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EFFECTS OF ACID pH ON EMBRYONIC AND JUVENILE FRESHWATER FISH

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ABSTRACT

The effects of sulfuric acid on embryos, larvae, and juvenile fish were examined using standard bioassay techniques, as well as in a preference/avoidance behavioral test. The objectives were to compare the sensitivities of the various ages of fish to acid and to assess the use of the behavioral test in a hazard assessment program. In an 8-day static renewal bioassay, embryos and larvae of the fathead minnow were not affected at a pH of 4.92 and above, but pH 3.57 produced complete mortality prior to hatching. In 96-hr acute bioassays, 8-wk juvenile fathead minnows survived 100% at pH's of 5.02 to 7.38, while complete mortality occurred at pH's below 3.90. At pH 4.29, only 15% of the population survived. Similar results were obtained with 12-wk animals. Juvenile bluegill sunfish (8 wk) and fathead minnows (6 and 14 wk) also were exposed to various concentrations of sulfuric acid in a preference/avoidance bioassay. Both 14-wk fathead minnows and 8-wk bluegill sunfish avoided acid pH's below pH 6.0, while the 6-wk fathead minnows avoided all acid levels tested (i.e., pH 6.19 and below). Therefore, the 6-wk fathead minnows appeared to be the most sensitive to acid stress. Based on these findings, juvenile fish, given a choice, would tend to avoid acid levels that might not prove lethal to them. Therefore, the preference/avoidance bioassay should not be used alone but could be an important tool in evaluating sublethal effects in a multistage hazard assessment program.

Descriptors: Acidic Streams
Aquatic Life
Fish Toxins
Hydrogen Ion Concentration

Identifiers: Acute Bioassays
Embryo-Larval Bioassays
Fish Behavior
Preference/Avoidance
Sulfuric Acid

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

One of the more serious environmental problems currently facing maintenance of acceptable water quality in Kentucky and other regions of the United States is that of the increasing acidification of surface waters. Acid mine drainage has lowered the pH of some coal-field streams to less than 3.5 (Swarts, et al., 1978), while acidic precipitation in the northeastern United States and southeastern Canada often ranges between 4.0 and 4.4 (Haines, 1981). Evaluation of effects of this increased acidity on aquatic organisms is essential in determining long-term impact of acid on aquatic ecosystems. Recent reviews by Swarts, et al. (1978), Drablos and Tollan (1980), Fromm (1980), Haines (1981), and D'Itri (1982) have examined physical, chemical, and biological effects of acid. Studies utilizing adult fish have shown that pH values away from neutrality often result in mortality of fish as well as deleterious changes in migration and distribution patterns (Rahel and Magnuson, 1980; Rosseland, et al., 1980; Haines, 1981; Spry, et al., 1981).

In light of the need for additional information on the effects of acid on freshwater environments, the current study was designed to examine the toxic and behavioral effects of low pH on embryonic and juvenile stages of fish. Both acute and sublethal effects were studied. The approach taken was based upon principles observed and put forth by a number of investigators. Currently, aquatic toxicity testing methods center on the determination of toxicant concentrations which produce mortality and otherwise harm aquatic organisms (NAS-NAE, 1973; Cairns, et al., 1978; Rand and Petrocelli, 1985).

However, due to dilution and dispersion, toxicants most likely are present in the aquatic ecosystems at low, subacute concentrations (Sprague, et al., 1965; Kleerekoper, 1976). These low levels of pollutants may exert stresses on fish that result in harmful effects on their growth and development, as well as their physiology and behavior (Ishio, 1966; Sprague, 1968; Mount, 1973; Kleerekoper, 1976; Fromm, 1980; McDonald and Wood, 1981; Alabaster and Lloyd, 1982; Rand and Petrocelli, 1985).

A toxicant which alters the behavior of fish may cause deleterious changes in their reproductive behavior (i.e., courtship), as well as migration and distribution patterns (Rice, 1973; Rand, 1985). Such changes can reduce or limit habitats suitable for spawning and growth, as well as feeding activities. Moreover, embryonic and juvenile fish are usually more sensitive than adults to toxicant stresses (McKim, 1977; Birge, et al., 1981). Therefore, it is essential to maintain water quality at a level which will insure the maintenance and propagation of not only adults but also early developmental stages of fish.

Numerous studies have been performed to ascertain the toxic effects of acidic waters on a wide variety of adult fish species. It is generally accepted that the pH range of 5 to 9 is not usually lethal to most fish (Alabaster and Lloyd, 1982). However, concentrations below 3.5 likely do not support viable fish populations. Between a pH of 4.0 and 4.5, most fish, including salmonids, goldfish, bream, common carp, and fathead minnow, are harmed and usually do not reproduce (Mount, 1973; Dunson, et al., 1977; Swarts, et al., 1978; Fromm, 1980; Alabaster and Lloyd, 1982).

Although the toxicity of acidic natural waters usually is attributed to

reduced pH, other factors should be taken into account. Natural waters with a high bicarbonate content will liberate free carbon dioxide as pH decreases (Fromm, 1980; Alabaster and Lloyd, 1982). This situation often results in levels of carbon dioxide lethal to fish, and sublethal concentrations may increase their sensitivity to a variety of other toxicants. In addition, the toxicity of several pollutants are altered by changes in pH, including nickelocyanide (Doudoroff, 1956), aluminum (Haines, 1981), and possibly iron (Alabaster and Lloyd, 1982).

As has been shown by Birge and Black (1979), Birge, et al. (1974; 1979a, b; 1981), Black, et al. (1982), and McKim (1977), developmental stages of fish are usually more sensitive than adults to a variety of aquatic pollutants. This also appears to be true for acid contamination. Kwain (1975) reported complete mortality of rainbow trout eggs below a pH of 4.5, while yearling trout were less sensitive. Similar results were observed by Menendez (1976) for brook trout when egg viability was reduced below a pH of 5.1, but adult death occurred only below 4.5. Fathead minnow eggs did not survive exposure to a pH of 5.2 or below and survival was reduced significantly at a pH of 5.9 (Mount, 1973). All surviving eggs at this level were abnormal.

Behavioral responses of fish to a variety of toxicants have been reviewed by Jones (1948), Sprague, et al. (1965), Ishio (1966), Sprague (1968), Weir and Hine (1970), Rice (1973), Black and Birge (1980), Hocutt, et al. (1982), Beitinger and Freeman (1983), Giattina and Garton (1983), and Rand (1985). The majority of these studies reported a net avoidance or attraction to the specific toxicant and that this behavior was concentration-

dependent, with the threshold often below the lethal threshold for adult fish. For example, Sprague (1968) found an avoidance threshold value of 5.6 ug zinc/L with rainbow trout. This level was 1% of the lethal threshold concentration. Similar results were reported by Ishio (1966) for goldfish. Black and Birge (1980), utilizing juvenile stages of bluegill sunfish, largemouth bass, and rainbow trout, evaluated preference/avoidance responses to several inorganic and organic toxicants. Results indicated that juvenile fish were significantly attracted to chloroform, dioctyl phthalate, and mercury, while significant avoidance was observed with cadmium, phenol, and zinc. These variations in response have obvious implications on potential distribution of fish. Avoidance behavior may restrict animals to unsuitable habitats for growth, feeding, and spawning, while attraction may lure fish into unhealthy regions, thereby affecting growth, reproduction, or even survival. Although more difficult to document precisely, such behavior has been found in natural populations responding to a variety of pollutants (Sprague, et al, 1965; Saunders and Sprague, 1967; Cherry, et al., 1977; Hocutt, et al., 1982). Stokes and Birkhead (1986) reported that the cyprinid Notropus venustus routinely avoided a pH range of 3.5 to 5.3 in a natural confluence between two creeks of varying pH, and that pH levels of 4 and below were toxic. Such comparisons of toxicity data and sublethal behavioral responses to acid are limited.

The principle objectives of this study included 1) determination of the threshold concentration of acid which elicited avoidance behavior in juvenile fish, 2) identification of effects of acid on the survival of embryonic and juvenile fish, and 3) comparison of a sublethal behavioral test with

traditional embryo-larval and acute bioassays for sensitivity and reliability.

RESEARCH PROCEDURES

Selection and Maintenance of Animal Test Species

Two species of fish were used in this study. Juvenile fathead minnows (Pimephales promelas) and bluegill sunfish (Lepomis macrochirus) were employed in a preference/avoidance study to examine the sublethal behavioral effects of acidic waters. To provide a basis of comparison for behavioral responses, traditional bioassays using embryos, larvae, and juveniles of the fathead minnow also were conducted (see below). Fathead minnow eggs and juveniles were obtained from the Aquatic Biology Section, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Newtown, Ohio, and bluegill sunfish were supplied by the Frankfort National Fish Hatchery, Frankfort, Kentucky. This selection of species was made in part on the basis of economic importance, seasonal availability, the data base available for comparison of test results, and general acceptance as test organisms (Adelman and Smith, 1976; Horning and Weber, 1985; Peltier and Weber, 1985).

Juvenile fish were transported to the laboratory in insulated coolers and were placed in either 10 or 30 gal holding aquaria containing dechlorinated tap water. The fish were maintained for 2 wk in the holding tanks and the dechlorinated tap water was gradually replaced with dilution water (see below). Fish were fed twice daily with #1 trout chow (Ziegler Brothers) ground with a mortar and pestle. Prior to either 96-hr acute bioassays or preference/avoidance studies, the required number of fish were

taken randomly from various holding tanks and were pooled in a common aquarium, where they were held for 24 hr without food.

Dilution Water

Dilution water used in all phases of this study was a synthetic fresh water of moderate hardness (i.e., 80-100 mg/L as $CaCO_3$) recommended by the U.S. Environmental Protection Agency (Peltier and Weber, 1985; Horning and Weber, 1985). Distilled, deionized water was reconstituted by the addition of appropriate quantities of reagent-grade sodium, potassium, magnesium, and calcium salts. This synthetic water was used to hold juvenile fish during the acclimation period as well as to provide the dilution water for 96-hr acute and embryo-larval bioassays and preference/avoidance studies. Physicochemical characteristics of the dilution water are presented in Table 1.

Selection and Analysis of Toxicant

Sulfuric acid (H_2SO_4) was chosen as the toxicant because it is the predominant compound in acid precipitation in the United States. In both 96-hr acute and embryo-larval bioassays (see below), a stock solution of 0.1N was prepared from concentrated sulfuric acid (Fisher Scientific Company). This stock solution was used to titrate dilution water to the appropriate pH for each exposure concentration. Measurements of pH were made just prior to and after a renewal of the test solution using a Corning pH meter (model 10).

Table 1. Physicochemical characteristics of synthetic fresh water used as dilution water in bioassays with sulfuric acid.

Test Condition	Mean[1]	Standard Deviation
pH	7.45	-
Alkalinity (mg/L as $CaCO_3$)	62.0	1.5
Hardness (mg/L as $CaCO_3$)	88.1	4.2
Conductivity (μmhos/cm)	319	30

[1]Means determined from analysis of control water used in each bioassay.

The mean pH of the test solution was based on the average exposure over a 24-hr period and was mathematically determined by conversion of all pH values to hydrogen ion normalities prior to averaging (Kinney, 1973). This approach was taken after noting that only those test solutions near neutrality were buffered to any great extent during a 24-hr period (Figure 1).

In preference/avoidance studies, test solutions were prepared by addition of concentrated sulfuric acid to 40 L of dilution water prior to testing. To determine mean pH exposure, samples of water were taken from three regions of both control and test channels prior to and immediately following the experimental run (see below). These six values for each channel were averaged, and served as the mean test and control exposures for each preference/avoidance study.

The hydrogen ion concentration of the test solutions altered both the alkalinity and conductivity. For all three bioassays, alkalinity and conductivity ranged from 0 to 61 mg/L as $CaCO_3$ and 300 to 600 umhos/cm, respectively, depending upon pH. Other water quality parameters (i.e., temperature, hardness, dissolved oxygen) were unaffected by pH and these data are presented in Table 2.

Procedures for Conducting Preference/Avoidance Tests

Exposure concentrations. Two species of fish were employed in the preference/avoidance study. Juvenile bluegill sunfish, approximately 2 months old (mean length 22.5 mm), were tested at four pH levels ranging from 3.50 to 6.60. Preference/avoidance responses of fathead minnows at

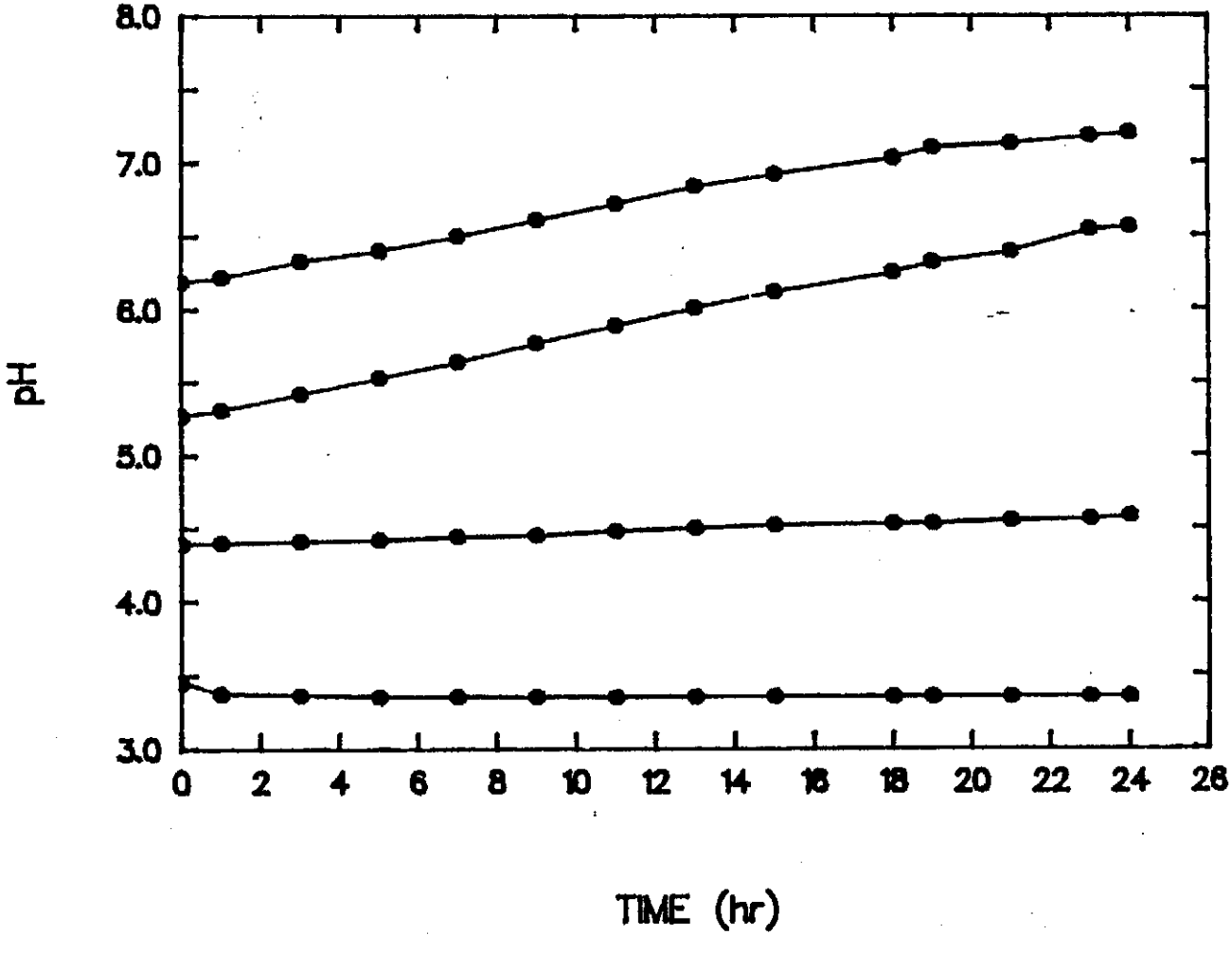

Figure 1. Changes in pH of acidified dilution water over a 24-hr period. Four concentrations, representative of the pH levels tested in 96-hr acute and embryo-larval bioassays, were initiated at 0 hr and monitored for 24 hr, the typical static-renewal period.

Table 2. Water quality parameters observed during 96-hr acute, embryo-larval, and preference/avoidance bioassays.

Bioassay	Test Animal	Age (wk)	Water Quality Parameters (Mean ± S.D.)		
			Temperature (°C)	Hardness (mg/L as $CaCO_3$)	Dissolved Oxygen (mg/L)
96-hr Acute	Fathead Minnow	8	23.4 ± 0.5	84.4 ± 4.6	9.4 ± 1.1
		12	23.8 ± 0.4	83.9 ± 3.7	9.3 ± 0.8
Embryo-Larval	Fathead Minnow	4DPH[1]	24.4 ± 0.1	84.8 ± 0.4	10.2 ± 0.03
Preference/ Avoidance	Bluegill Sunfish	8	22.1 ± 0.3	91.5 ± 6.6	9.5 ± 0.3
	Fathead Minnow	6	24.1 ± 0.4	84.3 ± 6.0	10.6 ± 0.7
		14	24.1 ± 0.3	83.3 ± 5.4	10.5 ± 0.7

[1]Values calculated through 4 days posthatching (4DPH).

approximately 6 wk of age (mean length 12.7 mm) were studied at three different pH levels (i.e., 3.61, 6.05, 6.91), while 14-wk fathead minnows (mean length 29.6 mm) were exposed to four pH levels ranging from 3.76 to 6.68.

Avoidance test system. The design of the overall test system used to evaluate avoidance responses was modified from that described by Black and Birge (1980). The avoidance test chamber was a fluviarium constructed from plexiglass with overall dimensions of 60 cm x 30 cm x 6.5 cm, and a total capacity of 10 L. The size of the fluviarium easily accommodated the test animals.

As shown in Figure 2, the fluviarium was operated on a flow-through principle. Both control and diluent water was synthetic fresh water (see above) and, therefore, had the same physicochemical characteristics. Plexiglass containers (13 gal) served as reservoirs for both control and test waters. A variable speed peristaltic pump (Cole Parmer Masterflex model 7553) was used to deliver both control and test waters to the appropriate channel of the fluviarium at an average rate of 249 mL/min. Flow rate was confirmed by timed volumetric measurements.

Avoidance response test. Prior to testing, juvenile fish were maintained in glass aquaria supplied with synthetic fresh water and recirculating carbon filters. Temperature was maintained between 21-25^{o}C, and stock cultures were continuously aerated, thereby keeping dissolved oxygen at or near saturation. Avoidance tests were conducted at the same temperature at which stock populations were maintained. Water was monitored at regular intervals for temperature, dissolved oxygen, pH, and specific

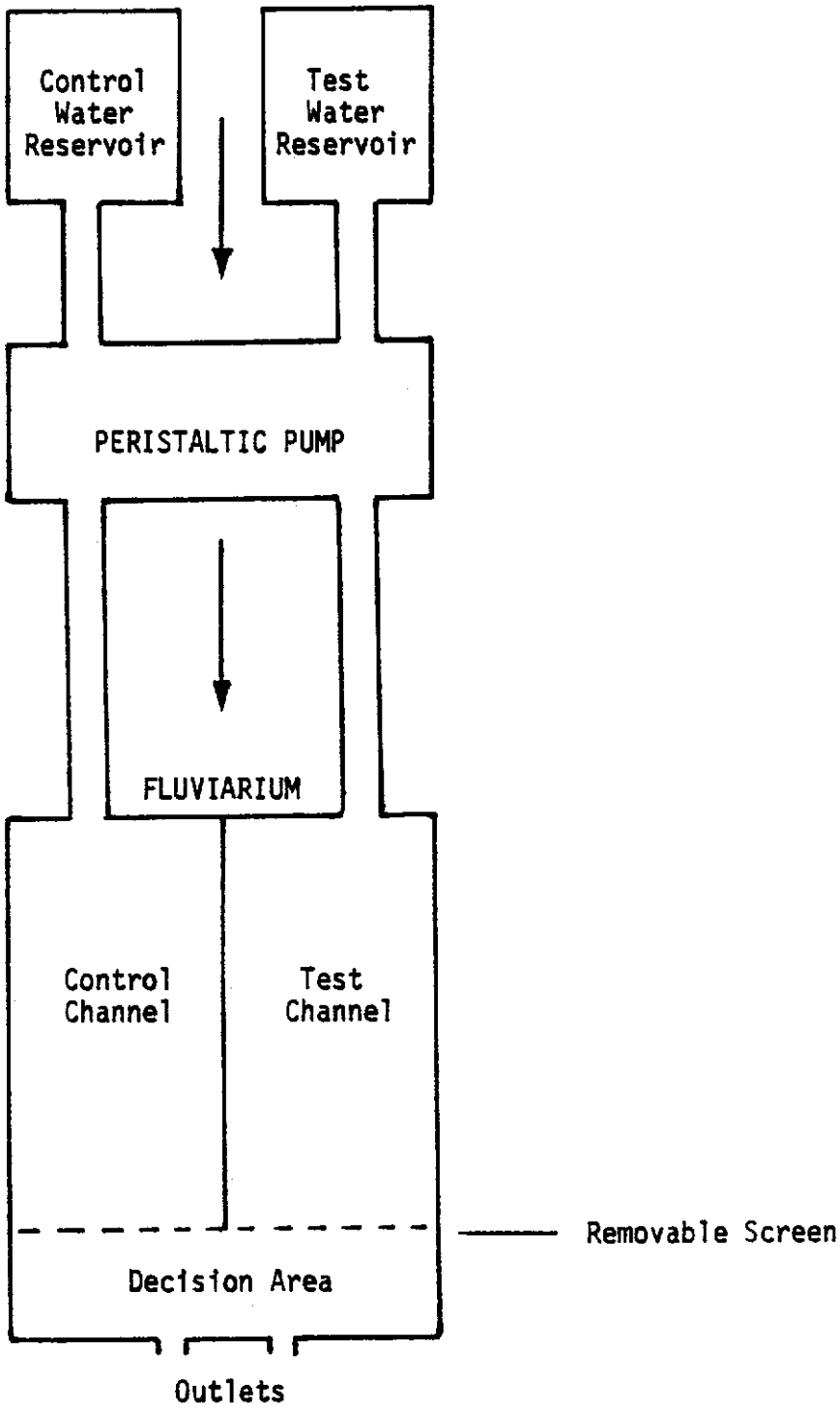

Figure 2. Avoidance test system. Control and test water were supplied to the fluviarium by controlled flow from a peristaltic pump. Arrows indicate direction of flow.

conductivity utilizing a mercury thermometer, YSI oxygen meter (model 54), Corning pH meter (model 10), and Markson conductivity meter (model 10). Measurements on alkalinity and hardness were accomplished using the methyl orange and EDTA titrimetric procedures described in Standard Methods (APHA, 1985).

Avoidance tests were performed using a sample size of 10 fish per exposure, as described by Black and Birge (1980) and modified from Sprague (1968). A summary of the procedures is given below.

1. After initiating the flow of only control water in both control and test channels at a rate of approximately 250 mL/min, fish were introduced into the decision area of the fluviarium for 20 min of acclimation. This time period allowed for one complete turnover of water in each of the 5-L channels.

2. To ascertain any preference for right or left channels (i.e., no toxicant effect), the screen was removed and the distribution of fish was recorded every 30 sec for 10 min.

3. Fish were relocated into the decision area and the low pH solution was delivered to the test channel for 20 min (one turnover per channel) to allow stabilization of exposure concentration.

4. The screen was removed again and distribution of animals was recorded at 30-sec intervals for 20 min. As shown by dye studies conducted by Black and Birge (1980), the interface between control and test channels is maintained in the decision area. Therefore, fish residing in the decision area during this 20-min period were counted as selecting either control or test channel (Figure 2). Water samples were collected from three regions of

each channel at the beginning and end of the 20-min test period and were analyzed for pH.

5. Test and control channels were reversed and the experiment was repeated at the same exposure level with 10 untested fish.

All tests were recorded on a Panasonic videocassette recorder (model 6500). Subsequent to the completion of these tests, the videotapes were reviewed and the position of the animals was scored according to four locations within the test chamber. The number of animals in the control or test decision areas and the partitioned control and test channels (Figure 2) was recorded every 30 sec. The percent time in each location was then determined.

Analysis of avoidance data. Results were expressed as percent gross avoidance (A) for each pH level tested. This value was calculated by using the scores for fish distribution in the control (C) and test (T) channels. The following formula was applied:

$$A = (C - T)/(C + T) \times 100$$

Negative avoidance was defined as preference. Similarly, any left- or right-handed preference was detected by applying the same analysis to data collected during the 10-min trial period (i.e., no toxicant present; step 2 above). For each pH level tested, data from the experimental trial (i.e., 20-min test; step 4 above) was examined in two ways. Gross avoidance (A) was compared to an expected theoretical distribution of 1:1 using the chi-square test (Sokal and Rohlf, 1969) on the assumption that if fish could not discriminate between control and test water, they would distribute equally in the two channels (Folmar, 1976). The chi-square procedure also was used to

compare the experimental data to the corresponding control trial (i.e., 10-min test; step 2 above). The no observed effect concentration (NOEC) was based on the lowest hydrogen ion concentration which did not elicite a significant gross response with pooled data, while the lowest observed effect (LOEC) was the lowest concentration which did show a significant response.

Procedures for Conducting Embryo-Larval Bioassays

Embryo-larval test system. To determine the toxic effects of low pH on embryonic stages and to provide a baseline for evaluating the sensitivity of avoidance test responses, embryo-larval bioassays were conducted following static-renewal procedures described by Horning and Weber (1985) and Birge, et al. (1981). Fertilized fathead minnow eggs were placed in deep Pyrex petri dishes (400 mL capacity), with a sample size of approximately 100 eggs per dish for both experimental and control populations. Test and control water were changed at regular 24-hr intervals and pH was determined just prior to and after each renewal. Aeration was supplied directly to each test chamber. Water quality parameters, including temperature, dissolved oxygen, conductivity, alkalinity, and hardness were determined daily. Test organisms were monitored daily to gauge extent of development and to remove dead specimens.

Exposure concentrations and test responses. The effect of pH was tested at five exposure concentrations ranging between a pH of 3.57 and that of control water (i.e., pH 7.68), using at least two replicates. Bioassays were initiated soon after fertilization and continued through 4 days posthatching.

Average hatching time was approximately 4-5 days for fathead minnows at a mean temperature of 24.4°C.

Test responses in embryo-larval bioassays were limited to mortality and teratogenesis. Percent survival at hatching and 4 days posthatching was expressed as frequency in experimental populations/frequency in controls. Teratogenic defects were determined at hatching and frequencies were based on survivors affected by gross debilitating anomalies (Birge and Black, 1977; Birge, et al., 1983). Counting teratic larvae as dead organisms, median lethal concentrations (LC_{50}) were calculated using the Trimmed Spearman-Karber method (Hamilton, et al., 1977) and no observed effect concentrations (NOEC) were determined using the methods of Dunnett (1955) and chi-square/Fisher's exact test (Sokal and Rohlf, 1969). The lowest observed effect concentration (LOEC) was determined to be the lowest hydrogen ion concentration which showed significant response.

Procedures for Conducting 96-Hr Acute Bioassays

Acute test system. To provide an additional basis for evaluating the preference/avoidance test, acute bioassays were conducted with two ages of juvenile fathead minnows. The 96-hr acute tests were performed using a static-renewal procedure described by Peltier and Weber (1985). Six concentrations, including controls, were used to test the toxicity of acid to two ages of juvenile fathead minnows (i.e., 8-wk and 12-wk old animals). The pH ranged from means of 3.14 to 7.38, and all experiments were conducted in replicate. Ten fish were placed in 2-L Pyrex beakers containing 1500 mL of

test solution. Approximately 1100 mL of solution were replaced every 24 hr and pH was determined just prior to and after each renewal. Water quality parameters, including temperature, dissolved oxygen, alkalinity, hardness, and conductivity were measured daily. During the first 8 hr of exposure, animals were monitored every 1 to 2 hr to remove dead specimens. Subsequent observations were made every 24 hr, at time of solution renewal.

Data analysis. Using both probit analysis (Finney, 1971) and the procedure of Spearman-Karber (Hamilton, et al., 1977), median lethal concentrations (LC_{50}) were determined for both 8- and 12-wk old fish. In addition, the median time to mortality (LT_{50}) was calculated for selected test levels following the procedures of Litchfield (1949). The no observed effect concentrations (NOEC) were determined by the procedures of Dunnett (1955) and chi-square/Fisher's exact test (Sokal and Rohlf, 1969). The lowest observed effect concentration (LOEC) was the lowest hydrogen ion concentration which produced statistically significant responses.

DATA AND RESULTS

Preference/Avoidance Study

Preference/avoidance sublethal bioassays were conducted with 8-wk bluegill sunfish and 6- and 14-wk fathead minnows using various levels of acid pH. Tests were conducted in duplicate and, based on analytical monitoring data of pH, exposure levels were reproducible (Tables 3-5). Differences in ion concentrations between test and control channels were maintained throughout the test run. Response data from replicate experiments were analyzed separately and then pooled. Using distribution of animals in control (C) and test (T) channels, percent gross avoidance (A) was determined at each ion concentration as $A = (C - T) / (C + T) \times 100$. Negative avoidance was defined as preference. To take into account any innate preference for the right or left channel, the gross response was adjusted using data from experiments conducted without acid (Research Procedures; Procedures for Conducting Preference/Avoidance Tests). These control-adjusted response values were defined as net response. As can be seen in Tables 3-5, this control-adjustment could result in rather sweeping changes in percent response. Because of this, only gross responses determined to be significant (chi-square, $P < 0.05$) were indicated by an asterisk (Table 3-5).

In general, both species of fish responded to low pH levels by avoiding the test channel. As part of the reaction to the acid, the fish usually became very hyperactive and swam in an agitated and erratic manner. When making excursions into the test channel, the fish often would reverse their

direction and appear somewhat disoriented when compared to fish in the control channel. These behavioral responses were concentration dependent and diminished as the pH in the test channel approached neutrality. Since acid is known to impair osmoregulatory mechanisms and oxygen uptake across the gills (Muniz and Leivestad, 1980; Rosseland, 1980; Spry, et al., 1981), as well as chemoreception (Lemly and Smith, 1985), such behavior is not surprising. Similar changes in locomotor activities have been shown to be concentration dependent with such toxicants as DDT (Ellgaard, et al., 1977), cadmium, chromium, and zinc (Ellgaard, et al., 1978), and a variety of herbicides (Folmar, 1976).

Avoidance responses with 8-wk bluegill sunfish were tested at four pH levels ranging from means of 3.50 to 6.60 (Tables 3 and 6). Based on gross responses, pH's of 5.94 and 3.50 consistently elicited significant avoidance. At a pH level of 6.20, one replicate demonstrated a strong avoidance while the second replicate showed non-significant preference, thereby indicating no significant pooled gross preference or avoidance. Both replicates at pH 6.60 showed a non-significant preference for the test channel. At these two pH levels, the inconsistent results may be attributed to the inability of the fish to discriminate low hydrogen ion concentrations. When statistical analyses were made of gross responses, significant avoidance was observed at a pH of 5.94 and below (Table 3), based on pooled data. Therefore, the no observed effect concentration (NOEC) was a pH of 6.20. When response data were analyzed by the amount of time spent in either control or test channel proper or in the respective decision areas, it was noted that as acid levels decreased (i.e., approached neutrality), percent time in the control channel

proper decreased from 81.3% to 36.0% (Table 6; Figure 3).

Juvenile fathead minnows at 6- and 14-wk of age were both exposed to various concentrations of acid to examine and compare their behavioral responses. Six-wk fathead minnows displayed gross avoidance at both pH 6.05 and 3.61 (Table 4; Figure 4). The results of replicate tests at a pH of 6.91 were variable, with one replicate showing significant avoidance and the other non-significant preference. As with the bluegill, these responses may reflect an inability to discriminate decisively low hydrogen ion concentrations. However, when both replicates were combined, the resulting gross avoidance response of 46.2% was significant ($P < 0.05$). The percent gross response was moderate at a pH of 6.91 and very strong at 6.05 and 3.61. When results were control-adjusted (i.e., net response), all pooled avoidance responses were statistically significant ($P < 0.05$), and ranged from 45.7% to 130.0% (Table 4). Therefore, the NOEC at 6-wk of age was a pH †6.91.

When data on percent time spent in the various regions of the chamber were analyzed, it was noted that at pH 6.05 and 3.61, the fish spent 92.0% and 89.4% of their time, respectively, in the control channel proper (Table 7; Figure 5). In both instances they spent virtually no time in the test channel decision area and less than 1% of the time in the test (low pH channel). At a test pH of 6.91, one replicate avoided the test channel while the other replicate spent approximately equal amounts of time in all regions of the exposure chamber. When data were pooled, the control channel was occupied by the fish 55.8% of the time. Based on these data, 6-wk fathead minnows were very sensitive to acid environments and responded by avoiding a pH of 6.91 and lower.

Fourteen-wk juvenile fathead minnows appeared to be more tolerant of low pH levels. Both gross and net avoidance responses at pH's of 5.61 and 3.76 were significant (Table 5; Figure 4). However, responses at pH's of 6.12 and 6.68 were variable between replicates. At a pH of 6.12, the gross response was 81.5% avoidance and 81.0% preference in the two replicates, while the net (control-adjusted) responses indicated non-significant avoidance. At a pH closer to neutrality (pH 6.68), the gross responses also were varied; however, control-adjustment gave significant avoidance. Based on these results, at pH's of 6.12 and above, 14-wk fathead minnows showed no definitive avoidance or preference to acid pH and the NOEC was determined to be pH 6.12.

As with the 6-wk animals, the 14-wk fathead minnows exposed to pH 3.76 and 5.61 spent the majority of the time in the control channel proper (Table 8; Figure 6). However, if percent time in the various portions of the chamber was examined at pH 6.12, it must be noted that the fish attracted to the acid test channel (i.e., replicate 1) actually spent the majority of the time (81.5%) in the acid decision area, and this attraction probably is not as significant as it might appear.

Embryo-Larval Bioassay

Eggs and embryos of the fathead minnow were exposed to four levels of acid. Although there were minor fluctuations in pH over a 24-hr period, the overall exposure concentrations of replicates were comparable (Table 9). Combined data indicated that percent survival at hatching and 4 days

posthatching was not significantly affected at a pH of 4.92 or above. However, pH 3.57 produced complete mortality prior to hatching in both replicates. Based on statistical analyses utilizing Dunnett's and chi-square procedures, the NOEC was a pH of 4.92. The median lethal concentration (LC_{50}) value at 4 days posthatching as determined by the Spearman-Karber procedure was a pH of 4.56. These results are in essential agreement with those reported by Mount (1973), where only 1% of fathead minnow eggs survived exposure to a pH of 4.5 while 67% hatched at pH 5.2.

Although there were few abnormal animals at hatching (Table 9), the acid appeared to cause some deformities during the early larval period. The increased frequency of terata in all surviving populations is reflected in the decreased number of normal survivors at 4 days posthatching (Table 9). The most commonly encountered deformities included edema and alterations in the alignment of the vertebral column.

96-Hr Acute Bioassays with Juvenile Stages of Fish

The acute toxicity of low pH to juvenile fathead minnows provided another basis of comparison for the sensitivity of the preference/avoidance bioassay. Juvenile fathead minnows at 8-wk of age showed 100% survival at pH's of 5.02 to 7.38, and complete mortality at pH's of 3.90 and 3.14 (Table 10). At a pH of 4.29, only 15% of the population survived. Similar results were obtained with 12-wk animals, except that exposure to a pH of 4.46 resulted in 55% survival (Table 10). Based on combined replicates, the LC_{50}'s determined with 8-and 12-wk fathead minnows were pH 4.57 and 4.46,

respectively. These two values were not statistically different from each other (Sprague and Fogels, 1977; Peltier and Weber, 1985). Therefore, both ages of juvenile fatheads were equally sensitive to acidic pH.

The no observed effect concentrations (NOEC) also were calculated at both ages using both Dunnett's (1955) procedure and the chi-square/Fisher's exact test (Sokal and Rohlf, 1969). At 8-wk, both statistical analyses resulted in an NOEC of 5.02 pH, while at 12-wk the NOEC was pH 4.46 based on Dunnett and 5.10 based on chi-square.

The time required to cause mortality appeared to be concentration dependent. The median lethal time (LT_{50}) with 8-wk fathead minnows for pH 3.90 and 4.29 were 3.6 and 39.9 hr, respectively (Table 11). With 12-wk animals, comparable levels of pH gave LT_{50} values of 2.4 and †96 hr (Table 11). Therefore, 12-wk animals appear to be somewhat more tolerant than 8-wk fatheads to pH levels in the range of 4.3 - 4.5, but essentially are equally sensitive to a pH of 3.9. At both ages, increases of an order of magnitude in hydrogen ion concentration resulted in at least an order of magnitude decrease in median survival time.

Comparison of Results from Three Bioassays

Juvenile 96-hr acute and embryo-larval bioassays were compared using NOEC and LC_{50} values. On the basis of LC_{50} values ranging from 4.46 to 4.57 (Table 12), both ages of juveniles and embryo-larval stages appear to be equally sensitive to acid. However, when no observed effect concentrations were determined, the 12-wk juveniles were considered to be slightly more

tolerant to acid levels, with an NOEC of pH 4.46 compared with the NOEC°s with 8-wk juveniles and embryo-larval stages of 5.02 and 4.92, respectively. Despite these minor differences, it appears that both tests are equally sensitive when examining the toxic effects of acids. However, when toxic effects of complex mixtures of industrial effluents are considered, these two bioassays have not been found to be equally sensitive (Birge and Black, 1981; Birge, et al., 1985). The embryo-larval bioassay consistently has been able to detect toxic responses at lower concentrations and often has been as sensitive as long-term chronic tests (Birge, et al., 1985).

The juvenile 96-hr acute and embryo-larval bioassays also were performed to provide a baseline of comparison for the avoidance test. The most striking result was that the behavioral response was a much more sensitive indicator of acid stress than were results from 96-hr acute bioassays. The NOEC for pH with 6-wk and 14-wk fathead minnows was >6.91 and 6.12, respectively (Table 12). These levels were one to two orders of magnitude different from the NOEC's determined in acute juvenile bioassays, indicating that, when given a choice, juvenile fathead minnows will avoid subacute acid levels. This behavioral response may provide the fish with the opportunity to avoid acidic environments that could prove harmful to feeding, maturation, and, eventually, reproduction. Although there was little difference in avoidance response between the two ages of fish, the 14-wk animals appeared to be slightly more tolerant to acid environments, with an NOEC of pH 6.12 compared to an NOEC of >6.91 with 6-wk animals. The bluegill responses at 8 wk of age were somewhat comparable to those of the 14-wk fathead minnows, with an NOEC of pH 6.20. As with the fathead minnow, bluegill strongly

avoided an acid environment that would not be lethal to them. The no observed effect concentration was a much lower hydrogen ion concentration than that reported by Ellgaard and Gilmore (1984) for bluegill. In 96-hr acute bioassays with bluegill sunfish of approximately 40 mm T.L., the LC_{50} for sulfuric acid was 3.5 and the NOEC was 4.0 (Table 12). Therefore, bluegill were sensitive to sublethal levels of acid and, given a choice, avoided even low concentrations of hydrogen ions.

Although the behavioral bioassay appears to be more sensitive, based on statistical analyses, than acute tests, the variability in responses makes it a very difficult test to evaluate. Until further information is available on typical behavioral patterns for standard aquatic test species, the accuracy of this bioassay may be limited. Further study will be required before definitive use can be made of this test in hazard assessment programs.

Table 3. Preference/avoidance responses of 8-wk bluegill sunfish to various concentrations of sulfuric acid.

Replicate	Mean Test pH	Range	Mean Control pH	Range	Percent Gross Response[1] (Avoidance [+] or Preference [-])	Percent Net Response[2] (Avoidance [+] or Preference [-])
1	6.63	6.50 - 6.77	7.58	7.10 - 7.70	- 6.5	-38.5
2	6.58	6.45 - 6.70	7.29	7.05 - 7.75	-26.0	-33.0
1 & 2 (combined)	6.60	6.45 - 6.77	7.37	7.05 - 7.75	-16.3	-35.8
1	6.25	6.05 - 6.55	7.68	7.55 - 7.80	+73.5*	+109.5
2	6.15	5.90 - 6.75	7.23	6.90 - 7.75	- 5.0	- 7.0
1 & 2 (combined)	6.20	5.90 - 6.75	7.40	6.90 - 7.80	+34.3	+51.3
1	5.87	5.65 - 6.40	7.48	7.25 - 7.80	+78.0*	+98.0
2	6.02	5.75 - 6.70	7.52	7.15 - 7.80	+66.5*	+84.5
1 & 2 (combined)	5.94	5.65 - 6.70	7.50	7.15 - 7.80	+72.3*	+91.3
1	3.57	3.30 - 5.65	6.53	6.20 - 7.75	+80.0*	+39.0
2	3.44	3.10 - 5.65	6.47	5.90 - 7.70	+88.9*	+53.9
1 & 2 (combined)	3.50	3.10 - 5.65	6.50	5.90 - 7.75	+84.2*	+46.2

[1]Percent calculated by formula (C-T)/(C+T) X 100, where C and T represent mean distribution of fish in control (C) and test (T) channels, respectively. Asterisks indicate statistically significant responses ($P < 0.05$, chi square test).

[2]Percent calculated as percent gross response minus percent response in corresponding control (non-acid) run.

Table 4. Preference/avoidance responses of 6-wk fathead minnows to various concentrations of sulfuric acid.

Replicate	Mean Test pH	Range	Mean Control pH	Range	Percent Gross Response[1] (Avoidance [+] or Preference [-])	Percent Net Response[2] (Avoidance [+] or Preference [-])
1	6.95	6.75 - 7.40	7.47	7.15 - 8.05	-11.5	+82.5
2	6.87	6.65 - 7.05	7.38	7.25 - 8.00	+98.6*	+12.2
1 & 2 (combined)	6.91	6.65 - 7.40	7.42	7.15 - 8.05	+46.2*	+45.7
1	6.08	5.85 - 6.60	7.19	6.70 - 8.00	+99.5*	+188.5
2	6.03	5.80 - 6.65	7.33	6.75 - 7.75	+97.5*	+74.5
1 & 2 (combined)	6.05	5.80 - 6.65	7.25	6.70 - 8.00	+98.5*	+130.0
1	3.52	3.20 - 5.60	6.32	5.85 - 8.30	+100.0*	+139.0
2	3.73	3.25 - 6.10	6.48	6.05 - 8.20	+96.5*	+23.5
1 & 2 (combined)	3.61	3.20 - 6.10	6.39	5.85 - 8.30	+98.3*	+81.3

[1]Percent calculated by formula (C-T)/(C+T) X 100, where C and T represent mean distribution of fish in control (C) and test (T) channels, respectively. Asterisks indicate statistically significant responses ($P < 0.05$, chi square test).

[2]Percent calculated as percent gross response minus percent response in corresponding control (non-acid) run.

Table 5. Preference/avoidance responses of 14-wk fathead minnows to various concentrations of sulfuric acid.

Replicate	Mean Test pH	Range	Mean Control pH	Range	Percent Gross Response[1] (Avoidance [+] or Preference [-])	Percent Net Response[2] (Avoidance [+] or Preference [-])
1	6.59	6.50 - 6.70	7.17	6.90 - 7.45	+85.0*	+112.0
2	6.81	6.70 - 6.85	7.39	7.20 - 7.50	-14.5	+71.5
1 & 2 (combined)	6.68	6.50 - 6.85	7.26	6.90 - 7.50	+35.3	+91.8
1	6.10	5.85 - 6.60	6.80	6.40 - 8.00	-81.0*	+ 3.0
2	6.14	5.85 - 6.65	7.49	7.15 - 8.00	+81.5*	+43.5
1 & 2 (combined)	6.12	5.85 - 6.65	7.02	6.40 - 8.00	+ 0.3	+23.3
1	5.62	5.30 - 6.45	6.58	6.10 - 8.10	+100.0*	+116.0
2	5.59	5.00 - 6.65	7.50	7.10 - 7.95	+97.5*	+148.5
1 & 2 (combined)	5.61	5.00 - 6.65	6.83	6.10 - 8.10	+98.8*	+132.3
1	3.64	3.10 - 6.05	6.15	5.65 - 8.40	+89.5*	+178.5
2	3.94	3.45 - 6.00	7.00	6.50 - 8.45	+100.0*	+21.0
1 & 2 (combined)	3.76	3.10 - 6.05	6.39	5.65 - 8.45	+94.8*	+99.8

[1]Percent calculated by formula (C-T)/(C+T) X 100, where C and T represent mean distribution of fish in control (C) and test (T) channels, respectively. Astericks indicate statistically significant responses ($P < 0.05$, chi square test).

[2]Percent calculated as percent gross response minus percent response in corresponding control (non-acid) run.

Table 6. Distribution of 8-wk bluegill sunfish within exposure chamber in preference/avoidance studies with sulfuric acid.

Mean Test pH[1]	Replicate	Percent Time by Location Within Chamber			
		Test	Test Decision	Control Decision	Control
6.60	1	45.3	8.0	10.5	36.3
	2	56.3	6.8	1.3	35.7
	1 & 2 (combined)	50.8	7.4	5.9	36.0
6.20	1	11.8	1.5	14.0	72.8
	2	39.3	13.3	10.5	37.0
	1 & 2 (combined)	25.5	7.4	12.3	54.9
5.94	1	10.5	0.5	5.8	83.3
	2	11.5	5.5	10.5	72.5
	1 & 2 (combined)	11.0	3.0	8.1	77.9
3.50	1	8.8	1.3	9.8	80.2
	2	3.9	1.7	11.9	82.5
	1 & 2 (combined)	6.4	1.5	10.8	81.3

[1]Value represents mean pH of combined replicates. Mean pH in the control channel ranged from 6.50 - 7.50 (see Table 3).

Table 7. Distribution of 6-wk fathead minnows within exposure chamber in preference/avoidance studies with sulfuric acid.

Mean Test pH[1]	Replicate	Percent Time by Location Within Chamber			
		Test	Test Decision	Control Decision	Control
6.91	1	29.5	26.3	21.3	23.0
	2	0.7	0.0	13.6	85.7
	1 & 2 (combined)	15.1	13.1	17.5	54.4
6.05	1	0.3	0.0	2.5	97.3
	2	0.8	0.5	12.0	86.8
	1 & 2 (combined)	0.5	0.3	7.3	92.0
3.61	1	0.0	0.0	19.5	80.5
	2	1.8	0.0	0.0	98.3
	1 & 2 (combined)	0.9	0.0	9.8	89.4

[1]Value represents mean pH of combined replicates. Mean pH in the control channel ranged from 6.39 - 7.42 (see Table 4).

Table 8. Distribution of 14-wk fathead minnows within exposure chamber in preference/avoidance studies with sulfuric acid.

Mean Test pH[1]	Replicate	Percent Time by Location Within Chamber			
		Test	Test Decision	Control Decision	Control
6.68[2]	1	0.0	7.5	7.0	85.5
	2	-	-	-	-
6.12	1	9.0	81.5	9.5	0.0
	2	9.3	0.0	24.8	66.0
	1 & 2 (combined)	9.1	40.8	17.1	33.0
5.61	1	0.0	0.0	10.8	89.3
	2	0.0	1.3	27.5	71.3
	1 & 2 (combined)	0.0	0.6	19.1	80.3
3.76	1	0.8	4.5	2.8	92.0
	2	0.0	0.0	28.3	71.8
	1 & 2 (combined)	0.4	2.3	15.5	81.9

[1]Value represents mean pH of combined replicates. Mean pH in the control channel ranged from 6.39 - 7.26 (see Table 5).

[2]Videotape data from replicate 2 was unavailable.

Table 9. Survival of embryo-larval stages of fathead minnows exposed to various concentrations of sulfuric acid.

Replicate	Mean pH	Range	Percent Hatchability[1]	Percent Normal Survival[2]	
				H	4DPH
1	7.65 (control)	7.50 - 7.94	84(1)	84	69
	6.49	6.05 - 7.59	85	85	76
	5.61	5.15 - 7.30	91	91	76
	4.87	4.20 - 7.00	81	81	64
	3.56	3.38 - 3.86	0	0	0
2	7.71 (control)	7.51 - 8.00	83	83	76
	6.50	6.06 - 7.47	85	85	79
	5.70	5.20 - 7.15	90(1)	89	80
	4.98	4.36 - 6.95	88	88	77
	3.58	3.42 - 3.85	0	0	0
1 & 2 (combined)	7.68 (control)	7.50 - 8.00	83	83	72
	6.50	6.05 - 7.59	85	85	77
	5.67	5.15 - 7.30	90	90	78
	4.92	4.20 - 7.00	85	85	71
	3.57	3.38 - 3.86	0	0	0

[1]Percent hatchability includes all animals which successfully hatched. Numbers in parentheses indicate percent of hatching population with gross, debilitating anomalies.

[2]Percentage of initial population that was normal at hatching (H) and 4 days posthatching (4DPH).

Table 10. Survival of juvenile fathead minnows exposed to various concentrations of sulfuric acid for 96 hr.

Age (wk)	Replicate	Mean pH	pH Range	Percent Survival
8	1	7.42	7.17 - 7.85	100
		6.26	6.00 - 6.67	100
		5.01	4.67 - 5.81	100
		4.30	4.03 - 5.00	10
		3.89	3.80 - 4.00	0
		3.10	-	0
	2	7.34	7.05 - 7.90	100
		6.23	6.00 - 6.68	100
		5.02	4.70 - 5.80	100
		4.27	4.05 - 4.82	20
		3.92	3.85 - 4.00	0
		3.18	-	0
	1 & 2 (combined)	7.38	7.05 - 7.90	100
		6.24	6.00 - 6.68	100
		5.02	4.67 - 5.81	100
		4.29	4.03 - 5.00	15
		3.90	3.80 - 4.00	0
		3.14	-	0
12	1	7.33	7.05 - 7.80	100
		6.34	6.10 - 6.75	100
		5.12	4.80 - 5.78	100
		4.52	4.20 - 5.45	90
		3.96	3.86 - 4.10	0
		3.15	-	0
	2	7.40	7.20 - 7.75	100
		6.33	6.10 - 6.75	100
		5.09	4.70 - 5.80	100
		4.40	4.10 - 5.05	20
		3.87	3.85 - 3.90	0
		3.20	-	0
	1 & 2 (combined)	7.36	7.05 - 7.80	100
		6.33	6.10 - 6.75	100
		5.10	4.70 - 5.80	100
		4.46	4.10 - 5.45	55
		3.92	3.85 - 4.10	0
		3.17	-	0

Table 11. Time-course of survival of juvenile fathead minnows exposed to various concentrations of sulfuric acid for 96 hr.

Age (wk)	Mean pH [1]	Percent Survival at Hour										LT_{50} (hr)
		0.5	1.0	2.0	4.0	6.0	8.0	24.0	48.0	72.0	96.0	
8	7.38	100	100	100	100	100	100	100	100	100	100	-
	6.24	100	100	100	100	100	100	100	100	100	100	-
	5.02	100	100	100	100	100	100	100	100	100	100	-
	4.29	100	100	100	100	100	100	80	40	15	15	39.9
	3.90	100	100	90	45	15	5	0	0	0	0	3.6
	3.14	0	0	0	0	0	0	0	0	0	0	-
12	7.36	-	100	100	100	100	100	100	100	100	100	-
	6.33	-	100	100	100	100	100	100	100	100	100	-
	5.10	-	100	100	100	100	100	100	100	100	100	-
	4.46	-	95	95	95	95	95	90	65	60	55	>96
	3.92	-	95	50	25	15	10	0	0	0	0	2.4
	3.17	-	50	0	0	0	0	0	0	0	0	1.0

[1]Mean pH and survival data are taken from combined replicates.

Table 12. Comparison of results from 96-hr acute, embryo-larval, and preference/avoidance bioassays with sulfuric acid.

Bioassay	Test Animal	Age (wk)	NOEC[1]	LOEC[2]	LC_{50}[3]
96-hr Acute	Bluegill Sunfish[4]	-	4.00	-	3.50
	Fathead Minnow	8	5.02	4.29	4.54
		12	4.46	3.92	4.46
Embryo-Larval	Fathead Minnow	4DPH[5]	4.92	3.57	4.56
Preference/ Avoidance	Bluegill Sunfish	8	6.20	5.94	-
	Fathead Minnow	6	>6.91	6.91	-
		14	6.12	5.61	-

[1]The pH value which produced no observed effects.

[2]The pH value which produced the lowest observed effects.

[3]The pH level calculated as the median lethal concentration (LC_{50}) in 96-hr acute and embryo-larval bioassays.

[4]Data taken from a study by Ellgaard and Gilmore (1984) with juvenile bluegill sunfish.

[5]Values calculated at 4 days posthatching (4DPH).

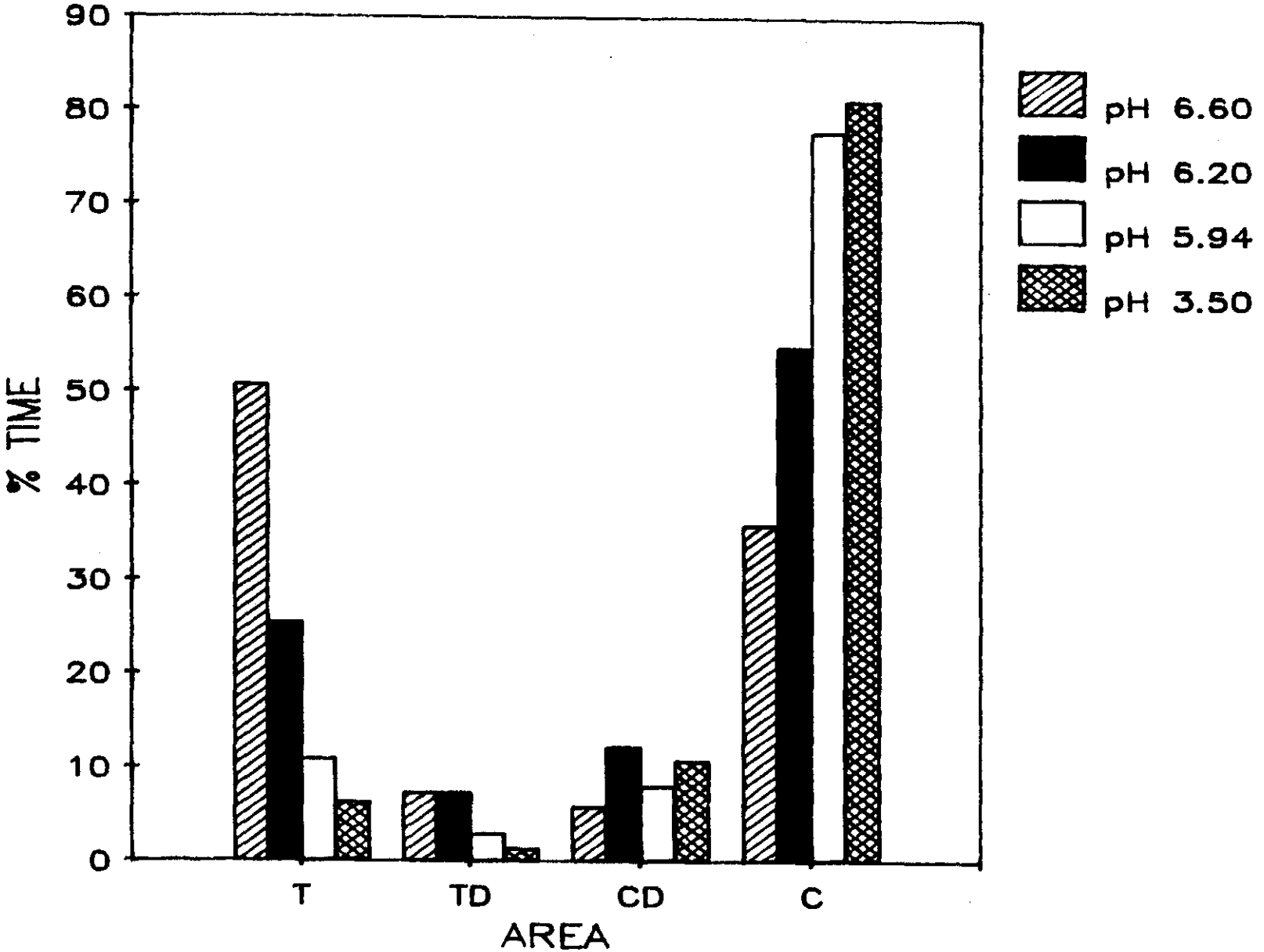

Figure 3. Distribution of bluegill sunfish in avoidance chamber. Values reflect percent time spent by the fish in the test channel proper (T), test channel decision area (TD), control channel decision area (CD), and control channel proper (C).

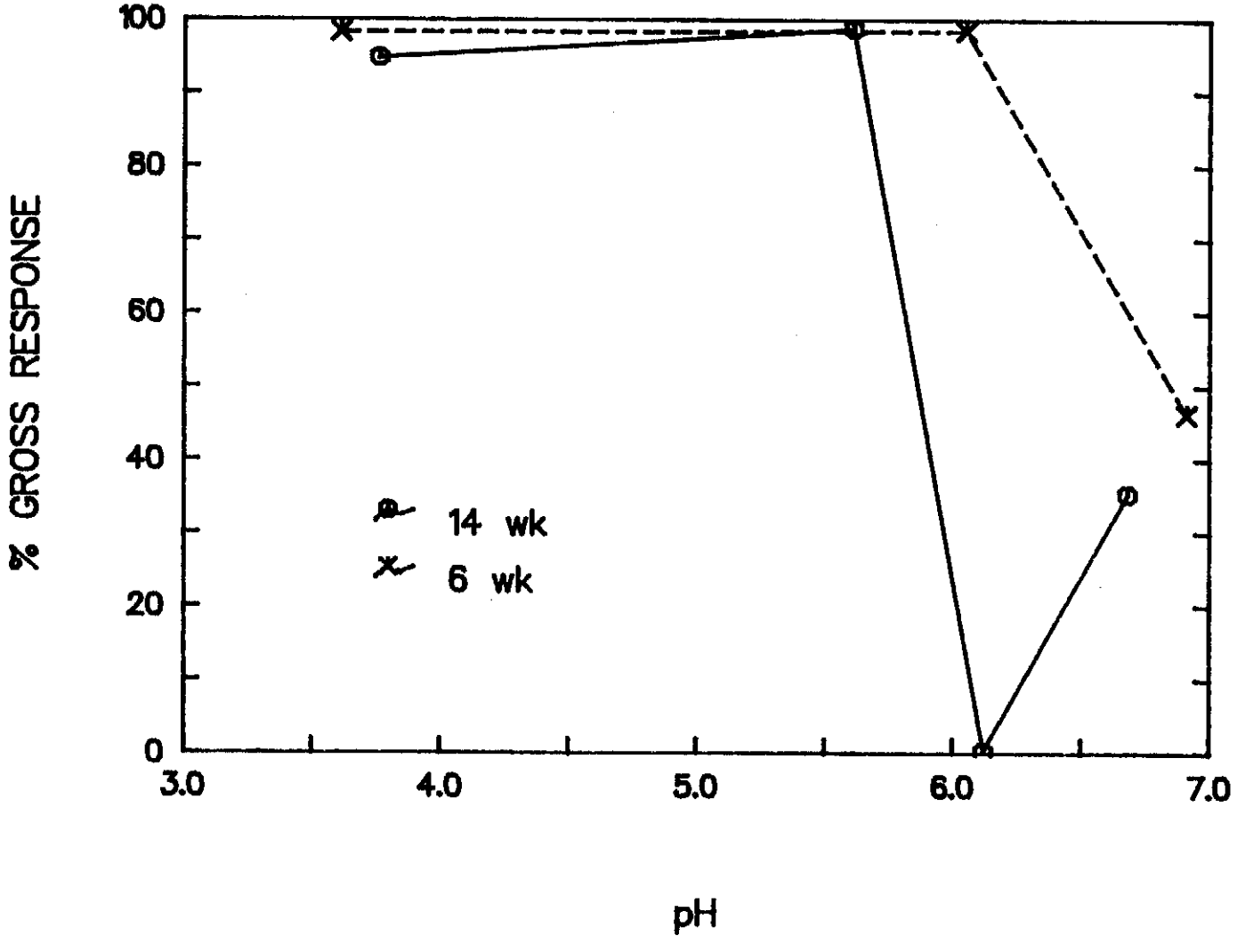

Figure 4. Avoidance responses of 6-wk (X---X) and 14-wk (o——o) fathead minnows exposed to sulfuric acid.

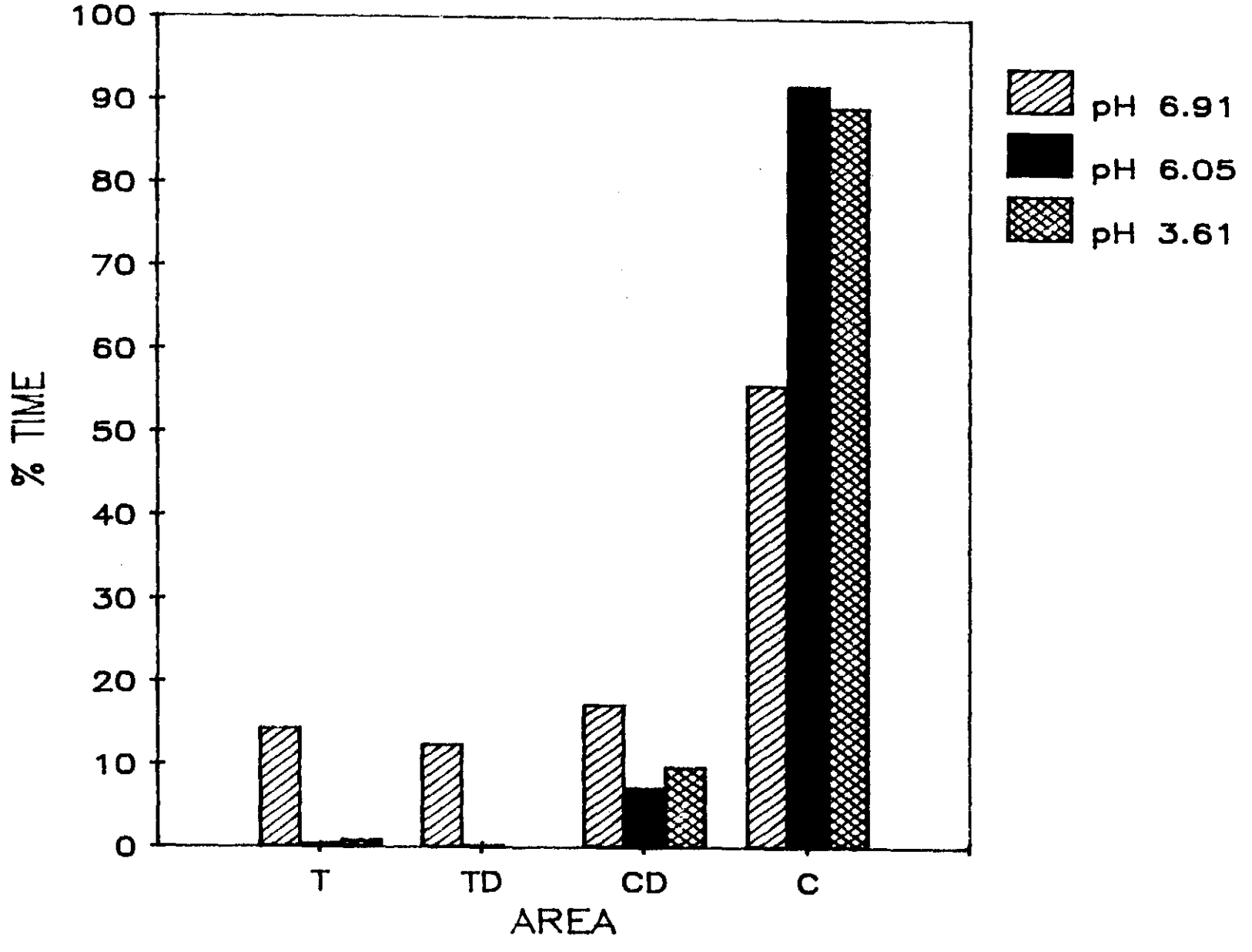

Figure 5. Distribution of 6-wk fathead minnows in avoidance chamber. Values reflect percent time spent by the fish in the test channel proper (T), test channel decision area (TD), control channel decision area (CD), and control channel proper (C).

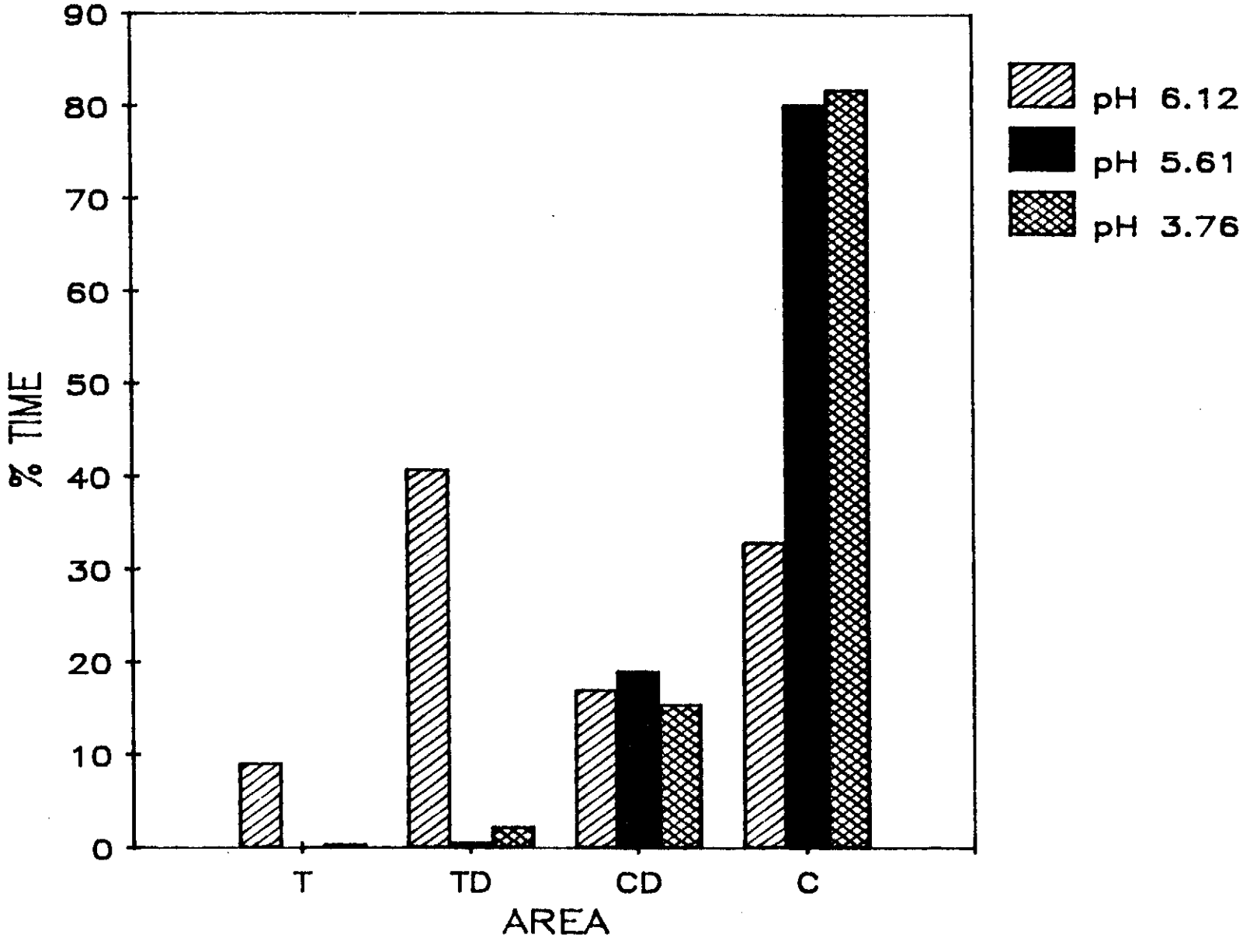

Figure 6. Distribution of 14-wk fathead minnows in avoidance chamber. Values reflect percent time spent by the fish in the test channel proper (T), test channel decision area (TD), control channel decision area (CD), and control channel proper (C).

CONCLUSIONS

Results of this study have provided an opportunity to compare the sensitivities to acid of several early life history stages of fish using a variety of bioassay techniques. Eggs, embryos, larvae, and juveniles were exposed to various concentrations of sulfuric acid in 96-hr acute, embryo-larval, and behavioral bioassays. Based on median lethal concentrations (LC_{50}), the embryo-larval and juvenile stages of fathead minnows appear to be equally sensitive to sulfuric acid. However, the no observed effect concentrations (NOEC) indicated that 12-wk juveniles may be slightly more tolerant to low pH levels than are larval and younger (i.e., 8-wk) juvenile stages. In the preference/avoidance study, juvenile fathead minnows and bluegill sunfish avoided pH's that would not necessarily prove to be acutely lethal. They actually avoided acid levels one to two orders of magnitude less than the hydrogen ion concentrations which produced no observed acutely lethal effects. Therefore, the behavioral test proved to be a more sensitive indicator of pH stress than did traditional 96-hr acute and embryo-larval bioassays. However, due to some rather wide variations in results that, when pooled, still produced statistically significant responses, the overall accuracy of this test will require further study.

From these findings it is obvious that a behavioral bioassay cannot be used alone in the process of decision-making in hazard assessment. It would not be practical to establish water quality criteria based solely on threshold levels obtained in behavioral bioassays. In so doing, environmentally unrealistic toxicant concentrations may be required.

However, a behavioral bioassay most certainly could compliment and be an integral part of an effective multistage testing program. This is especially true when toxicants are present in sublethal concentrations. If low levels of pollutants cause behavioral modifications, the end result may not be death but alterations in reproductive success for the animal and imbalance of the aquatic ecosystem. The major problem in implementing a behavioral bioassay in a multistage testing program is the current lack of standardization of procedures, including test design, test animals, duration of test, and criteria to be evaluated. If a behavioral bioassay is to be developed for routine use in hazard assessment, then basic information is needed on behavior of species commonly used in standard aquatic toxicity testing.

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