard water was calculated using the data collected in experiment I. The LC50 and associated fiducial limits were: pH 4.06 (3.95, 4.13). Data were pooled from experiments I and II (excluding treatments that exhibited 100 percent mortality) to estimate the LC50 in soft water. Pooling the data resulted in a minimal change in the LC50 estimate (0.03 pH units), and a reduction in the range of the fiducial limits. The LC50 and associated fiducial limits for soft water were: pH 4.67 (4.49, 4.80).

The potency analysis revealed that the slopes of the probit regression lines for hard and soft water treatments were -2.337 and -2.248, respectively and that they were not different ($P < 0.05$) from one another (Figure 9). The pooled slope (-2.344) was, therefore, used to estimate the potency (and associated fiducial limits) of acid to embryo-larval bluegill in hard water relative to soft water, and was 0.62 (0.46, 0.81) pH units. Mortality rates in hard equivalent to soft water require a solution that is 0.62 pH units more acidic. A change of 0.62 pH units represents a tripling in the hydrogen ion activity. Because the fiducial limits for the estimate of potency did not included zero, the LC50 estimates in hard and soft water were considered to be different ($P < 0.05$) from one another (Daum 1970).

The LC1 values provide an estimate of the MATC for bluegill in hard and soft waters. The LC1 and associated fiducial limits for soft water were pH 5.66 (6.50, 5.35) and is the estimated MATC. Various other test parameters support the the use of the LC1 estimates for indicating maximum acceptable levels. A decline in yolk-sac volume occurred pH levels below 5.5 and abnormal behavior (reduced swimming ability) was observed at pH 5.5 and below. These observations suggest that bluegill populations in soft water may be adversely affected by acid levels below pH 5.7.

In hard water, the LC1 and associated fiducial limits were 5.04 (5.38, 4.48) and is the estimated MATC for bluegill in hard water. The yolk-sac volume of the larvae declined at pH levels below pH 5.1 and abnormal swimming behavior was observed in larvae held at pH 5.10 and below. This evidence suggests that in hard water, bluegill population will be affected by acid levels below pH 5.0.

Growth and Yolk-Sac Analysis

High mortality of larvae in experiment I prevented growth analysis. In laboratory experiment II, analysis of larval growth over four days was not pH related (Table 11). Growth in the hard water treatment at pH 4.3, appeared to be reduced but variances were large and no statistical differences among acid treatments was found. High mortality observed in treatments below pH 5.25 in soft water (Table 10) may have resulted in mortality of smaller, larvae since only those alive at the termination of the test were measured.

The mean yolk-sac volume of larvae declined as pH declined at both water hardness levels (Table 12). Larvae held in soft water showed a decline ($P < 0.05$) in yolk-sac volume below pH 5.5, whereas a comparable decline in yolk-sac volume was evident only below pH 5.1 in hard water.

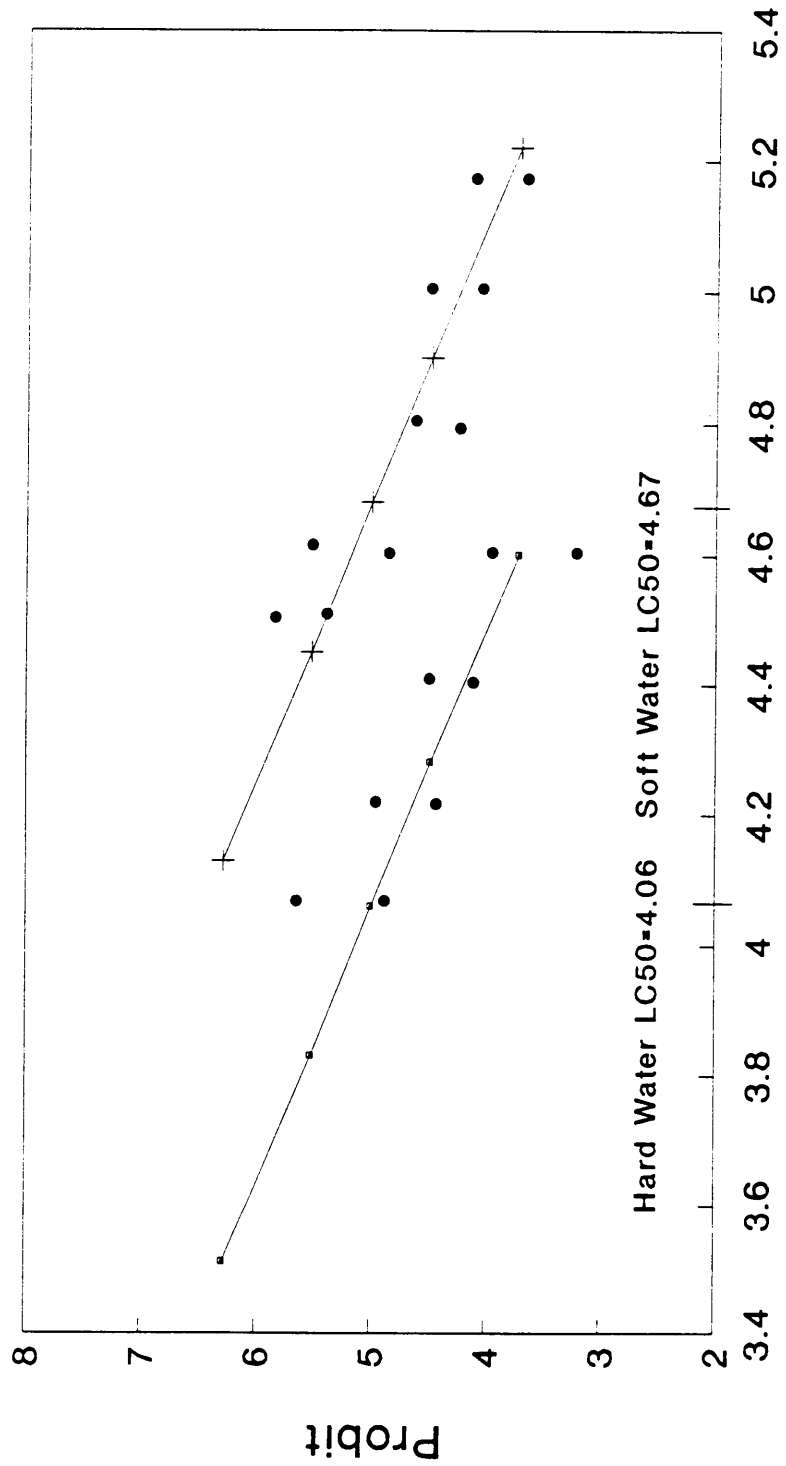

Figure 9. Probit regression analysis of mortality in hard and soft water conditions.

Table 11. Mean standard length (mm) of bluegill larvae on days one and five of experiment laboratory II and growth (mm) for four days as a function of acid and water hardness levels. Standard deviations are shown in parentheses.

Soft Water						Hard Water					
Nominal pH	Mean(SD) Initial length (mm)	N	Mean(SD) Final length (mm)	N	Growth (mm)	Nominal pH	Mean(SD) Initial length (mm)	N	Mean(SD) Final length (mm)	N	Growth (mm)
7.00	4.05(0.14)	6	5.35(0.26)	34	1.30	8.00	3.96(0.10)	9	5.35(0.22)	64	1.39
5.50	3.99(0.11)	7	5.29(0.27)	21	1.30	5.10	4.03(0.10)	10	5.35(0.26)	70	1.32
5.25	4.06(0.09)	10	5.33(0.25)	27	1.27	4.90	4.02(0.16)	10	5.27(0.23)	86	1.25
5.10	4.08(0.10)	9	5.22(0.25)	46	1.14	4.70	4.02(0.07)	10	5.41(0.26)	82	1.39
4.75	4.07(0.08)	9	5.34(0.22)	60	1.27	4.50	3.97(0.09)	10	5.37(0.26)	94	1.40
4.50	3.96(0.12)	10	5.32(0.20)	18	1.36	4.30	4.13(0.14)	9	5.33(0.26)	74	1.20

Table 12. Mean yolk sac volume of larvae on day five of laboratory experiment II. Standard deviations are shown in parentheses.

Soft Water			Hard Water		
Nominal pH	Yolk Sac (n)	Mean(SD) Volume (μm^3)	Nominal pH	Yolk Sac (n)	Mean(SD) Volume (μm^3)
7.00	24	5.7(3.5)[a]	8.00	67	9.1(5.3)[a]
5.50	14	3.8(3.1)[a]	5.10	72	7.3(4.7)[a]
5.25	25	2.1(1.5)[b]	4.90	83	4.2(3.2)[b]
5.00	53	2.3(2.3)[b]	4.70	83	4.1(3.1)[b]
4.75	54	2.9(2.3)[b]	4.50	89	4.4(3.8)[b]
4.50	10	1.3(0.4)[b]	4.30	68	2.8(2.1)[b]

Treatment comparisons in columns with different superscripts are significantly different at ($p < 0.05$).

Behavior and Physical Aberrations

Qualitative differences in swimming activity and behavior were observed for larvae in treated and control aquaria. Throughout days one through four larvae in all pH and hardness treatments, and the controls moved only when disturbed. When disturbed, larval swimming movements were erratic and without orientation; they were not capable of changing their direction. The movements were unidirectional in a curved path. Swimming speed gradually increased then stopped abruptly. Depth distribution and swimming was largely benthic, a few millimeters from the bottom of the aquaria.

By day four, larvae in control aquaria were distributed well off the bottom and active without stimulus. Their movements seemed directed and controlled, and they altered speed and direction frequently and smoothly. Larvae at low pH's ($\leq$5.5) also were swimming throughout the aquaria even when undisturbed. While the swimming movements of the larvae were more directed than on previous days, they remained erratic and halting. Swimming speed of the larvae in treatment aquaria appeared to be slower and distances traveled shorter than those in control aquaria. At the termination of the test, larvae in treatment aquaria ($\leq$5.5) were more lethargic and were much easier to capture at both water hardness levels.

Physical abnormalities were found upon microscopic examination (40X) of the larvae after they were preserved in buffered formalin (10 percent). Three main types of deformities were observed: 1) spinal curvatures mostly lordosis but some scoliosis, 2) fin erosion, as determined by dorsal fin size and uneven fin margins, 3) exophthalmos. In general, pH was inversely related to physical aberrations at both hardness levels (Table 13). Higher occurrences of all three types of aberrations were

observed in larvae held in soft water than hard water at pH 4.5. Exophthalmos consistently increased as pH decreased for larvae tested in hard water.

Field Toxicity Tests

In all treatment aquaria, the mean pH observed for 24 hour periods was consistently higher than the nominal pH (the pH acid level of reservoir water used to refresh treatment solutions), however, variation among replicates was low (Table 14). The pH levels fluctuated daily, especially at a nominal pH of 5.5. This treatment level was at the threshold of bicarbonate alkalinity, and changes in the CO_2 concentration in the water altered pH. The hardness level in the renewal water averaged 3.4 $\pm$0.42 mg/L $CaCO_3$. Lake water temperature during the seven day experiment ranged from 24.3C to 26.9C; temperatures in the treatment aquaria ranged from 20.4 C to 26.9 C. The greater temperature fluctuations in treatment aquaria occurred as a result of the exposure of renewal (reservoir) water to air temperatures for 24 hours (to allow stabilization after acid addition) prior to use in the experiment. Dissolved oxygen in the experimental units was never below 90 percent of saturation.

Hatching of bluegill eggs began during the first 24 hours of the experiment and was completed in all aquaria 72 hours after the test began (Figure 9). On day one, different ($P < 0.05$) hatching rates occurred at all pH treatment levels. Hatching decreased with pH averaging 88 percent in pH 7.3, 75 percent in pH 5.7, 35 percent in pH 4.6, and 0.67 percent in pH 3.5 (Figure 9). On day two, cumulative hatching rates at pH 3.5 (0.6 percent) and 4.6 (88.9 percent) were different ($P < 0.05$) from pH 7.3 (98.7

Table 13. Incidence (%) of abberations in bluegill larvae on day five of laboratory experiment II.

Soft Water					Hard Water				
Nominal pH	Larvae Tested (n)	Aberrations Spine (%)	Fin (%)	Eye (%)	Nominal pH	Larvae Tested (n)	Aberrations Spine (%)	Fin (%)	Eye (%)
7.00	22	0.0	4.3	0.0	8.00	63	1.6	0.0	0.0
5.50	16	0.0	6.3	0.0	5.10	71	2.4	2.4	2.4
5.25	27	3.7	18.5	14.8	4.90	81	0.0	9.9	7.4
5.00	50	8.0	14.0	6.0	4.70	79	1.3	15.0	8.9
4.75	53	1.8	1.8	1.8	4.50	88	2.3	8.0	9.1
4.50	10	20.0	50.0	40.0	4.30	69	1.4	2.9	10.1

Table 14. Mean hydrogen ion concentrations for 24 hour periods over eight days for each treatment replicate, and mean pH for each treatment in the on-site field experiment.

Nominal pH	Mean Replicate pH (n=8)	Range pH	Mean Treatment pH (n=3)
3.5	3.51*	3.50-3.53	3.5
	3.51	3.50-3.53	
	3.52	3.50-3.55	
4.5	4.61	4.47-5.07	4.6
	4.57	4.47-4.86	
	4.58	4.47-4.81	
5.5	5.67	4.48-6.32	5.7
	5.67	4.49-6.27	
	5.67	4.49-6.31	
7.3	7.30	7.22-7.41	7.3
	7.32	7.21-7.49	
	7.31	7.23-7.45	

* n=2 due to mortality of all eggs at pH 3.5 within 24 hours.

percent). No differences ($P > 0.05$) between pH 5.7 (94.7) and pH 7.3 were detected. On day three, total cumulative hatching success was at least 90 percent in all pH treatments except pH 3.5. Total cumulative hatching success was decreased ($P < 0.05$) at pH's 3.5 and 4.6, but not at 5.7 (Table 15). Hatching was delayed in low pH treatments, cumulative hatching success increased to 80 percent on days one, two and three in pH 7.3, 5.7 and 4.6, respectively (Figure 9).

The delay in hatching at the lower pH levels was reflected by an increased incidence of partial hatching (Table 15). Partial hatching rates averaged 1.3 percent, 5.3 percent and 14.7 percent in pH 7.3, 5.7 and 4.6, respectively. Incidences of partial hatching were higher ($P < 0.05$) in pH 5.7 and pH 4.6, than in the control aquaria at pH 7.3 (Table 15). Since hatching success was minimal, (< 1 percent) in pH 3.5 observations on partial hatching at this pH were precluded. Most of the partially hatched eggs at all pH treatment levels were able to free themselves from the chorion within three days.

Mortality included the cumulative number of dead embryos, dead larvae, and deformed larvae observed during the test, and in pH 3.5, 4.6, 5.7 averaged 99.3 percent, 54.7 percent and 13.4 percent, respectively (Table 16). Because sections of the incubation chambers were not sealed securely, some larvae escaped or were caught between sections and injured. This was especially a problem in the control chambers (pH 7.3) where larvae were more active; mortality averaged 42.3 percent. Survival was greater than 80 percent in pH 5.7, therefore, it was used as the reference for comparison among pH treatment levels of 4.6 and 3.5. Mortalities at pH 4.6 and pH 3.5 treatments were significantly higher ($P < 0.05$) than in pH 5.7 (Table 16).

Examination of effects of acid on mean total length of larvae at hatching (day one), and on the final day of the experiment (day seven), and on increase in length over

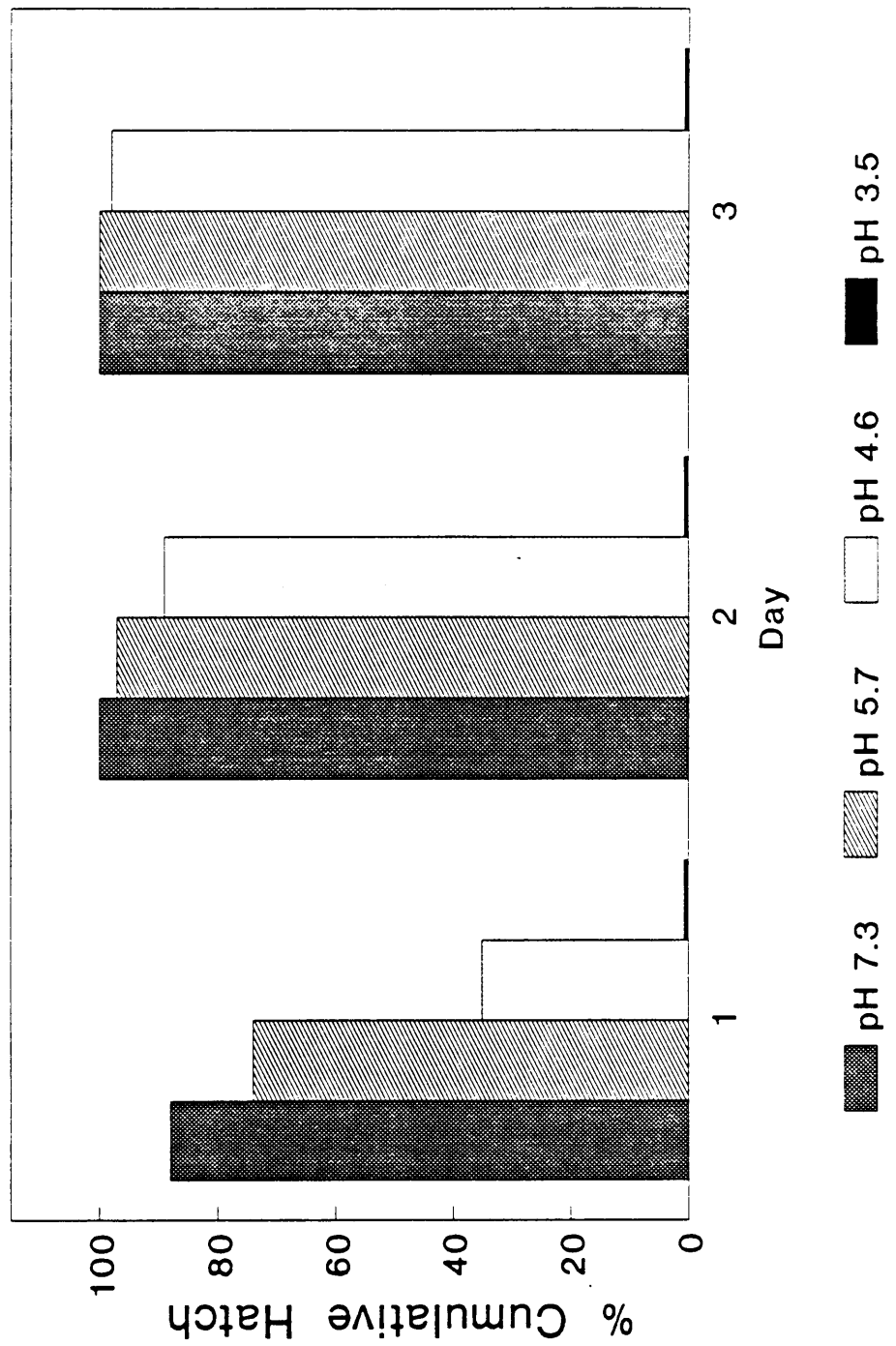

Figure 10. Cumulative hatching rates (%) of bluegill eggs for days one to three of the field experiment.

Table 15. Total hatching and partial hatching observed in the field experiment.

Mean pH	Eggs Tested N	Eggs Hatched (n)	Eggs Hatched (%)	Partially Hatched (n)	Partially Hatched (%)
7.3	150	148	98.7[c]	2	1.3[a]
5.7	150	142	94.7[c]	8	5.3[b]
4.6	150	135	90.0[b]	22	14.7[c]
3.5	150	1	0.6[a]	0	0.0[a]

Treatments with different superscripts were significantly different ($P < 0.05$).

Table 16. Mortality of bluegill larvae observed in the field experiment over seven days.

Mean pH	Embryo-larval Tested (N)	Embryo-larval Mortality (N)	Embryo-larval Mortality (%)
7.3	52	22	42.3 [b]
5.7	97	13	13.4 [c]
4.6	95	52	54.7 [b]
3.5	150	149	99.3 [a]

Treatments with different superscripts are significantly different at ($P < 0.05$).

six days were tested (Table 17). None of these indices of growth was pH dependent. High variability within treatments, compromised detection of treatment effects. Size dependent mortality may have occurred, resulting in survival of only the largest larvae to the final day of the experiment. Only larvae that were alive at the termination of the test were measured.

Table 17. Mean total length of bluegill larvae on day one and day seven, and growth in length over six days in the field experiment. Standard deviations are shown in parentheses.

Mean pH	N	Mean(SD) Initial Length (mm)	Mean(SD) Final Length (mm)	Growth (mm)
7.3	3	3.06(0.06)	5.06(0.11)	2.00(0.08)
5.7	3	3.62(0.41)	5.07(0.10)	1.44(0.31)
4.6	3	2.92(0.33)	4.92(0.09)	2.00(0.28)

All measurements were unrelated to pH ($P < 0.05$).

Discussion

Hatching

The embryonic stage of various fish species show a wide range of acid tolerances (Table 18). Species that are reportedly acid tolerant, such as European perch (Vinogradov and Komov 1985), brown trout (Johansson et al. 1977), and brook trout (Baker and Schofield 1982) show increased egg mortality in a pH range of pH 3.5 to pH 4.6. More acid sensitive species such as Atlantic salmon (Johansson et al. 1977), lake trout (Kennedy 1980), and walleye (Hulsman et al. 1983) have increased egg mortality in a pH range of pH 5.0 to pH 6.7. The results of this study suggest bluegill eggs are acid tolerant. They exhibited 50 percent egg mortality between pH 4.0 and 4.2 in soft water and between pH 4.0 and 3.8 in hard water.

Reduced hatchability in soft acidic water frequently has been attributed to reduced hatching enzyme activity (Mount 1973, Johansson et al. 1977, Runn et al. 1977, Peterson et al. 1980, Brown 1981, Brown and Lynam 1981, Haya and Waiwood 1981,

Table 18. Egg mortality as a result of acid toxicity. Ionic levels are denoted as: H = water hardness in mg/L $CaCO_3$, Ca = calcium in mg/L. C = Conductivity in μS/cm. Egg development stage of initial acid exposure were FF = freshly fertilized, Wt = water hardened at treatment pH levels, P = parents exposed. Effective pH levels are those that elicited the reported percent mortality.

Species	Ionic Level	Egg Stage	Duration (Days)	Effective pH Level	Mortality (%)	Reference
Bluegill	10(H)	FF	6	7.0	97	This Study
				4.4	2	
				4.2	7	
				4.0	79	
				3.8	100	
Bluegill	180(H)	FF	6	8.0	2	This Study
				4.4	4	
				4.2	15	
				4.0	26	
				3.8	100	
Yellow perch	6.4(Ca)	FF	12	4.0	0	Vinogradov and Komov 1985
				3.5	100	
Walleye	3-5(Ca)	WT	30	6.0	32	Hulsman et al. 1983
				5.4	93	
White sucker	2.2(Ca)	WT	23	7.1	26	Baker and Schofield 1982
				5.4	37	
				5.1	70	
				4.8	100	
Atlantic salmon	100(C)	FF	210	7.2	4	Daye and Garside 1979
				5.5	6	
				5.3	10	
				5.0	90	
				4.8	99	
Brown trout	100(C)	FF	210	7.2	3	Johansson et al. 1977
				5.3	3	
				5.0	10	
				4.8	6	
				4.4	34	
				4.1	93	

Table 18 continued.

Species	Ionic Level	Egg Stage	Duration (Days)	Effective pH Level	Mortality (%)	Reference
Steelhead	4.2(Ca)	FF	4	4.5	50	Rombough and Jensen 1985
		WT	4	4.5	100	
Brook trout	72(H)	P	150	7.0	15	Menendez 1976
				6.5	24	
				6.1	40	
				5.5	26	
				5.0	69	
Brook trout	2.2(Ca)	WT	58	7.0	6	Baker and Schofield 1982
				4.6	8	
				4.4	46	
				4.2	100	
Arctic Char	13(C)	WT	8	6.7	14	Jagoe et al. 1984
				5.0	50	
				4.5	100	
Lake trout	10(H)	WT	60	6.6	7	Kennedy 1980
				5.8	94	
Fathead minnow	200(H)	P	390	7.5	21	Mount 1973
				6.6	20	
				5.9	58	
Flagfish	28(H)	WT	20	6.8	56	Craig an Baksi 1977
				6.0	43	
				5.5	49	
				5.0	83	

Vinogradov and Komov 1985, Thomsen et al. 1988). Hatching is initiated in teleosts eggs by the release of hatching enzymes into the perivitelline fluid that separates the developing embryo from the chorion. (The terms chorion, zona radiata, and egg capsule have been all used to denote the outer membrane surrounding fish eggs. I will use chorion). The hatching enzyme is released from hatching glands located on the anterior surface of the embryonic body and yolk sac in most fishes (Yanai 1966). The enzyme degrades only the inner proteinaceous layer of the chorion. Vigorous movements of the embryo erupts the outer mucopolysaccharide layer and permits hatching. The precise mechanisms of how the hatching enzyme functions are unknown; however, a general view is apparent from current research.

Hatching enzymes probably are choriolytic proteases that have a maximum activity at approximately pH 8.0 (Yamagami 1988). Maximum chorionase activity was between pH 7.5 and pH 8.0 in chum salmon (*Oncorhynchus keta*) (Bell et al. 1969). Hagenmaier (1974) found rainbow trout hatching enzyme has a pH optimum of 8.5. In Atlantic salmon chorionase activity at pH 4.6 was 49% of the activity found at pH 6.5 (Haya and Waiwood 1981). At pH 5.2 the activity of the enzymes degrading the chorion of Atlantic salmon eggs was reduced 10% of the optimal rate (Peterson et al. 1980). Microscopic examination of sectioned chorions of European perch (*Perca fluviatilis*) showed that those eggs incubated at pH 4.5 had intact chorions, on the day that eggs reared at pH 7.3 had chorions that were partially dissolved or absent (Runn et al. 1977).

In acid environments, measurements of the pH of the perivitelline (pv) fluid of salmonids have shown rapid equilibration with the pH of the surrounding medium, and depressed hatching success through reduced activity of hatching enzymes (Daye

and Garside 1980, Johansson et al. 1977, Peterson et al. 1980). Peterson et al. (1980) found the pH of the perivitelline fluid of Atlantic salmon in acidic water approached steady state in 8 hours. The pv fluid of eggs transferred to solution of pH 5.5 equilibrated at a pv fluid pH of 5.5; however, those transferred to pH 4.0 stabilized at pH 4.5. Apparently the pv fluid has some capacity to buffer acidic inputs. This buffering capacity has been shown to vary among species and may partially explain differential sensitivities among species. Johansson et al. (1977) noted that Atlantic salmon were more sensitive to acid than brook trout or brown trout when reared at pH levels ranging from pH 4.1 to pH 5.0. They attributed such differences to the capacity of trout to stabilize their perivitelline fluid at 0.8 pH units above ambient, whereas Atlantic salmon could maintain theirs only 0.4 pH units above the surrounding water.

Reduced hatching enzyme activity at low pH probably does not fully explain the effect of acid on embryos. Structural changes in the chorionic tissue might also contribute to reduced hatching. Haya and Waiwood (1981) observed that the chorions of eggs incubated at low pH were hard and opaque, and suggested that alteration of the mucopolysaccharide layer makes it difficult for the embryos to free themselves once the inner layer is digested by enzymes. Furthermore, a general decrease in the embryonic activity at low pH was observed for Atlantic salmon reared at pH 4.0 to pH 4.5. Reduced activity could result in a lowered ability to escape the chorion; or it might reduce the distribution of the hatching enzyme throughout the perivitelline fluid, thus reducing the enzymes effectiveness.

Delayed hatching and partial hatching, evident in my study, provides further evidence that implicates low pH of the pv fluid in reduced hatching success. In this study, the

percent of bluegill embryos observed in a partially hatched condition increased at low pH (<4.6) in both hard and soft water. Other investigators also have observed delayed and/or partial hatching in acid exposed eggs and have implicated reduced enzyme activity due to low pH of the pv fluid (Brown and Lynam 1981, Johansson et al. 1977, Mount 1973, Nelson 1982, Thomsen et al. 1988, and Vinogradov and Komov 1985).

In this study, an extension of the hatching period was apparent in eggs exposed to low pH. At high pH levels, (pH's 7.0 and 8.0, 4.6 hard and soft water), the hatching period extended for two days while at pH ≤4.0 (hard and soft water) almost no hatching occurred on day one and hatching was not completed until day four (Figures 2 and 3). Peterson et al. (1980) reported that in Atlantic salmon the median hatching date was delayed approximately 6 days at pH's 4.5 to pH 5.0, and that no hatching occurred at pH's 4.2 and 4.0. However, normal hatching resulted when eggs were transferred from pH 4.0 to neutral pH levels for up to 10 days past the normal (50 percent hatching in controls) hatching date, indicating that hatching was prevented by reduced enzyme function not larval damage. Bluegill eggs in this study, exposed to low pH levels were not transferred to neutral media, eggs in pH's 3.8 and 4.0 had either hatched or died in four days and hatching in 4.2 was only slightly reduced over controls. At pH levels below 4.2, hatching in soft water was depressed rather than delayed. The death of embryos exposed to low pH levels probably was not the result of hatching enzyme failure, but rather was caused by erosion of the epidermal cells at a rate that exceeded replacement. Other investigators also have attributed egg mortality to embryonic damage rather than to low pH of the perivitelline fluid (Daye and Garside 1977, Kwain and Rose 1985, Peterson et al. 1980). The effects of acid on hatching in salmonids and bluegill may differ because of the lengths of their

incubation periods. Bluegill hatch in 48 to 72 hours, whereas and most salmonids hatch in 30 to 60 days. During the prolonged incubation and exposure period salmonid eggs may absorb more acid than do bluegill eggs. These comments on bluegill egg pathogenesis, however are conjectural and demonstration of mode of mortality needs to be detailed in future research.

The duration of the hatching period was influenced by low water hardness. Initiation of hatching of bluegill eggs was delayed several hours in soft water compared to hard water, even at near neutral pH levels (Figure 5). Similarly, Thomsen et al. (1988) found a reduction in calcium level from 150 mg/l to 1 mg/l delayed hatching period of rainbow trout from 1.2 days (hard water) to 4.5 days (soft water) without altering the pH. Low pH levels were more lethal to bluegill eggs in soft water than in hard water. Hatching was reduced at pH 4.0 in both hard and soft water, but hatching was significantly lower in soft than in hard water (Figure 4). Other investigators working with a variety of fish species, largely salmonids, reported similar results. Brown and Lynam (1981) found that freshly fertilized eggs of brown trout incubated at pH 4.5 survived longer if the calcium ion concentration was raised from 1 to 10 mg/L. Atlantic salmon, brown trout, sea run brown trout held at pH 4.0, all exhibited an increase in egg mortality when the ionic strength of the test water was reduced by 50 percent (Carrick 1979). Nelson (1982) found that hatching success of rainbow trout eggs increased by about 40 percent by the addition of calcium sulfate (118 mg/L, calcium) to acidified (pH's 4.8, 4.6) water. Conversely, Trojnar (1977) found that white sucker embryos were sensitive to low pH regardless of the ambient ion levels. He found no eggs survived to hatch at a pH levels near 4.5, regardless of conductivity, hatching at pH 5.0 was unaffected.

Two physiological mechanisms: 1) reduced hatching enzyme activity and, 2) increased membrane permeability at low water hardness levels may explain the observed interactions of water hardness and pH on hatching rates. Hatching enzymes were inhibited by ethylenediamine tetracetic acid (EDTA) in medaka (*Oryzias laptipes*) (Yamagami 1973) and in chum salmon (*Oncorhynchus keta*) (Bell et al. 1969), suggesting that divalent ions, (i.e. calcium ions), are necessary for maximum enzyme function and optimum egg hatching. Dissolution of the chorion by enzymes occurs at a slower rate in soft water due to a deficiency in calcium ions (Bell et al. 1969, Yamagami 1966). High water hardness decreases the permeability of cellular membranes to ions and water (Leivestad 1982). Nelson (1982) found that calcium levels of 112 mg/L could improve hatching in rainbow trout held at pH levels of 4.6 to 4.8 and attributed the increased hatching to a reduced permeability of the chorion to hydrogen ion in the presence of high calcium.

The effects of acid on eggs can be affected by low ambient pH at egg water hardening (Rombough and Jensen 1985), and parental exposure to low pH (Menendez 1976, Mount 1973), exert significant influence over egg mortality. Because neither of these effects were examined in this study, the pH levels found to cause 50 percent egg mortality, (pH 4.0 to pH 4.2, and pH 3.8 to pH 4.0, in soft and hard water, respectively), may have underestimated acid toxicity in bluegill embryos.

Bluegill eggs in this study, were water hardened at approximately pH 7.3, the ambient pH of Flat Top Lake. Some studies have shown that mortality is increased when eggs are water hardened at low pH. Steelhead eggs water hardened at neutral pH levels had 50 percent survival at pH 4.5 in 96 hours, whereas none of the eggs water hardened at pH 4.5 survived over 24 hours (Rombough and Jensen 1985). Desert

pupfish (*Cyprinodon nevadensis*) are extremely acid sensitive and normally inhabit water with an ambient pH near 8.0. Lee and Gerking (1980) found greater hatching success in eggs water hardened at pH 8.3 than for those water hardened at treatment pH levels (7.0, 6.5, 6.0, 5.5).

Absorption of acidic, low ionic strength water during water hardening may account for the observed increase in acid sensitivity of eggs. The perivitelline fluid that separates the chorion from the embryo is formed during water hardening. Osmotically active colliodal material, released from the cortical alveoli, initiates water absorption and formation the perivitelline fluid (Peterson 1984). The colloid has a net negative charge at near neutral pH levels, and concentrates cations at levels above that of the surrounding medium (Rudy and Potts 1969). Cation concentration is reduced as pH declines to 5.0 and is absent at pH 4.0 in Atlantic salmon (Peterson 1984). A reduction in the amount of calcium, needed as hatching enzyme cofactors (Bell et al. 1963, Yamagami 1966), in the pv fluid as a result of low ambient hardness and pH levels could significantly depress hatching success.

Increased mortality in embryos water hardened at low pH levels may also result from their heightened sensitivity during organogenesis. Corrosion of the epidermal cells in the early embryo stage can readily disrupt organ and tissue differentiation. Older embryos undergoing organ development have a relatively thick rudimentary integument that can act as an ion gradient along with the chorion and pv fluid (Peterson et al. 1982), which probably accounts for the observed increased acid tolerance of Atlantic salmon embryos exposed in later stages of development (Daye and Garside 1977)

Exposure of the parental stock to low pH, was not assessed in this study. The pH of Flat Top Lake was circumneutral ranging from pH 6.9 to pH 7.7 during March-August,1987 and the eggs from wild stocks were used in all experiments. Some studies have observed significant effects due to the exposure of the parental stock to acidic conditions (Mount 1973, Menendez 1976). In a full life cycle toxicity test, Mount (1973) reported that adult fathead minnows (*Pimephales promelas*) held at pH 5.2 produced eggs but did not spawn; whereas eggs from unexposed parental stock, held at pH 5.2 had a hatching rate of 67 percent.

The exposure of parental stock to acid may be influenced by the levels of calcium in the water. Vitellognin, the source of yolk in mature eggs, is transported from the liver to the ovaries through the blood bound to calcium which facilitates its dissolution in plasma. In female salmonids, a seasonal change in the levels of calcium and vitellognin occurs in plasma that is related to oogenesis (Mount et al. 1988). Observation of female white sucker from an acid lake did not show an increase in serum calcium levels as expected, suggesting vitellogenesis was affected by low pH levels (Beamish et al. 1975). Mount et al. (1988) tested the affect of different levels of acid, calcium and aluminum on serum vitellognin in brook trout and found a decrease only in the most severe treatment (pH 5.04, 0.05 mg/L calcium, and 172 ug/L monomeric Al). However, fecundity was reduced at pH 4.8. Weirner et al. (1986) found that progeny of rainbow trout exposed to acid conditions had lower survival than controls through seven days of development. Conversely, Menendez (1976) found that survival of brook trout alevins from exposed and unexposed parents was similar. The effect of acid and calcium levels on the survival of the prodgeny of acid exposed parents is still being assessed. A more substantial understanding of the processes

involved is needed to assess the role of pH and calcium on vitellognin formation and egg production and survival.

The eggs of bluegill were more tolerant of acidic conditions than were larvae. The pH which caused 50 percent mortality in eggs was between pH 3.8 and pH 4.0 in hard water and pH 4.0 and pH 4.2 in soft water (Figure 4), whereas the 50 percent mortality in larvae was pH 4.02 and 4.67 in hard and soft water, respectively (Figure 7). In a review of acid toxicity tests, Fritz (1980) concluded that egg and post yolk sac larvae were generally more tolerant than yolk sac larvae. Toxicity varies with life stage in a similar fashion for other toxicants as well (Smith et al. 1976, McKim 1977). There is general agreement that the chorion and perivitelline fluid protect the embryo from environmental extremes. The chorion has been shown to sequester hydrogen ions (Daye and Garside 1980) and the pv fluid exhibited some buffer capacity (Peterson et al. 1980). These observations led Rombough (1983) to suggest that the inherent sensitivity of the organism does not change, rather the environment, upon hatching, is more extreme.

Embryo-Larval Mortality

Acid Effects

Many difficulties are encountered when making comparisons among fish toxicity studies. Investigators use a wide variety parameters to quantify acid toxicity, such as LT50's, LC50's, and percent mortality at various pH levels, or subjective

comparisons of median period of survival. Differing environmental conditions and parameters not statistically analyzed make comparing results from various experiments difficult. Major factors complicating comparisons among studies include the differences between the acids and life stages used, testing times, and varying water chemistry parameters, especially water hardness. Although such inconsistencies make interpretation difficult, inter-experimental comparisons reveal some important trends.

Previous studies have estimated that adult and juvenile bluegill show only minimal mortality at pH 4.0 in four day tests (Trama 1954, Ultsch 1978, Ellgaard and Gillmore 1985). In comparison to other species, bluegill adults are probably intermediate in their tolerance to acid (Table 19). Bluegill are not as tolerant as European perch which apparently can survive at an ambient pH of 3.5 (Vinogradov and Komov 1985). However, bluegill are more tolerant than brook trout or fathead minnow which are limited by pH's 5.0 and 4.5, respectively (Mount 1973, Menendez 1976). The adult stages of various fish species show differences in acid tolerance, but most are affected within a pH range of 3.5 to 4.5 (Table 19).

Variation in the reported pH tolerance of adult fishes probably has two main sources; one arising from the test conditions, the other from inherent physiological differences among species. Test conditions such as temperature (Kwain 1975) and water hardness (LLoyd and Jordan 1964) have been shown to influence acid toxicity. The 96 hour LC50's of fingerling rainbow trout were 3.86 and 4.04 at 5 C and 20 C, respectively (Kwain 1975). Lloyd and Jordan (1964) found 50 percent mortality in juvenile rainbow trout at pH 4.18 and 4.25 for hard (320 mg/L $CaCO_3$) and soft (12 mg/L $CaCO_3$) water, respectively.

Table 19. Acid toxicity as a function of life stage. The pH values were the lowest acid levels that caused an effect in each life stage, and estimates the tolerance range for the species. Various ionic level parameters are denoted as: H = water hardness in mg/L $CaCO_3$, Ca = calcium in mg/L. All tests used H_2SO_4 except tests of bluegill done by other investigators which all used HCL.

Species	Ionic Level (mg/L)	Duration (Days)	Adults or Juveniles (pH)	Eggs or Larvae (pH)	Mortality (%)	Reference
Bluegill	10(H)	5	--	5.7	1	This Study
	180(H)	5	--	5.0	1	This Study
	-	4	3.5	--	50	Ellgaard & Gillmore 1985
	46-47(H)	4	4.0	--	0-30	Trama 1954
	-	4	4.0	--	10	Ultsch 1978
White sucker	2.2(Ca)	60	--	5.6	30	Baker & Schofield 1982
	43.4(H)	120	4.2[a]	--	0	Beamish 1972
Brook trout	76(H)	150	--	6.5	24	Menendez 1976
	76(H)	150	5.0	--	25	Menendez 1976
Rainbow trout	10(H)	33	--	5.5	20	Weiner et al. 1986
	8.8(Ca)	4	4.1	--	50	Kwain 1975
Fathead minnow	200(H)	390	--	5.9	58	Mount 1973
	200(H)	390	4.5[a]	--	0	Mount 1973
Flagfish	28(H)	45	--	6.0[b]	0	Craig & Baksi 1977
	28(H)	20	5.5	--	21	Craig & Baksi 1977
European perch	41(Ca)	12	--	5.3	29[c]	Vinogradov & Komov 1985
	-	-	3.5	--	-	Vinogradov & Komov 1985
Smallmouth	18.6(Ca)	33	--	5.7	30[c]	Hill et al. 1988
Largemouth	1.8(Ca)	7	--	5.0	62	Swenson et al. 1989
Rock bass	1.6(Ca)	7	--	5.6	54	Swenson et al. 1989

[a] increased spinal deformaties. [b] decreased growth. [c] The percent mortality over the controls.

Inherent differences among species are probably attributable to differences in the ability to adapt to ionoregulatory stress (Leino et al. 1987) There is a general agreement among researchers that the physiological mode of death, at environmentally important pH levels (4.0-6.0), is failure in body salt regulation leading to hemoconcentration and circulatory collapse (Packer and Dunson 1970,1972, DeRenzis and Maetz 1973, Dively et al. 1977, Swarts 1978, McWilliams and Potts 1978, Vinogradov and Komov 1985, and Mount et al. 1988). In freshwater fish, specialized cells (probably chloride cells) located in the branchial epithelium in adults and in the integument in prolarvae are responsible for the active uptake of salts that are needed to maintain internal ionic levels (Alderdice 1988). In the fathead minnow, damage to chloride cells resulted from exposure to pH 5.0, but in the more acid tolerant yellow perch damage to chloride cells did not occur until pH 4.1 (Leino et al. 1987). Since adult bluegill are relatively acid tolerant, it would be instructive to know if their chlorides cell are similar to those of yellow perch or if another mechanism is used to resist ionic imbalance, such as excessive mucus secretion by the gills as suggested by McDonald (1983a).

The results from my study indicated that embryo-larvae bluegill were much more sensitive to low pH than reported for later stages of larvae (Palmer et al. 1988), juveniles (Ellgaard and Gillmore 1985), or adults (Trama 1954, Ultsch 1978), all of which exhibit a tolerance level near pH 4.0. In this study, the estimated MATC of embryo-larvae bluegill in soft water was pH 5.7. Life stage has a large influence on acid toxicity (Lee and Gerking 1980, Baker and Schofield 1982, Kwain and Rose, 1985). A comparison of acid tolerance between early and adult stages among various species indicated that reduced survival occurred in adults at levels that were generally at least one pH unit lower, (i.e. solutions that are 10 times more acidic)

than those that reduce survival of embryo-larval stages (Table 19). The pH levels listed in Table 19 are the highest pH levels reported to have caused an effect (usually mortality) in adult and early life stages and are representative of the range of tolerance exhibited by each species.

Bluegill embryo-larvae appear to be intermediate in their acid tolerance in comparison with other species. The embryo-larval of species, such as brook trout, flagfish (*Jordanella floridae*), and fathead minnow are particularly sensitive and are intolerant of ambient pH levels below 6.5, 6.0, and 5.9, respectively (Mount 1973, Menendez 1976, Craig and Baksi 1977). Embryos and larvae of others species exhibit an acid tolerance of about pH 5.6; bluegill, as well as, white sucker (Baker and Schofield 1982), rainbow trout (Weiner et al. 1986), smallmouth bass (Hill et al. 1988), and rock bass (*Ambloplites rupestris*) (Swenson et al. 1989) are included in this intermediate sensitivity range. Early life stages of largemouth bass (Swenson et al. 1989) and yellow perch (Vinogradov and Komov 1985) demonstrate a higher degree of acid tolerance, intolerant of pH 5.0 and 5.3, respectively. Although the acid tolerance of early life stages of various species differ, a pH range of 5.3 to 6.5 includes many North American sportfish species (Table 19).

The reduced acid tolerance in early life stages probably results from the increased quantity and quality of exposure sites in embryos and larvae (Birge et al. 1985); developing gill and brain tissues are particularly sensitive to acid effects (Daye and Garside 1980, Hill et al. 1988). Sensitive tissues in adults are largely confined to the gill epithelium which is in close contact with the external environment (Wood and McDonald 1982). Embryo and larvae are more exposed and integument, eye, kidney, spleen, erythrocytes and, gill tissue of Atlantic salmon exhibited sublethal and lethal

damage at pH levels 3.7-5.0 (Daye and Garside 1980). They noted that some regeneration of sensitive tissues and cells was evident at pH levels between 4.2-5.0, however, gill or brain tissue did not regenerate even at pH 5.0.

As in adults, ionoregulatory failure accounts for a significant portion of larval fish mortality in acid environments (Peterson and Martin-Robichaud 1986), and acidic conditions are particularly stressful as larvae undergo transition from integumental to branchial ionoregulation (Daye and Garside 1980). The integument is relatively robust in comparison to the highly permeable gill epithelium (McWilliams and Potts 1978). As the gill becomes more important in ion and gas exchange it also becomes the major factor in the survival of the fish (Daye and Garside 1980). In brook trout, a rapid expansion of the gill filaments, which generally occurs in late stages of alevin development at pH 5.0 and above did not occur at pH 4.5, and an increase in mortality was observed at this time period (Kwain and Rose 1985). Many investigators have found that increased mortality in acid-stressed larvae is related temporally with the enlargement of the gill system (Daye and Garside 1980, Peterson et al. 1982, Rombough 1983, Hill et al. 1988). A more accurate assessment of the toxic effects of acid on larval fishes is obtained, therefore, if this critical stage of gill development is included in the toxicity test.

Coughlan and Gloss (1984) found that a complete gill apparatus including arches, filaments and secondary lamellae was not present in smallmouth bass until swim-up (15 days after fertilization). I assume that other centrarchid species with short incubation periods, such as bluegill, have a branchial development period similar to smallmouth bass. Swim-up occurred at five days post-hatch in the bluegill I tested, therefore it is likely that my tests included this sensitive period of initial gill system

enlargement. In the present study, survival after hatching remained fairly constant up to day four or five, then decreases in survival were evident at most treatment pH levels (Figure 7).

Water Hardness Effects

While life stage is a significant biotic factor affecting acid toxicity in fishes, water hardness probably is one of the more influential abiotic factors. Even at circumneutral pH levels, water hardness can increase survival of egg and larval fishes (Stickney 1979, Grizzle et al. 1985, Thomsen et al. 1988). Aquaculturists have long recognized the hazards of extremely soft waters, and recommend hardness levels of 20 to 150 mg/L to increase survival of freshwater fish species (Stickney 1979). A scarcity of ions in soft water increases the concentration gradient and the energy expenditure required for the ionoregulation (Eddy 1975, Stickney 1979, Thompsen et al. 1988). Thomsen et al. (1988) found that the greatest reductions in survival of rainbow trout larvae were related to the calcium concentration of the test media. He observed a 24 percent decrease in survival as the calcium concentration was reduced from 150 to 1 mg/L without lowering the pH.

In acidified waters, hardness has been shown to directly increase survival of juvenile stages of rainbow trout (Lloyd and Jordan 1964, Graham and Wood 1981, McDonald et al. 1980, Nelson 1982), brown trout (Brown 1981, Brown and Lynam 1981), and white sucker (Trojnar 1977). Rainbow trout juveniles in a four day test exhibited approximate 50 percent mortality rates at increased pH levels as water hardness was decreased; approximately 50 percent mortality occurred at pH 4.18 at 320 mg/L

$CaCO_3$, pH 4.22 at 40 mg/L $CaCO_3$, and pH 4.25 at 12 mg/L $CaCO_3$(Lloyd and Jordan 1964). Brown trout larvae survived in pH 4.5 at calcium levels of 10 mg/L, but not at 1 mg/L.

The LC50 values for various species exposed to acid in soft water averaged between pH 4.25 and 5.0, whereas, in hard water for most species was pH 4.06 to 4.56 (Table 20). Of the species listed in Table 20 Pacific salmon (Rombough 1983), smallmouth bass (Holtze and Hutchison 1989), and flagfish (Hutchison and Sprague 1989) were the least tolerant in soft, acidified water, whereas rainbow trout (Graham and Wood 1981), bluegill, and white sucker (Holtze and Hutchison 1989) were the most acid tolerant.

The ameliorating effect of water hardness on acid toxicity varied among species. The LC50 for bluegill decreased 0.61 pH units as the water hardness increased from 18 to 197 mg/L $CaCO_3$. In rainbow trout, a similar increase in water hardness resulted in a decrease in the LC50 of 0.13 pH units. As the conductivity of the test media increased from 30 to 60 uS/cm, the LC50 of Atlantic salmon decreased 0.40 pH units.

The variable response in toxicity to the combined effects of water hardness and acid may be the result of interspecific physiological differences. Bluegill appear more tolerant of low pH in hard water than are rainbow trout, with LC50's of pH 4.06 and 4.12 in hard water, respectively. However in soft water, rainbow trout exhibit greater acid tolerance than bluegill, exhibiting soft water LC50's of pH 4.25 and pH 4.65, respectively. Atlantic salmon acid tolerances reported by Daye and Garside (1979) showed greater acid sensitivity in hard water than did bluegill in this study, but in soft water Atlantic salmon acid sensitivity was similar to bluegill in this study (Lacroix 1985). Interspecific variation in factors such as, the ability of the gills to bind calcium,

Table 20. LC50's of the early life stage of fish species as a function of water hardness. Various ionic level parameters are denoted as: H = water hardness in mg/L $CaCO_3$, Ca = calcium level in mg/L, C = conductivity in uS/cm.

Species	Duration (days)	Ionic Level (mg/L)	LC50 (pH)	Reference
Bluegill	5	10(H)	4.67	This Study
	5	180(H)	4.06	This Study
Smallmouth bass	4	4(Ca)	4.98	Holtze and Hutchison 1989
White sucker	14	2(Ca)	5.02[a]	Baker and Schofield 1982
	4	4(Ca)	4.67	Holtze and Hutchison 1989
Fathead minnow	4	20(H)	4.50[b]	Palmer et al. 1988
	4	81(H)	4.56	Ramey and Colten 1986
Rainbow trout	7	14(H)	4.25	Graham and Wood 1981
	7	140(H)	4.12	Graham and Wood 1981
Atlantic salmon	210	30(C)	4.70	Lacroix 1985
	4	60(C)	4.30	Daye and Garside 1979
Flagfish	4	5.5(H)	4.82	Hutchison and Sprague 1989
Coho salmon	10	11(H)	4.86	Rombough 1983
Chinook salmon	10	11(H)	4.85	Rombough 1983
Sockeye salmon	10	11(H)	5.02	Rombough 1983
Pink salmon	10	11(H)	5.01	Rombough 1983
Chum salmon	10	11(H)	5.00	Ronbough 1983

[a] Approximately 50 percent mortality occurred at this pH in white sucker (Baker and Schofield 1981).
[b] pH 4.50 caused 100 percent mortality in fathead minnows in soft water (Palmer et al. 1988).

and physiology of chloride cells probably accounts for the differential effects of water hardness on acid toxicity among species (McDonald 1983b, Leino et al. 1987).

The physiological mechanisms underlying the mitigative effects of water hardness are not precisely known, but apparently are related to changes in cellular membrane permeability (Wood and McDonald 1982). Hemoconcentration and circulatory collapse which is the ultimate source of mortality in acid exposed fish is dependent on the rate of salt lost rather than the total amount lost (Packer and Dunson 1972, McDonald et al. 1983). Mortality increased as did the rate of salt loss when pH was lowered from 3.25 to 2.0, but the total amount of salt lost was lower at pH 2.0 (Packer and Dunson 1972). In general, the effect of water hardness is to reduce the rate of sodium and chloride loss and, thus increase survival (McDonald et al. 1980). When investigating the effects of water hardness on the toxic response of fish in acid media McDonald et al. (1980) found that the ionic disturbance in rainbow trout exposed to acid in hard water differed from that in soft water, even though the ultimate cause of death was similar. In acidified hard water, large declines in blood pH and plasma HCO_3^- concentrations (metabolic acidosis) occurred, while sodium and chloride levels declined only slightly. In contrast, fish held in soft acid water exhibited slight acidosis, and large declines in sodium and chloride. McDonald et al. (1983) found similar results when rainbow trout were exposed to low pH and three concentrations of calcium ions. During the initial hours of acid exposure there was a large influx of acid across the gill at all calcium levels. However, after 40 hours there was a small, but significant net excretion of H^+ in low calcium media, whereas in high calcium media the net inward diffusion of H^+ persisted.

The reason that gills are less permeable to hydrogen ions in soft water compared to hard water is unknown but may be related to structural alteration of the membrane diffusion channels, from cation to neutral or anion selecting in low calcium environments (McDonald 1983b). Fish acclimated to low calcium prior to acid exposure showed initially greater efflux of chloride ions than did fish acclimated to high calcium. Thus, it appears neutral or anion selective membrane channels inhibit the uptake of hydrogen ions and so, blood acidosis is less favored in low calcium environments (McDonald et al. 1983).

Sodium and cloride ion uptake is inhibited in acidified waters, with greater inhibition occurring in soft water than in hard water (McDonald et al. 1983). The mechanism behind the reduction of ion uptake in low pH and low calcium environments is not precisely known; but it is likely that calcium affects the transport mechanisms, or the general membrane stability and protein structure in the ion transport channels (McDonald 1983b).

Regardless of the mechanisms involved, it is clear that water hardness exerts significant effects on acid toxicity in many species. In the studies cited, increased water hardness resulted in increases in LC50's of 0.09 to 0.61 pH units. The magnitude of the increase in pH may not seem large, but the effect is notable in the natural environment. Field surveys have demonstrated that at a given pH the percentage of fishless lakes is always higher in low calcium than in high calcium aquatic environments (Brown 1982). In a survey of 37 lakes in Quebec, with pH values ranging from from 4.6 to 7.0, catch per unit effort was found to be more highly correlated with calcium ion concentration than with pH level (Frenette and Richard 1986). Bluegill in my study have demonstrated greater acid tolerance in hard water

that in soft water at all pH levels tested. Recruitment in natural populations will undoubtedly be dependent on both the pH and the water hardness level.

Growth and Yolk-sac Absorption

Growth was unaffected by pH and water hardness levels through day four post-hatch (Table 11). Because bluegill yolk-sac larvae were not fed during the experiment, growth was due solely to the conversion of yolk to tissue. The mean length at hatching (4.0 mm) and on day 5 (5.3 mm) are similar to those found by investigators of bluegill embryology, suggesting that growth during the yolk-sac period is not affected by low pH. In other laboratory studies, some of which included exogenous feeding, yolk-sac larvae of Atlantic salmon (Peterson and Martin-Robichaud 1986), brook trout (Menendez 1976, Baker and Schofield 1982, Kwain and Rose 1985), rainbow trout (Nelson 1982, Thomsen et al. 1988) and white sucker (Baker and Schofield 1982) exhibited decreased growth in acid media.

The short test duration (four days) used in this study may be inadequate to detect acid effects on growth. Other studies investigating the effects of low pH on growth have used salmonid species. Salmonids take up to four weeks to absorb their yolk sacs (Johansson et al. 1977), whereas in bluegill, exogenous feeding begins at less than one week from hatching. Salmonids, because they rely on yolk metabolites much longer than bluegill, and use their yolk reserves for differentiation and growth, may more sensitive to acid related anomalies in yolk absorption than are bluegill. A more realistic estimate of growth in bluegill larvae in acidic conditions may result from

extending the test duration to include exogenous feeding and examine the lengths of larval bluegill at maximium growth.

Other investigators reported that growth at low pH was affected by calcium levels. A temporary reduction in growth of adult brook trout in pH 5.0 in soft water did not occur in hard water (Mount et al. 1988). Thomsen et al. (1988) found that as acidity was reduced from pH 7.0 to 5.0 growth of rainbow trout was reduced by 6 percent in hard water (150 mg/l Ca) and 9 percent in soft water (1 mg/L Ca). Conversely, differences in growth of yolk-sac rainbow trout could not be attributed to differences in water hardness, growth was reduced equally in soft and hard water at pH levels below 5.0 (Nelson 1982).

The response in growth of fish to low pH appears variable and subject to the length of the acclimation period and life stage. Kwain and Rose (1985) reported that a persistent decrease in the growth of brook trout alevins occurred only at pH 4.5; at pH 5.0 the reduction in growth observed on day 50 was only temporary and did not exist in juveniles on day 123 of the experiment. In other studies with brook trout, Mount et al. (1988) and Menendez (1976) also found only temporary reductions in growth at pH 5.0. Reduced growth has been attributed to increased energy demand associated with ionoregulatory difficulties in acid water (Lee et al. 1983) and low calcium water (Eddy 1975). Mount et al. (1988) reported that initial depressions in blood osmolality and Na concentration observed on day 41 were apparently compensated for by day 97 in all but the most severe treatment (pH 4.97, 47 ug/L Al, 0.05 mg/L Ca). Growth compensation may be related to acclimation of the ionoregulatory systems to sublethal levels of pH.

Inter and intraspecific competition is an important growth regulating factor that can be as important as acid toxicity for adults of acid-tolerant species at sublethal pH levels. In six circumneutral (6.7 to-7.5) lakes and five acidic (5.1 to 6.0) lakes, in northern Wisconsin, bluegill growth and condition were found to be related to density and not to pH (Weirner and Hanneman 1982). Acidic conditions can provide for a competitive release of acid tolerant species by eliminating more sensitive ones. Increases in growth at low pH has been reported in laboratory (Hill et al. 1988) and field studies (Beamish 1976, Ryan and Harvey 1980, Lappalainen et al. 1988) presumably as a result of reduced competition among surviving acid-tolerant individuals or species.

Although growth of yolk-sac bluegill larvae was not reduced as pH or water hardness declined, decreased yolk sac volumes were evident under these conditions in experiment II (Table 13). Bluegill larvae held at low pH and water hardness levels consumed more energy (yolk) while achieving lengths comparable to larvae reared in circumneutral pH levels and hard water. Ion regulation consumes more energy in acid water (Lee et al. 1983), and in soft water (Eddy 1975) than in neutral, hard water conditions. Regeneration of acid damaged tissues, especially gill epithelium is associated with increased energy costs (Daye and Garside 1980, McDonald 1983b). The decreased yolk sac volume observed for larvae exposed to a combination of acid and soft water in this study probably was related to the increased cost of ionoregulation, and perhaps the regeneration of acid damaged tissues.

In contrast, other investigators reported increased yolk sac size at low pH (Johannessen and Kilstrom 1975, Menendez 1976, Brown and Lynam 1981, Baker and Schofield 1982, Kwain and Rose 1985, Peterson and Martin-Robichaud 1986). Poor

yolk-sac utilization indicates decreased capacity to metabolize yolk or transfer yolk metabolites to developing tissues (von Westernhagen 1988). Increased yolk-sac size in acid exposed larvae may indicate that the ability of these larvae to mediate the effects of acid through mobilization of their energy reserve (Lee et al. 1983) had been surpassed. A decrease in the ability of larvae to utilize their yolk sac may be directly related to holding them at lethal pH levels, whereas increased consumption of the yolk reserve may be indicative of sublethal pH levels. In experiment I, visual observations revealed that bluegill held at lethal pH levels (below 4.2) and soft water had larger yolk-sacs than controls, whereas larvae held at sublethal levels of pH (pH's 5.5 to 4.3) had smaller yolk-sacs than those of the controls. Atlantic salmon alevins reared at pH 4.5 had one-third of their yolk-sac left after 90 days, while those held in pH 5.1 had only four to five percent of their yolk sacs remaining (Peterson and Martin-Robichaud 1986). A pH of 4.5 is below the LL50 (pH 4.7) reported for Atlantic salmon in soft water conditions (Lacroix 1985). Brook trout at pH 5.0 took 21 more days than did the controls to absorb their yolk sacs and at this pH there was 100 percent mortality in three months. Northern pike sac fry reared at pH 4.2 had larger yolk sac than those reared at pH 5.2 and 6.8, but 96 percent mortality was observed in eight days (Johannessen and Kihlstrom 1975). Brown and Lynam (1981), however, report that brown trout survival was nearly 100 percent at pH 4.5, and also report that prolarvae had larger yolk sac than controls at this pH.

Yolk absorption is affected by low pH, apparently it is increased at sublethal levels and inhibited at lethal pH levels. Premature consumption of the yolk reserve may result in decreased larval survival under natural conditions (von Westernhagen 1988). Larvae have a limited time to encounter and capture suitable prey following yolk absorption (Blaxter 1969). The ability of larvae to efficiently use yolk reserves is a

prerequisite for survival; larvae unable to use these reserves for differentiation and growth may be unprepared for exogenous feeding at the completion of yolk absorption (von Westernhagen 1988). The results of the present experiment are inconclusive because testing throughout the conversion to exogenous feeding was not examined. However, early consumption of yolk reserves by bluegill larvae at pH levels below 5.5 and 5.1 in hard and soft water, respectively, combined with decreased activity suggests bluegill held at low pH levels may be less successful in initiating exogenous feeding.

Behavior and Physical Abnormalities

In this study, bluegill larvae at pH levels of 5.5 or lower were less active than those in controls. The larval swim-up period was not delayed, but the swimming movements of larvae in treated aquaria were more halting and erratic than those of the controls. Yolk-sac larvae of rainbow trout also exhibited a decrease in activity at pH 5.5 (Weiner et al. 1986). Ellgaard and Gilmore (1985) measured the activity of bluegill (4 cm T.L.) ten days after exposure to pH 4.0 and found they were only 25 percent as active as they were when exposed to pH 7.5. In a preference/avoidance test, eight-week old bluegill actively avoided pH levels as high as 5.94 (Ramey and Colten 1986). White sucker (Beamish 1972), Atlantic salmon (Daye and Garside 1977,1979), Pacific salmon (Rombough 1983), Arctic char (Jones et al. 1987), and wild and domestic brook trout (Jorhal and Benson 1987), all have exhibited lethargic and/or uncoordinated swimming behavior in response to acid exposure. Other reported behavioral responses to acid conditions include: decreased attraction to

food (Jones et al. 1987), reduced feeding (Beamish 1972, Jones et al. 1987), hyperactivity when disturbed (Jones et al. 1987) or prior to death (Beamish 1972), and reproductive failure by gravid females (Mount 1973).

Behavioral responses result from the integration of various systems within the organism, and represent a powerful tool in assessing subtle (sublethal) physiological or biochemical effects. Moreover, because most animal behavioral activities are highly adaptive to environmental variables, significant alteration in these patterns could adversely affect survival and reproduction (Rand 1977). Studies have shown that behavior is a sensitive indicator of toxicity (Henry and Atchison 1979, Rand 1977); however, it has not been widely used in aquatic toxicology, due to a paucity of knowledge concerning the behavior of aquatic organisms. Even the behavior of species routinely used in acute, early life stage, or chronic tests has not been characterized. No standardized techniques are currently available for quantifying behavior (Rand 1977). Therefore, while some studies report locomotor abnormalities associated with sublethal levels of acid, a clear interpretation of the significance these observations has not been established (Beamish 1972, Daye and Garside 1977, 1979, Rombough 1983, Ellgaard and Gilmore 1985, Ramey and Colten 1986).

Ellgaard and Gilmore (1985) suggested that an observed reduction in the activity of bluegill exposed to an acid environment resulted from impaired oxygen uptake by reduced oxygen carrying capacity of hemoglobin or heavy mucus covering on the gills. Histological examinations of trout alevins by Daye and Garside (1980) revealed that acid exposure resulted in damage of brain and eye tissue. Since locomotion results from the integration of the nervous, sensory, and muscle systems (Rand 1977), depressed locomotor activity may resulted from damage to these systems,

impaired respiration or both. Few investigators have directly related aberrant behavior to physiological effects; Jones et al. (1987) found significant correlations between blood parameters (plasma protein and glucose levels) and activity in acid exposed Arctic char (*Salvelinus alpinus*).

The effects of adverse behavioral alterations to acidic conditions may have important implications for natural populations. Reduced activity in larvae could be particularly harmful when exogenous feeding begins. Food acquisition in bluegill larval is complex and involves five acts: orientation, fixate, lunge, snap, and bite (Brown and Colgan 1984). Fry at first feeding must make more attempts to capture prey than older fry because their success rate is generally lower (Brown and Colgan 1984). Reduction in activity and coordination at this life stage will therefore, probably result in lower prey consumption and survival in young bluegill. Feeding fry must also avoid predation by fleeing to shelter, performing evasive action, or showing aggressive defense posture (Rand 1977). All of these actions require complete locomotor ability, impairment of this ability adversely influences survival.

Larval bluegill (5.0 mm S.L) were examined microscopically for gross morphological abnormalities at the completion of experiment II. Developing reliable criteria for abnormal features was difficult at this stage of development, because the size of physical structures was small preventing the full expression of abnormality and its detection. Gross abnormalities were evident at low pH (4.5). Eroded fins were obvious in 50 percent of the larvae, exophthalmos was apparent in 40 percent, and lordosis of the spine was observed in 20 percent of bluegill larvae (Table 13). Clearly sublethal abnormalities would decrease the ability of the larvae to survive in the natural environment and may prove lethal over a longer duration. At high pH levels,

the proportion of physical aberrations was diminished and acid affects, if any existed, were not detectable.

Spinal deformities have been observed in yellow perch at pH 4.0 to 4.5 (Runn et al. 1977), and in white sucker at pH 5.0 (Trojnar 1977). The spinal deformities in yellow perch were thought to be a result of prolonged egg encasement, perhaps aggravated by smaller inner egg volume and reduction of the diffusion of metabolites through the perivitelline fluid, as a result of low pH levels, rather than disturbance of early organogenesis (Runn et al. 1977). Prolonged egg encasement probably was not the cause of bluegill larval spinal deformities in my study because incubation periods lasted only two days at lower pH levels, and many of these larvae were partially hatched, not confined in the egg capsule for extended periods. Except for Daye and Garside (1980), who found that salmon alevins incubated at pH 4.0 had less differentiated eye lens fibers and severe sloughing of lens epithelium, no other literature reports of exphthalmos in fish held at low pH were found.

While it is important to record obvious moribund deformities, it does not seem practical or particularly useful to try to evaluate more subtle deformities at this stage of bluegill development; mainly because bluegill hatch and absorb their yolk sac at a small size. Bluegill averaged 5.3 mm in length at yolk-sac absorption, while brook trout examined by Kwain and Rose (1895) were 30.5 mm in size. The criteria developed for physical abnormalities in this study were not, in themselves, particularly definitive, but in combination with behavioral observation and other test end points (depressed yolk-sac absorption) added useful information. Morphological examination would be more appropriate in 30 day partial-life cycle tests. In the larger juvenile fish, deformities would be more obvious, and, perhaps so to would be the

implications for survival. Histological assessments may yield more detailed information about toxic acid levels, ionoregulation, and the modes of acid toxicity and resistance in larval fish.

Field and Laboratory Comparison

There are important differences between whole lake acidification and the present field experiment which consisted of a short-term, embryo-larval toxicity test conducted *in situ*. The former is able to assess fate, and chronic effects on a fish assemblage (Sanders 1985), while the latter provided an estimate of the acid level that will cause irreversible harm to a single species, in this case bluegill, under conditions that were more representative of the natural environment; and provides a standardized method for evaluating effects of various dilution waters on acid toxicity (Parish 1985, Macek et al. 1978).

Field verification of acid tolerance in bluegill through whole lake acidification was impossible at Flat Top Lake, due to political, ethical, and practical considerations. *In situ* toxicity testing represented the next best approach for simulating the natural setting, and offered the advantage of conducting an experiment with treatments, controls, and replication. *In-situ* toxicity tests with early life stages of fish now are being used more frequently in an attempt to make experimental results more realistic and predictive in natural environments (de Lafontaine and Leggett 1987).

In the natural environment, the effect of acidification on water chemistry is influenced by the lithology and mineralogy of the surrounding watershed, and by factors such as reaction kinetics, the proportion of groundwater and overland flow inflows, vegetative cover, and land use practices (Norton 1982). The response of fishes to acidification is related to the dissolution of metals such aluminum, manganese, zinc, lead, and copper from the surrounding soils due to low pH (Baker and Schofield 1982), and mitigative effects of calcium (Hulsman et al. 1983, Johnson et al. 1987).

Lethal levels of acid predicted from laboratory experiments often deviate from those of field studies (Muniz and Leivestad 1980, Kennedy 1980, Hulsman et al. 1983, Jordahl and Benson 1987). In part, these deviations arise from differences in the chemical composition of the natural and the laboratory dilution water. The interdependence of acid toxicity and ion concentrations suggests that laboratory experiments should be performed with dilution water containing realistic levels of dissolved ions in order to accurately predict the effects of acid precipitation on natural ecosystems (Leivestad 1982). In my study, the on-site toxicity test was conducted using Flat Top Lake as a source of dilution water for establishing treatment pH levels. The Flat Top Lake watershed contains soils weathered from acid sandstone, siltstone and shale; the lake water is low in calcium and metal ions, and is considered to be acid sensitive (alkalinity<25 mg/L $CaCO_3$). Water from Flat Top Lake was similar to the reconstituted soft water used in laboratory experiments (Tables 2,3,4).

Hatching followed similar patterns in lake and soft water laboratory experiments. Delayed and partial hatching were observed in both experimental conditions at similar pH levels (Figures 2,3,4). In each case, hatching was reduced only in the most

severe treatments. In the on-site experiment, the pH which caused approximately 50 percent mortality (pH 4.7) was nearly identical to the laboratory LC50 (pH 4.67) in soft water. In the field experiment, growth parameters was independent of pH treatment levels (Table 17). The consistency between on-site and laboratory results estimates of acid toxicity in soft water suggests the study was representative of the impact of acidification on embryo-larval bluegill.

The level of acid that will cause significant mortality rates in aquatic organisms can vary as much as 1 to 2.5 pH units, depending on species, life stage, and water hardness (Tables 18,19). Protecting even a single species can not be done effectively only by considering a single pH level independent of other conditions. Spatial and temporal heterogeneity among aquatic environments provide a variety of conditions that can either intensify (metals and low water hardness) or mitigate (high water hardness) acid effects. Bluegills are adversely affected at a pH range of 4.0 to 5.7 depending on life stage and water hardness.

The results of the laboratory experiments in this study suggest that increased water hardness can improve embryo-larval bluegill survival in acid conditions (Figure 5). In an acidified stream, rainbow trout embryos incubated in limestone enclosures exhibited increased survival (Gunn and Keller 1984). Hutchison and Sprague (1989) added dolomite to a solution of acid and a combination of three metals (aluminum, zinc and copper) increasing hardness from 5.1 mg/L to 17.2 mg/L $CaCO_3$ and pH from 5.84 to 6.84 and found that in the neutralized solution, metal lethality was reduced by a factor of 2.4. In lakes where recruitment failure has been occurring due to the effects of acidification, liming (addition of $CaCO_3$) restored recruitment in resident fish populations (Nyberg 1984, Eriksson et al. 1983). Lakes that were fishless due to

acidification, supported self-reproducing populations of reintroduced fish species after liming (Fraser et al. 1985, Hasselrot and Hultberg 1984). Although liming acidified waters is only a temporary solution to the problem of acid deposition, it can help sustain some fish populations until the source of the ultimate problem, sulfur and nitrogen oxide emission, is reduced.

Toxicity Testing Considerations and Recommendations

In the field experiment, too many (50) eggs held in each egg incubation chamber made daily counts difficult, especially as larvae became more active. In laboratory experiments, eggs were separated and fewer (10) eggs were held in six small incubation chambers to facilitate efficient counting. In both field and laboratory experiments, closures (field tests) or lids (laboratory tests) on egg incubation chambers physically damaged some larvae. Upon hatching the larvae were small (4.0 mm) and thigmotactic, preferring to hide in crevasses created by the closures. Opening the incubation chambers for inspection caught and crushed the larvae in the crevasses. This problem was never completely solved and physical damage to larvae occurred in all experiments, although it occurred considerably less in laboratory tests. Egg incubation chambers that float at the surface with screening on the bottom would eliminate the need for closures and yet would keep groups of eggs and larvae separated, facilitating daily counts. While mortality due to physical damage made some calculations difficult, it did not compromise the results. Subjects removed from an assay because of accidental death not attributable to the toxicant, may be omitted

from the data without introducing bias or compromising the validity of the test according to Finney (1971).

The use of eggs that were naturally spawned in Flat Top Lake prevented water hardening at treatment pH levels, and this is potentially an important factor in characterizing acid toxicity. Also, the eggs collected from even a single nest were not always at the same stage of development. Identifying, separating, and staging the eggs was time consuming, involved some error, and probably injury. Separating eggs increased handling which could have represented an additional source of egg mortality. Artificial spawning of bluegill from a naturally reproducing population in a soft circumneutral or slightly acidic environment would be ideal. The effects of holding the parental stock in low ionic and low pH or circumneutral conditions, and water hardening eggs at treatment pH levels should be considered.

In soft water, accurate pH measurements were difficult to make because of the low electrical potential of the media. The sleeve-junction universal glass electrode (Fisher Model 13-639-61) was found to give stable readings in a relatively short time period (under three minutes), and is recommended.

High levels of sulfate in the hard water solutions resulted from the addition of sulfate salts and sulfuric acid. Ellgaard and Gilmore (1985) examined the effect of the anions liberated from acid solutions by reducing the pH to 3.0 with H_2SO_4 and then neutralizing the test solution to 7.5 with NaOH, and found no mortality in juvenile bluegill exposed to the neutralized water. In distilled water, sulfate ions can be toxic to fish. Goldfish died in five days when kept in deionized water and sulfate (1.0 meq/L Na_2SO_4), but survived in deionized water alone (DeRenzis and Maetz 1973). McWilliams (1983) found that sulfuric acid leached calcium from gill membranes at a

much faster rate than either nitric acid or hydrochloric acid in distilled water. Graham and Wood (1981) found that sulfuric acid was less toxic than hydrochloric acid in rainbow trout at all pH levels in hard water. In soft water, a similar relationship among acids was evident only for pH levels below 4.0; at pH 4.0 and greater sulfuric acid was more toxic in soft water. Apparently, in acidified soft water the sulfate ion is a potentiator of other stress, because the control fish were unaffected by similar sulfate levels (Graham and Wood 1981). Control larvae in my experiments appeared unaffected by the sulfate levels in hard or soft water, therefore, it is assumed that the sulfate ion itself was not toxic, although it can increase acid toxicity in distilled or extremely soft water.

Estimates of partial hatching showed wide variability between experiments. The variability may have been a function of the examination interval (24 hours). More accurate assessments of partial hatching incidence may be obtainable through the use of more frequent examinations.

Several attempts were made to conduct on-site toxicity tests with yellow perch eggs at Flat Top Lake. Yellow perch spawn in the spring at ice-out (March or April in West Virginia). Perch were caught in trap nets and sexes were held separately in floating enclosures at the lake until eggs were ripe. Artificially spawned eggs were used to begin on-site tests, however, cold weather and unseasonal snow storms resulted in the death of all embryos. A more practical method of characterizing yellow perch embryo-larval acid toxicity would be to conduct tests in environmentally controlled rooms in a laboratory setting given the variable weather condition during the spawning season.

Literature Cited

Alderdice, D.F. 1988. Osmotic and ionic regulation in teleost eggs and larvae. pp. 163-251. *In* W. S. Hoar, and D. J. Randal (eds.). Fish physiology: The physiology of the developing fish. A. Eggs and larvae. Academic Press Inc., San Diego, Ca.

American Public Health Association, American Water Works Association and Water Pollution Control Federation. 1975. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, Washington, D.C.

ASTM (American Society for Testing and Materials). 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. No. E 729-80. Annual book of ASTM Standards. 11.01:285-309.

ASTM (American Society for Testing and Materials). 1988. Standard guide for conducting early life-stage toxicity tests with fishes. No. E 1241-88 Annual Book of ASTM Standards. 11.04:769-794.

Arnold, D. E., R. W. Light, and E. A. Paul. 1985. Vulnerability of selected lakes and streams in Middle Atlantic region to acidification: A regional survey. U. S. Fish and Wildlife Service Biological Report 80(40.19).

Baker ,J. P., and C. L. Schofield. 1982. Aluminum toxicity to fish in acidic waters. Water Air Soil Pollut. 18:289-309.

Beamish, R. J. 1972. Lethal pH for the white sucker *Catostomus commersoni* Lacepede. Trans. Am. Fish. Soc. 101:355-358.

Beamish, R. J. 1976. Acidification of lakes in Canada by acid precipitation and the resulting effects on fishes. Water Air Soil Pollut. 6:501-514.

Beamish, R. J., W. L. Lockhart, J. C. Van Loon, and H. H. Harvey. 1975. Long-term acidification of a lake and resulting effects on fish. Ambio 4:98-102.

Bell, G. R., G. E. Hoskins, and J. W. Bagshaw. 1969. On the structure and enzymatic degradation of the external membrane of the salmon egg. Can. J. Zool. 47:146-178.

Benoit, D. A., F. A. Puglisi, and D. L. Olson. 1982. A fathead minnow, *Pinephales promelas*, early life stage toxicity test method, evaluation and exposure to four organic chemicals. Environ. Pollut. 28A:189-197.

Birge, W. J., L. A. Black, and A. G. Westerman. 1985. Short- term fish and amphibian embryo-larval tests for determining the effects of toxicant stress on early life stages and estimating chronic value for single compounds and complex effluents. Environ. Toxicol. Chem. 4:804-821.

Blaxter, J. H. S. 1969. Development of eggs and larvae. pp. 177-252. *In* W. S. Hoar, and D. J. Randal (eds.). Fish physiology. Vol. 3. Academic Press, NY.

Brown, D. J. A. 1981. The effects of various cations on the survival of brown trout, *Salmo trutta*, at low pHs. J. Fish Biol. 128:31.

Brown, D. J. A. 1982. The effect of pH and calcium on fish and fisheries. Water Air Soil Pollut. 18:343-351.

Brown, D. J. A., and P. W. Colgan. 1984. The onotgeny of feeding behaviour in four species of centrarchids fishes. Behav. Process. 9:395-411.

Brown, D. J. A., and S. Lynam. 1981. The effect of sodium and calcium concentrations on the hatching of eggs and the survival of the yolk sac fry of brown trout, *Salmo trutta* L., at low pH. J. Fish Biol. 19:205-211.

Canter, L. W. 1986. Acid rain and dry deposition. Lewis Publishers, Inc. Chelsea, Mi.

Carrick, T. R. 1979. The effect of acid water on the hatching of salmonid eggs. J. Fish Biol. 14:165-172.

Cogbill, C. and G. Likens. 1974. Acid precipitation in Northeastern United States. Wat. Resourc. Res. 10:1133-1137.

Coughlan, D. J., and S. P. Gloss. 1984. Early morphological development of gills in smallmouth bass (*Micropterus dolomieui*). Can. J. Zool. 62:951-958.

Craig, G. R., and W. F. Baksi. 1977. The effects of depressed pH on flagfish reproduction, growth and survival. Wat. Res. 11:621-626.

Daum, R. J. 1970. A revision of two computer programs for probit analysis. Bull. Entomol. Soc. Am. 16:10-15.

Daye, P. G., and E. T. Garside. 1977. Lower lethal levels of pH for embryos and alevins of Atlantic salmon, *Salmo salar* L., Can. J. Zool. 55:1504-1508.

Daye, P. G., and E. T. Garside. 1979. Development and survival of embryos and alevins of the Atlantic salmon, *Salmo salar* L., continuously exposed to acidic levels of pH from fertilization. Can. J. Zool. 57:1713-1718.

Daye, P. G., and E. T. Garside. 1980. Structural alterations in embryos and alevins of the Atlantic salmon, *Salmo salar* L. induced by continuous or short term exposure to acidic levels of pH. Can. J. Zool. 58:27-43.

de Lafontaine, Y., and W. C. Leggett. 1987. Evaluation of *in situ* enclosures for larval fish studies. Can. J. Fish. Aquat. Sci. 44:54-65.

DeRenzis, G., and J. Maetz. 1973. Studies on the mechanism of chloride absorption by the goldfish gill relation with acid-base regulation. J. Exp. Biol. 59:339-358.

Dively, J., J. Mudge, W. Neff, and A. Anthony. 1977. Blood PO_2 , PCO_2 and pH changes in brook trout (*Salvelinus fontinalis*) exposed to sublethal levels of acidity. Comp. Biochem. Physiol. 57A:347-351.

Eddy, F. B. 1975. The effect of calcium on gill potentials and on sodium and chloride fluxes in the goldfish, *Carassius auratus*. J. Comp. Physiol. 96:131-142.

Ellgaard, E. G., and J. Y. Gillmore III. 1985. Effects of different acids on bluegill sunfish, *Lepomis machrochirus* Ratinesque. J. Fish Biol. 25:133-137.

Eriksson, F, E. Hornstrom, P. Mossberg, and P. Nyberg. 1983. Ecological effects of lime treatment of acidified lakes and streams in Sweden. Hydrobiologia 101:145-164.

Finney, D. J. 1971. Statistical methods in biological assay. Charles Griffin & Company LTD., London.

Fraser, J. E., W. P. Saunders, Jr., S. L. von Oettingen, D. L. Britt, and H. U. Sverdrup. 1985. Ecological effects of base addition to lake ecosystems. Reported to General Research Corporation, McLean, Va. International Science and Technology, Inc., Reston, Va.

Frenette, J. J., and Y. Richard. 1986. Fish responses to acidity in Quebec lakes: a review. Water Air Soil Pollut. 30:461-475.

Fritz, E. S. 1980. Potential impacts of low pH on fish and fish populations. Biological Services Program. Air Pollution and Acid Rain Report No. 2. FWS/OBS-80/40.2.

Graham, M. S. and C. M. Wood. 1981. Toxicity of environmental acid to the rainbow trout: Interactions of water hardness, acid type, and exercise. Can. J. Zool. 59:1518-1526.

Grizzle, J. M., A. C. Mauldin, D. Young, and E. Henderson. 1985. Survival of juvenile striped bass (*Morone saxatilis*) and Morone hybrid bass (*M. chrysops* x *M. saxatilis*) increased by addition of calcium to soft water. Aquaculture 46:167-171.

Gunn, J., and W. Keller. 1984. *In situ* manipulation of water chemistry using crushed limestone and observed effects on fish. Fisheries 9:19-24.

Hagenmaier, H. E. 1974. The hatching process in fish embryos. IV. The enzymological properties of purified enzyme (chorionase) from the hatching fluid of rainbow trout *Salmo gairdneri* Rich. Comp. Biochem. Physiol. 49B:313-324.

Haines, T.A. 1981. Acid precipitation and its consequences for aquatic ecosystems: A review. Trans. Am. Fish. Soc. 110:670-691.

Haines, T. A., and J. B. Baker. 1986. Evidence of fish population responses to acidification in the eastern United States. Water Air Soil Pollut. 31:605-629.

Harvey, H.H. 1982. Population responses of fishes in acidified waters. pp. 227-241. *In* T. A. Haines, and R. E. Johnson (eds.). Acid Rain/Fisheries. Proc. Int. Symp. Acid. Precip. Fish. New York. American Fisheries Society, Bethesda, Md.

Hasselrot B., and H. Hultberg. 1984. Liming Swedish Lakes and streams and its consequences for aquatic ecosystems. Fisheries 9:4-9.

Haya, K. and B. A. Waiwood. 1981. Acid, pH, and chorionase activity of Atlantic salmon (*Salmo salar*) eggs. Bull. Environ. Contam. Toxicol. 27:164-178.

Helfrich, L. A., and D. J. Orth. 1988. Flat Top Lake: Management considerations. Management series No. 6. Dept. Fish and Wildl. Sci. Virginia Polytechnic and State University Blacksburg, Virginia, unpubl. report.

Henry, M. G., and G. J. Atchison. 1979. Behavioral changes in bluegill (*Lepomis macrochirus*) as indicators of sublethal effects of metals. Environ. Biol. Fish. 4:37-49.

Hill, J., R. E. Foley, V. S. Blazer, R. G. Werner, and J. E. Giannon. 1988. Effects of acidic water on young-of-the-year smallmouth bass (*Micropterus dolomieui*). Environ. Biol. Fish. 21:23-29.

Holtze, K. E., and N. J. Hutchison. 1989. Lethality of low pH and Al to early life stages of six fish species inhabiting precambrian shield waters in Ontario. Can. J. Fish. Aquat. Sci. 46:1188-1202.

Horning, W. B., and Weber C. I. 1985. Short-term methods for estimationg the chronic toxicity of effluents and receiving waters to freshwater organisms. U. S. Environmental Protection Agency. Report PB86-158474, National Technical Information Service, Springfield, Va.

Hulsman, P. E., P. M. Powels, and J. M. Gunn. 1983. Mortality of walleye eggs and rainbow trout yolk-sac larvae in low pH water of the La Cloche mountain area, Ontario. Trans. Am. Fish. Soc. 112:680-688.

Hutchison, N. J., and S. B. Sprague. 1989. Lethality of trace metal mixtures to American flagfish in neutralized acid water. Arch. Environ. Contam. Toxicol. 18:249-254

Jagoe, C. H., T. A. Haines, and F. W. Kircheis. 1984. Effects of reduced pH on three life stages of sunapee char *Salvelinus alpinus* Bull. Environ. Contam. Toxicol. 33:430-438.

Johannessen, N., and J. Kihlstrom. 1975. Pikes (*Esox lucius* L.) shown to be affected by low pH values during first weeks after hatching. Environ. Res. 9:12-17.

Johansson. N., P. Runn, and P. G. Milbrink. 1977. Early development of three salmonid species in acidified water. Zoon 5:137-132.

Johnson, D. W., H. A. Simonin, J. R. Colquhoon, and F. M. Flack. 1987. *In situ* toxicity tests of fishes in acid waters. Biogeochemistry 3:181-208.

Jones, K. A., S. B. Brown, and T. J. Hara. 1987. Behavorial and biochemical studies of onset and recovery from acid stress in Arctic char (*Salvelinus alpinus*). Can. J. Fish. Aquat. Sci. 44:373-381.

Jordahl D. M., and Benson. 1987. Effect of low pH on survival of brook trout embryos and yolk-sac larvae in West Virginia streams. Trans. Am. Fish. Soc. 116:807-816.

Kane D. A., and C. F. Rabeni. 1987. The effects of aluminum and pH on the early life stages of smallmouth bass (*Micropterus dolomieui*). Wat. Res. 21:633-639.

Kennedy, L. A. 1980. Teratogenesis in lake trout (*Salvelinus namaycush*) in an experimentally acidified lake. Can. J. Fish. Aquat. Sci. 37:2355-2350.

Kim, Y. U., Y. S. Park, and D. S. Kim. 1987. Development of eggs, larvae and juveniles of bluegill, *Lepomis macrochirus* Rafinesque. Bull. Korean Fish. Soc. 20:24-32.

Kinney, E. C. 1973. Average or mean pH. Progres. Fish-Cult. 35:93.

Kwain, W. 1975. Effects of temperature on development and survival of rainbow trout, *Salvo gairdnerii*, in acid waters. J. Fish Res. Brd. Can. 32:493-497.

Kwain, W., R. W. McCauley, and J. A. MacLean. 1984. Susceptibility of starved, juvenile smallmouth bass, *Macropterus dolomieui*, (Lacepede) to low pH. J. Fish Biol. 25:501-504.

Kwain, W., and G. A. Rose. 1985. Growth of brook trout, *Salvelinus fontinalis*, subject to sudden reduction of pH during their early life history. Trans. Am. Fish. Soc. 114:564-570.

Lacroix, G. L. 1985. Survival of eggs and alevins of Atlantic salmon (*Salvo salar*) in relation to the chemistry of interstital water in redds in some acidic streams of Atlantic Canada. Can. J. Fish. Aquat. Soc. 42:292-299.

Lappalainen, A., M. Rask, and P. J. Vuorinen. 1988. Acidification affects the perch, *Perca fluviatilis*, populations in small lakes of southern Finland. Environ. Biol. Fish. 21:231-239.

Lee, R. M., and S. D. Gerking. 1980. Sensitivity of fish eggs to acid stress. Wat. Res. 14:1679-1681.

Lee, R. M., S. D. Gerking, and B. Jezierska. 1983. Electrolyte balance and energy mobilization in acid stressed rainbow trout, *Salmo gairdneri*, and their relation to reproductive success. Environ. Biol. Fish. 8:115-123.

Leino, R. L., J. H. McCormick, and K. M. Jensen. 1987. Changes in gill histology of fathead minnows and yellow perch transferred to soft water or acidified soft water with particular reference to chloride cells. Cell Tissue Res. 250:389-399.

Leivestad, H. 1982. Physiological effects of acid on fish. pp.157-164. *In* T. A. Haines, and R. Johnson (eds.). Acid Rain/Fisheries. Proc. Int. Symp. Acid. Precip. Fish., New York. American Fisheries Society, Bethesda, Md.

LLoyd R., and D. H. M. Jordan. 1964. Some factors affecting the resistance of rainbow trout (*Salmo gairdnerii* Richardson) to acid waters. Int. J. Air Wat. Pollut. 8:393-403.

Macek, K., B. W. Birge, F. L. Mayer, A. L. Buikema Jr., and A. W. Maki. 1978. Toxicological effects. pp. 27-32. *In*: J. Cairns Jr., K. C. Dickson, A. W. Maki (eds.). Estimating the hazards of chemical substances to aquatic life. American Society for Testing and Materials. Philadelphia, Pa.

Macek, K. J., and B. H. Sleight. 1977. Utility of toxicity tests with embryos and fry of fish in evaluating hazards associated with the chronic toxicity of chemicals to fishes. pp. 137-146. *In* F. L. Mayer and J. L. Hamelink, (eds.). Aquatic Toxiclolgy and Hazard Evaluation, ASTM STP 634, American Society for Testing and Materials. Philadelphia, Pa.

Marking, L. L., and V. K. Dawson. 1973. Toxicity of quinaldine sulfate to fish. Investigation in fish control. U. S. Fish and Wildlife Service. Report 48. Washington D. C.

McDonald, D., H. Hobe, and C. Wood. 1980. The influence of calcium on the physiological response of the rainbow trout, *Salmo gairdneri* to low environmental pH. J. Exp. Biol. 88:109-131.

McDonald, D. G. 1983a. The interaction of the physiology of the rainbow trout, *Salmo gairdneri*, I. branchial and renal net ion and H+ fluxes. J. Exp. Biol. 102:123-140.

McDonald, D. G. 1983b. The effect of H+ upon the gills of fresh water fish. Can. J. Zool. 61:691-703.

McDonald, D., H. Hobe, and C. Woods. 1980. The influence of calcium on the physiological response of the rainbow trout, *Salmo gairdneri*, to low environmental pH. J. Exp. Biol. 88:109-131.

McDonald, D. G., R. L. Walder, and P. R. H. Wilkes. 1983. The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*, II. Branchial ionregulatory mechanisms. J. Exp. Biol. 102:141-155.

McKim, J. M. 1977. Evaluation of tests with the early life stages of fish for predicting long-term toxicity. J. Fish. Res. Board Can. 34:1148-1154.

McWilliams, P. G. 1983. An investigation of the loss of bound calcium from the gills of the brown trout, *Salmo trutta*, in acid media. Comp. Biochem. Physiol. 74A:107-116.

McWilliam, P., and W. Potts. 1978. The effects of pH and calcium concentrations on gill potentials in brown trout, *Salmo trutta*, J. Comp. Physiol. 126:227-286.

Menendez, R. 1976. Chronic effects of reduced pH on brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 33:118-123.

Miller, R. G. 1981. Simultaneous statistical inference. Springer-Verlag, New York.

Milliken, G., and D. Johnson. 1985. Analysis of messy data volume I. Designed Experiments. Marcel Dekker Inc., New York.

Moriarity, F., A. A. Bell, and H Hanson. 1986. Does p,p'-DDE thin eggshells? Environ. Pollut. 40A:257-286.

Mount, D. I. 1973. Chronic effects of low pH on fathead minnow survival, growth and reproduction. Wat. Res. 7:987-993.

Mount, D. I., and C. E. Stephan. 1976. A method for establishing acceptable toxicant limits for fish - malathion and the butoxyethanol ester of 2,4 D. Trans. Am. Fish. Soc. 96:185-193.

Mount, D. I., J. R. Hockett, and W. A. Gern. 1988. Effects of long-term exposure to acid, aluminum, and low calcium on adult brook trout(*Salvelinus fontinalis*). 2. Vitellogensis and osmoregulation. Can. J. Fish. Aquat. Sci. 45:1633-1642.

Muniz, I. P. and H. Leivestad. 1980. Acidification effects on freshwater fish. pp. 84-92. *In* D. Drablos, and A. Tollan (eds.). Proc. Int. Ecol. Impact Acid Precip., Norway 1980, SNSF project.

Nelson, J. A. 1982. Physiological observations on developing rainbow trout, *Salvo gairdneri*, Richardson, exposed to low pH and varied calcium ion concentrations. J. Fish Biol. 20:359-372.

Norton, S. A. 1982. The effects of acidification on the chemistry of ground and surface waters. *In* T. A. Haines, and R. E. Johnson (eds.). Acid Rain/Fisheries Proc. Inter. Symp. Acid. Precip. Fish. New York. American Fisheries Society, Bethesda, Md.

Nyberg, P. 1984. Effects of liming on fisheries. Phil. Trans. Royal Soc. Land. B305:549-560.

Packer, R. K., and W. A. Dunson. 1970. Effects of low environmental pH on blood pH and sodium balance of brook trout. J. Exp. Zool. 174:65-72.

Packer, R. K., and W. A. Dunson. 1972. Anoxia and sodium loss associated with the death of brook trout at low pH. Comp. Biochem. Physiol. 41A:17-26.

Palmer, R. E., R. J. Klauda, and T. E. Lewis. 1988. Comparative sensitivities of bluegill, channel catfish, and fathead minnow to pH and aluminum. Environ. Toxicol. Chem. 7:505-516.

Parish, P. R. 1985. Acute toxicity tests pp 31-57. *In* G. M. Rand, and S. R. Petrocelli (eds.). Fundamentals of aquatic toxicology. Hemisphere Publishing Corp., Washington, D.C.

Pearce, F. 1987. Acid Rain. New Sci. 5:1-2.

Peterson, R., D. Daye, and J. Metcalfe. 1980. Inhibition of Atlantic salmon (*Salmo salar*) hatching at low pH. Can. J Fish. Aquat. Sci. 37:770-774.

Peterson, R. H. 1984. Influence of varying pH and some inorganic cations on the perivitelline potential of eggs of Atlantic salmon, *Salmo salar* Can. J. Fish. Aquat. Sci. 41:1066-1069.

Peterson, R. H., P. G. Daye, G. L. Lacroix, and E. T. Garside. 1982. Reproduction in fish experiencing acid and metal stress. pp 177-196. *In* J. E. Raymond (ed.). Acid Rain/Fisheries Proc. Inter. Symp. Acid. Precip. Fish. New York. American Fisheries Society, Bethesda, Md.

Peterson, R. H., and D. S. Martin-Robichaud. 1986. Growth and major inorganic cation budgets of Atlantic salmon alevins at three ambient acidities. Trans. Am. Fish. Soc. 115:220-226.

Ramey, B. A., and L. A. Colten. 1986. Effects of acid pH on embryonic and juvenile freshwater fish. University of Kentucky Water Resources Research Institute. U. S. Dept. of Interior Agreement No:14-08-001-G1019-03 (FR 1985) PL 98-242. Research Report 164. Lexington, Ky.

Rand, G. M. 1977. Behavior aquatic toxicology and hazard evaluation. pp. 253-268. *In* F. L. Mayer, and J. L. Hamelink, (eds.). Aquatic Toxiclolgy and Hazard Evaluation, ASTM STP 634, American Society for Testing and Materials. Philadelphia, Pa.

Rand, G. M., and S. R. Petrocelli. 1985. Fundamentals of aquatic toxicology: Methods and applications. Hemisphere Publishing Corp., Washtington, D.C.

Robinson, G., W. Dunson, J. Wright, and G. Mamolito. 1976. Difference in low pH tolerance among strains of brook trout(*Salvelinus fontinalis*). J. Fish Biol. 8:5-17.

Rombough, P. J. 1983. Effects of low pH on eyed embryos and alevins of Pacific salmon. Can. J. Fish. Aquat. Sci. 40:1575-1582.

Rombough, P. J., and J. O. T. Jensen. 1985. Reduced water uptake and resistance to deformation in acie-exposed egg of steelhead, *Salmo gairdneri* Trans. Am. Fish. Soc. 114:571-576.

Rosseland, B. 1980. Physiological responses to acid water in fish. 2. Effects of acid water on metabolism and gill ventilation in brown trout *Salmo trutta* L., and brook trout *Salvelinus fontinalis* Mitchill. pp. 348-248. *In* D. Drablos, and A. Tollan (eds.). Proc. Int. Ecol. Impact Acid Precip., Norway 1980, SNSF Project.

Rudy, P. P., Jr., and W. T. W Potts. 1969. Sodium balance in eggs of the Atlantic salmon *Salmo salar*. J. Exp. Biol. 50:239-246.

Runn, P., N. Johansson, and G. Milbrink. 1977. Some effects of low pH on the hatchability of perch, *Perca fluviatilis* L. Zoon 5:115-125.

Ryan, P. M., and H. H. Harvey. 1980. Growth responses of yellow perch, *Perca flavescens*, to lake acidification in the La Cloche mountain lakes of Ontario. Environ. Biol. Fish. 5:97-108.

Sanders, W. M. III. 1985. Field validation pp.601-618 *In* G. M. Rand, and S. R. Petrocelli (eds.). Fundamentals of aquatic toxicology. Hemisphere Publishing Corp., Washington, D.C.

SAS Institute Inc. 1985. SAS User's guide: Statistics, version 5 edition. SAS Institute Inc., Cary, NC.

Schindler, D. W., 1988. Effects of acid rain on freshwater ecosystems. Science 239:149-157.

Schindler, D., R. Hesslein, R. Wagerman, and W. Broecker. 1980. Effects of acidification on mobilization of heavy metals and radioneuclides from sediments of a freshwater lake. Can. J. Fish. Aquat. Sci. 37:373-377.

Schofield C. L. 1982. Historical changes in the United States to decrease in surface water pH. pp. 57-67. In Haines, T. A., and R. E. Johnson (eds). Acid Rain/Fisheries Proc. Inter. Symp. Acid. Precip. Fish. New York. American Fisheries Society, Bethesda, Md.

Seip H. M. 1980. Acidification of freshwater - sources trout, *Salvelinus fontinalis* Mitchill. pp. 348-349. *In* Drablos D. and A. Tollan (eds.). Proc Int. Ecol. Impact Acid Precip., Norway 1980, SNSF project.

Sheehan, R J., and P. Leonard. 1986. An assessment of the sport fishery, water quality, and weed problem of Flat Top Lake-West Virginia. Dept. of Fisheries and Wildlife Sciences. Virginia Polytechnic and State University, Blacksburg, Va., Unpul. Report.

Smith, L. L. Jr., D. M. Oseid, and L. E. Olson. 1976. Acute and chronic toxicity of hydrogen sulfide to fathead minnow *Pimephales promeles* Environ. Sci. Technol. 10:565-568.

Snyder, D. E. 1983. Fish eggs and larvae. pp 165-197. *In* L. A. Nielsen and D. J. Johnson (eds.). Fisheries techniques. American Fisheries Society, Bethesda, Md.

Stickney, R. R. 1979. Principles of warmwater aquaculture. John Wiley and Sons, Inc., New York.

Swarts, F. A., W. A. Dunson, and J. E. Wright. 1978. Genetic and environmental factors involved in increased resistance of brook trout to sulfuric acid solutions in mine acid polluted waters. Trans. Am. Fish. Soc. 107:651-677.

Swenson, W. A., J. H. McCormick, T. D. Simonson, K. M. Jensen, and J. G. Eaton. 1989. Experimental acidification of Little Rock lake (Wisconsin): Fish research approach and early responses. Arch. Environ. Contam. Toxicol. 18:167-174

Thomsen, A., B. Korsgaard, and J. Joensen. 1988. Effects of aluminium and calcium ions on survival and physiology of rainbow trout, *Salmo gairdneri*, (Richardson) eggs and larvae exposed to acid stress. Aquat. Toxicol. 12(4):291-300.

Thurston, R. V., R. C. Russo, C. M. Fetterolf, Jr., T. A. Edsall, and Y. M. Barber, Jr. (eds.). 1979. A review of the EPA red book: Quality criteria for water. Water Quality Section, American Fisheries Society, Bethesda, Md.

Trama, F. B. 1954. The pH tolerance of the common blue gill *Lepomis macrochirus* Rafinesque. Not. Nat. 256:1-13.

Trojnar, J. 1977. Egg and larval survival of white sucker *Catostomus commersoni* at low pH. J. Fish. Res. Board Can. 34:262-266.

U. S. Dept. of the Interior, U.S. Fish and Wildlife Service. 1988. 1985 National survey of fishing, hunting, and wildlife associated recreation. Washington, D.C.

Ultsch, G. R. 1978. Oxygen consumption as a function of pH in three species of freshwater fishes. Copeia. 272-279.

Vinogradov, G. A., and V. T. Komov. 1985. Ion regulation in the perch, *Perca fluviatilis*, in connection with the problem of acidification of water bodies. J. Ichthyol. 25:53-61.

von Westernhagen, H. 1988. Sublethal effects of pollutants on fish eggs and larvae. pp.253-346. *In* W. S. Hoar, and D. J. Randall (eds.). Fish physiology. Vol.11. The physiology of developing fish. Part A: Eggs and larvae. Academic Press Inc., San Diego, Ca.

Webb, J. R., B. J. Cosby, J. N. Galloway, and G. M. Hornberger. 1989. Acidification of native brook trout streams in Virginia. Wat. Resour. Res. 25:1367-1377.

Weiner, G. S., C. B. Schreck, and H. W. Li. 1986. Effects of low pH on reproduction of rainbow trout. Trans. Am. Fish. Soc. 115:75-82.

Wiener, J. G., and W. R. Hanneman. 1982. Growth and condition of bluegills in Wisconsin lakes: Effects of population density and lake pH. Trans. Am. Fish. Soc. 111:761-767.

Wood C. M., and D. G. McDonald. 1982. Physiological mechanisms of acid toxicity to fish. pp. 197-226. *In* Haines, T. A., and R. E. Johnson (eds.). Acid Rain/Fisheries Proc. Inter. Symp. Acid. Precip. Fish. New York. American Fisheries Society, Bethesda, Md.

World Resources Institute. 1986. An assessment of the resources base that supports the global economy. The World Resources Institute and The International Institute for Environment and Development. SRI:R9455-1. Busil Book Inc., NY.

Yamagami, K. 1966. Some enzymological properties of a hatching enzyme (chorionase) isolated from the fresh water teleost, *Oryzias latipes*. Comp. Biochem. Physiol. 46B:603-616.

Yamagami, K. 1988. Mechanisms of hatching in fish. pp. 447-499. *In* W. S. Hoar, and D. J. Randal (eds.). Fish physiology: the physiology of developing fish. A. Eggs and larvae. Vol. 11. Academic Press, NY.

Yanai, T. 1966. Hatching. pp. 49-58. *In* M. Kume (ed.). Vertebrate embryology (in Japanese). Baifukan Publ. Co., Tokyo. Cited in: Yamagami, K. 1988. Mechanisms of hatching in fish. pp. 447-499. *In* W. S. Hoar, and D. J. Randal (eds.). Fish physiology: the physiology of developing fish. A. Eggs and larvae. Vol. 11. Academic Press, NY.

Zeba, S. G., D. Golomb, J. A. Fay. 1988. Wet sulfate and nitrate deposition patterns in eastern North America. Atmos. Environ. 22:2751-2761.

Appendix

Appendix A. The quantity of salts needed to reconstitute very soft and hard water, and the resultant equilibrium water quality.

	Very Soft	Hard
Salts Required		
$NaHCO_3$ (mg/L)	12.0	192.0
$CaSO_4$ (mg/L)	7.5	120.0
$MgSO_4$ (mg/L)	7.5	8.0
KCL mg/L	0.5	
Resulting Water Quality		
pH[a]	6.7 - 6.9	7.8 - 8.0
Hardness (mg/L $CaCO_3$)	10 - 13	160 - 180
Alkalinity (mg/L $CaCO_3$)	10 - 13	110 - 120

Source: ASTM 1980.

The vita has been removed from the scanned document