VALIDATION OF LABORATORY VERSUS FIELD AVOIDANCE BEHAVIOR OF SCHOOLING FATHEAD MINNOWS TO HEAVY METAL BLENDS RELATIVE TO ACUTE TOXICITY DURING LONG TERM EXPOSURE

by

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Zoology

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May, 1985

Blacksburg, Virginia

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ABSTRACT

Avoidance to a blend of four metals (relative proportions: 1.00 copper, 0.54 chromium, 1.85 arsenic, 0.38 selenium) was determined for schools of fathead minnows (Pimephales promelas) in a laboratory avoidance chamber, an artificial stream and in a natural stream setting on a seasonal basis during continuous exposure to low levels of the metal blend for up to 9 months. Laboratory avoidance responses were determined seasonally during 12 months of laboratory holding for unexposed (control) fish and two levels of metal blend exposure in a steep gradient laminar flow chamber. Toxicity of the metal blend was determined for laboratory and field control and metals acclimated fish.

Unexposed fish avoided very low levels of the blend (29 ug/L total metals). Fish exposed to low levels of the blend (49 ug/L total metals) for 3 months failed to avoid levels equal to 5X holding exposure levels. Fish exposed to higher levels of the blend (98 ug/L total metals) preferred elevated levels

(3X holding exposure) after 3 months exposure, mildly avoided 5X holding levels after 6 months exposure and were not responsive to levels approaching 10X holding exposure after 9 months continuous exposure. Activity was not affected by long term exposure.

Field avoidance responses were determined seasonally during 7 months of field laboratory holding in New River water for unexposed (control) fish and exposed (98 ug/L total metals) fish in a modified artificial stream supplied with raw New River water. Unexposed fish avoided 71.1 and 34.3 ug/L total metals in spring (3 months holding) and summer (6 months holding) respectively. After 3 months exposure in New River water, fish did not respond to metal blends as high as 1,470 ug/L total metals. In-stream avoidance responses were determined in the summer for unexposed (control) and exposed (98 ug/L total metals) fish in a Adair Run, a second order tributary to the New River. Unexposed fish avoided 73.5 ug/L total metals in Adair Run. After 3 months exposure, in New River water fish did not respond to metal blends as high as 2,940 ug/L in Adair Run. Water hardness, turbidity and physical setting are implicated as possible causative factors for differences between control fish responses tested in different seasonal and locations.

Fish exposed to the high level exposure in the laboratory had a 96-hr LC50 value 1.25X higher than laboratory control fish. Laboratory control fish avoided metals levels at 0.4% of their 96-hr LC50. Fish exposed to the metals blend in the field had a 96-hr LC50 value 1.41X higher than field control fish. Field control fish avoided metals levels between 0.7 and 2.5% of their 96-hr LC50 depending upon test location and season. There was no difference between the 96-hr LC50s of laboratory vs field control fish or between laboratory vs field exposed fish. Optimum statistical methods for analyzing avoidance behavior in schooling fish were developed.

ACKNOWLEDGMENTS

I would first like to thank my wife who was a source of constant support and encouragement as well as a considerable amount of insight into analytical chemistry, throughout the entire course of my studies. I also am grateful for the assistance and valuable input from the co-chairmen of my committee, Dr. John Cairns, Jr. and Dr. Donald S. Cherry and the other committee members, Dr. Alan Heath, Dr. Albert Hendricks and Dr. Robert Hoehn. Dr. Eric Smith provided guidance in statistical analyses. pro-
vided guidance and assisted in chemical analyses.

assisted in the field studies. I wish to thank . and
for kindly permitting the in-stream study to be conducted on their property. Valuable assistance in chemical analyses was provided by

and who also drew most of the figures.

This research was supported by a grant from the American Electric Power Service Corporation, Columbus, Ohio 43216-6631.

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INTRODUCTION

Responses of fish to various chemical and physical parameters in the aquatic environment are becoming increasingly important as a consequence of enhanced public concern for fish stocks as a recreational and economic resource, for water quality in general, and by public statute. The Toxic Substances Control Act of 1976 stipulated that new chemical substances, prior to manufacture and distribution, must be evaluated for health and environmental effects including toxicological and environmental fate, among others (Brungs & Mount, 1978). Laboratory toxicity bioassays have become a convenient test for screening chemical and physical parameters for potency, but predictive results and relevance to aquatic ecosystems in the "real world" have been inadequate in hazard assessment (Cairns et al., 1981). Acute toxicity bioassays cannot address questions of sublethal effects. Likewise, chronic exposure to some pollutants may result in cumulative or delayed effects. Simple acute toxicity tests cannot adequately resolve synergistic effects of various materials or easily incorporate variation of background water quality into the tests. In short, acute toxicity tests are useful as a yardstick for comparing relative toxicity, but results cannot be reliably extrapolated to predict environmental consequences.

Both sublethal and chronic exposure experiments have addressed a number of questions pertinent to environmental sensitivity to pollutants. It has been demonstrated that long term exposure may have severe impacts on growth and reproductive success of fish (Mount and Stephan,1967; Mount, 1968; White and Angelovic, 1968; Gardner and LaRoche, 1973; Servizi and Martens, 1978; Holcomb et al.,1979). It is generally observed that egg and larval stages of fish are the most sensitive stages of the life cycle (McKim et al., 1977). Chronic exposure can also result in direct toxicity or reduced resistance to disease and predators (George, 1970; Eaton, 1973; Hoss et al., 1974; Benoit et al., 1976; Knittel, 1980). In those studies where comparative data are available, chronic exposure effects often occur at concentrations well below acute levels. The implication of these observations is that the environment's ability to resist chemical insult is drastically lower than levels suggested by simple survival of organisms. The integrity of a robust ecosystem is crucial to its ability to assimilate stressful inputs (Cairns, 1977).

One outgrowth of the need for more accurate estimation of environmental response to pollutants has been the development of response criteria which vary from detailed physiological measurements to whole animal responses, specifically preference/avoidance behavior. Behavioral responses of fish

to chemicals have been examined for many years (Marcucella and Abramson, 1978). Many studies were designed to illuminate the process of learning in classical psychological conditioning responses. In recent years, water pollution researchers have used behavioral toxicology techniques as a tool for hazard assessment (Beitinger and Freeman, 1983).

Direct toxic action of pollutants may occur in limited areas but after the material in question has spread out and become diluted, vastly larger and more diverse habitats and their residents will be exposed to long term sublethal exposure. Materials which may affect the normal functioning of neurosensory systems are of interest since they may affect how fish move through the environment and respond to the normal range of cues which direct them toward food, shelter, spawning grounds, etc. The proper response to those cues and the ability to avoid harmful situations are of paramount importance to the survival of the population.

Our ability to predict what levels of a pollutant will cause harmful side effects in the organisms in a receiving system is limited by our ability to extrapolate laboratory derived results to the real environment. While a variety of researchers have attempted to contrast laboratory results with field distributions of aquatic animals exposed to pollution, no one has attempted to recreate their laboratory experiments

in a natural setting. This endeavor would generate information on the validity of laboratory studies and lead to information concerning how and why organisms respond in the way they do to effluents and normal environmental cues.

No factor, whether natural of man-made, can be assumed to elicit a response without that response being influenced by other stimuli. Ishio (1964) and Collins (1952) noted that sensitivity and behavioral responses are influenced by both the steepness of the gradient and the absolute value of the stimulus. Other studies have demonstrated specific interactions between environmental factors.

Validation and correlation of laboratory results with field studies are crucial to assessment of environmental impact predictions. Clear demonstration of laboratory avoidance at levels below those causing harmful effects may be meaningless from an environmental standpoint if the organisms fail to avoid those concentrations in the wild. Conversely, if organisms avoid field concentrations below that predicted in the laboratory the result is a de facto loss of otherwise suitable habitat. Highly variable conditions in the environment over seasonal time spans and the variable nature of industrial effluents may render laboratory results questionable as indicators of environmental harm without concurrent field validation. Well defined avoidance response bioassays

need to be developed and verified with field tests since aquatic organisms respond to the environment as a composite, with some stimuli more important than others. Because the "environment" cannot be completely duplicated in the laboratory, correlations between important stimuli in the laboratory and the field must be derived for purposes of hazard evaluation. Improved knowledge of the actual environmental effects of effluents, through generation of scientifically acceptable data, will lead to improved understanding of the environmental system and therefore more optimal use.

OBJECTIVES

The objectives of this study were: 1, Determine the acutly toxic conentrations of a combination of selected heavy metals in a laboratory 96-hour flow through bioassay on adult fathead minnows which had been maintained in either unadulterated laboratory or river water and in laboratory of river water dosed with two heavy metals (13-ug/L Cu and 7-ug/L Cr) and two metaloids (24-ug/L As and 5-ug/L Se) for nine months; 2, Determine laboratory avoidance concentrations to the metals combination in a steep gradient avoidance chamber on a seasonal basis, for each of three groups of laboratory maintained fish exposed constantly to either control, low or high metals concentration; 3, Determine field avoidance concentrations to the metal combination in an ar-

tificial stream supplied with raw river water, for each of two groups of fish maintained in river water and exposed constantly to either control or high metals concentration; and 4, to determine field avoidance concentrations to the metals combination in a natural stream, for each of two groups of fish maintained in river water and exposed constantly to either control or high concentrations of metals.

LITERATURE REVIEW

AVOIDANCE METHODOLOGY

Avoidance capabilities presume some limits of detection for the material in question. The sensory capabilities of fish have been shown to be quite precise and fish are behaviorally responsive to a large variety of parameters. They can detect sweet, sour, salt, and bitter substances. Through conditioned reflex training, it has been shown that some species can detect temperature changes as small as 0.03 C° (Bull, 1936), pH to 0.04 units (Bull, 1940), salinity to 0.06-mg/L (Bull, 1938), phenol to less than 0.0005 ppm (Hasler and Wisby, 1950), L-serine (from mammalian skin) diluted eight million times (Idler et al., 1956), morpholine down to $1x10^{-11}$ppm (Hasler, 1957) and seven different sugars and saccharin (Trudel, 1929). These are examples of materials for which limits of detection are extreme and there is variability between species, but they serve to demonstrate the lower range of potential detection limits. However, detection capabilities alone have not proven useful in predicting avoidance behavior, which is mediated by the central nervous system.

Fish are relatively sensitive to pesticides, being able to detect and avoid concentrations below 10 mg/L (Hansen et al., 1972; Hansen, 1969; Scherer, 1975; Folmar, 1976). A good body of data correlating avoidance levels to precise toxicity of pesticides also exists. Hiatt et al. (1953) tested a large variety of organic materials on fish and demonstrated that fish respond to skin and eye irritants and neural or respiratory poisons at much lower levels than to known olfactory or gustatory repellents of terrestrial animals.

Metals may be avoided at very low levels. Copper avoidance levels have been reported from 0.1 ug/l to 74 ug/l, depending on test species (Kleerekoper et al., 1973; Folmar, 1976; Black and Birge, 1980; Giattina et al., 1982). Zinc is avoided by largemouth bass (Micropterus salmoides) at concentrations at least as low as 7 mg/l, and 47 ug/l by rainbow trout (Salmo gairdneri), but not avoided by bluegill sunfish (Lepomis macrochirus) between 11 and 43 mg/l (Black and Birge, 1980). However, Sprague (1968) reported that rainbow trout avoided zinc levels as low as 5.6 ug/l. Whether this difference is due to methodology or background water characteristics cannot be determined at this time.

Preference/avoidance studies are a sensitive method for evaluation of sensory impacts of introduced chemicals (Giattina et al., 1982). The behavioral response of an

organism to pollutants is the primary, short term mechanism which will determine if the organism will remain in contact with potentially toxic materials or retreat to more dilute or uncontaminated areas. A variety of methods have been devised over the last several years for preference/avoidance testing of fish.

Laboratory avoidance studies of fish have used four basic types of apparatus: Y tanks (Fava and Tsai, 1976; Folmar, 1976; Hansen, 1969; B.F. Jones et al., 1956; Kynard, 1974; Whitmore et al., 1960), gradient tanks (Hansen et al., 1972; Ishio, 1964), boundary layer chambers (Cherry et al., 1977; J.R.E. Jones, 1948; Lubinski et al., 1980; Otto and Hartwell, 1981; Scherer, 1975; Sprague, 1964), and open field tanks with multiple inputs (Hoglund, 1951; Johnson and Webster, 1977 Kleerekoper, 1967; Whitmore et al., 1960). All the designs share some characteristics with certain others.

Parameters that are measured in behavioral tests are either residence time, sequential counts of location, activity, or some combination of these. Measurements of activity yield pertinent information on the effects of a substance on fish, but are not accurate predictors of preference/ avoidance behavior (Fava and Tsai, 1976; Sprague, 1968; Hartwell et al., 1985), especially when the test substance affects overall activity (Cherry et al., 1977; Dandy, 1972; J.R.E. Jones,

1948; Sprague and Drury, 1969). Residence time is the preferred measure of choice but lends itself best to situations in which a single or a pair of fish are tested. However, most fish species school at some point in their life history (Burgess and Shaw, 1979), and fish schools have been tested for behavioral responses to pollutants as often as individuals. The term 'school' here is loosly defined as an aggregation which may posess no, moderate or a high degree of polarization (Williams, 1964). Fathead minnows generally form loose schools where most but not all individuals are oriented with each other. Schools cannot be measured for residence time without utilizing complicated electronic apparatus since it is impossible to distinguish one fish from another without marking individuals in some way (Cripe, 1979; Kleerekoper, 1967). Sequential counts of fish location over time is the only other method which has been developed for this type of testing. Electronic and manual data-collection techniques have been reported from laboratory studies; however, manual techniques can be carried out in the field. Evaluation of laboratory data, as reported in the literature, is usually done by some form of graphical display, although regression, analysis of variance, and chi square or t-tests have been infrequently used.

FIELD VALIDATION

Hansen et al. (1972) reported that DDT increased salinity preference in mosquito fish (Gambusia affinis), Ogilvie and Anderson (1965) demonstrated that DDT affected temperature preference of Atlantic salmon (Salmo salar). Kleerekoper et al. (1973) suggested that copper and temperature selections are interrelated. Temperature selection has been shown to compete with light intensity avoidance (Sullivan and Fisher, 1953).

Field distribution studies have demonstrated avoidance or preference in wild organisms (Collins, 1952; Elson et al.,1970; Giattina et al., 1982). There are a limited number of studies which contrast field distributions with laboratory avoidance behavior and/or toxicity (Sprague et al., 1965; Saunders and Sprague, 1967; Geckler et al., 1976; Laughlin et al., 1978). There has been no specific avoidance study which critically compares laboratory and field avoidance responses in fish beyond gross population distribution studies (Cherry et al., 1982a).

POWER PLANT EFFLUENTS AND HEAVY METALS EXPOSURE

Coal-fired, electric-generating stations are among the larger users of fossil fuels. The increasing use of coal is rapidly generating fly ash far in excess of demand for such by-products. While some fly ash can be used as roadbed or construction material, the vast majority is used as land-fill or dumped at off-shore locations. Typical fly ash exhibits a variety of chemical constituents and physical attributes capable of chemically altering the aquatic environment. Fly ash contains significant concentrations of heavy metals and other trace elements which are present in fly ash disposal systems in an ionizable or toxic form. Also, since sluicing ash to a holding pond is a common practice, small fly-ash particles may remain in suspension and be released from the ponds with the accompanying heavy metal load to receiving waters. The physical particulate load in the receiving system has the potential to smother bottom life and eggs of aquatic organisms and/or alter organic productivity through increased turbidity (Guthrie and Cherry, 1976; Spect et al., 1985). Sudden changes in pH are also associated with fly ash basins which may be harmful or toxic in itself, or will alter the chemical state of metals and other chemicals in the water and sediment.

Metals accumulate in coal through two mechanisms. The first is the naturally occurring concentrations in decaying biological material from which the coal is formed. The second is chelation of trace elements by humic substances in the coal swamps (Mason, 1966). Burning the coal removes the organic material leaving behind a concentrated residue of silicate particles with non-volatile elements deposited on the surface. These elements are susceptible to leaching by rainfall or in disposal slurries. The leachate containing the metals may enter surface or ground waters through runoff and percolation and poses a threat to local aquatic biota.

A considerable body of knowledge has been developed concerning the toxicity of individual metals to aquatic organisms and there is some data on synergistic action of certain metal combinations. Trace metals may be directly toxic to fish at relatively low concentrations (N.A.S., 1972; E.P.A., 1976; A.F.S., 1979). At lower levels, they result in harmful sublethal effects, altering protein synthesis (Dixon and Sprague, 1981), osmoregulation (Stagg and Shuttleworth, 1982), reproduction (Shukla and Pandy, 1984), growth (Mount, 1968), respiration rate (O'Hara, 1971) and susceptibility to infection (Knittel, 1980). In addition, because they are obviously non-degradable, trace metals may tend to bioaccumulate up the food chain. Information on the effects of large assemblages of metals is very limited.

Sprague (1964) reported that copper and zinc were synergistic in eliciting an avoidance response. Rainbow trout avoided nickel at 23.9 ug/l (Giattina et al., 1982) and cadmium at 52 ug/l but were attracted to mercury at 0.2 ug/l (Black and Birge, 1980). Thus, the synergistic action of a metal blend will be highly complex, and will be influenced by blend composition, absolute concentration and test species. Toxic levels and behavioral response to the fly ash material itself is even less well studied (Cherry et al., 1977; Guthrie and Cherry, 1976; Theis and De Pinto, 1976; Cherry et al., 1982; Magnuson et al., 1980).

Long-term exposure to metals may result in variations in response over time in behavioral and metabolic parameters. Bengtsson (1974) reported a hyperactivity response to zinc in the minnow, Phoxinus phoxinus , followed by hypoactivity as zinc concentration and exposure duration increased. Scarfe et al. (1982) demonstrated that selected behavioral traits were altered by copper exposure which were both species specific and reversible following three weeks depuration. The respiratory rate of fish exposed to metals may return to normal or below normal levels over time (O'Hara, 1971), and apparent acclamation or compensation has been observed for ventilation rate and blood characteristics (McKim et al., 1970; Lewis and Lewis, 1971; Morgan and Kuhn, 1974; Brenner et al., 1976). The ultimate acclimation time

may be very long however. McKim et al. (1976) and Benoit et al. (1976) reported that up to 20 weeks exposure to a single metal was required to achieve a steady state body burden.

Long-term exposure to metals also affects the resistance of fish to elevated metals levels. This increased tolerance is under intensive investigation in other laboratories. The increased resistance appears to be due to metallothionein type proteins, which are normal components of the biochemical systems for regulating essential trace elements such as (Cu) and (Zn). When fish are exposed to abnormally high metals levels, metallothionein concentrations increase in the fish. This process has been shown to be inducible and temporary (Benson and Birge, 1985). When metals exposure is discontinued, metallothionein levels decrease as does tolerance. Furthermore, Dixon and Sprague (1981) reported that at low Cu exposure levels, rainbow trout are less tolerant than unexposed fish, but at higher exposure levels, presumably above the induction threshold for increased metallothionein production, tolerance increases. Holdway et al. (1983) have also demonstrated that the transition from low to high uptake of vanadium (V) correlates with the sublethal threshold concentration of V. Increased metallothionein levels due to higher levels of one metal may affect other metals in the fish's body as well. Muramoto (1983) exposed fish to elevated Cu and found that cadmium (Cd) concentrations rose and

fell with Cu concentrations as metallothionein scavenged for several metals. Thus, elevated metallothionein resulting from exposure to one metal can fortuitously provide protection from another, by complexing the second metal species in the fish's body. Roch and McCarter (1984a) demonstrated that metallothionein levels were correlated with Cu body burdens, and not Zn or Cd when rainbow trout were exposed to a mixture of all three metals

Thus, the potential importance of the metals composition, concentration and duration of exposure of fish to metals, relative to their behavioral response to metal laden effluents, is significant.

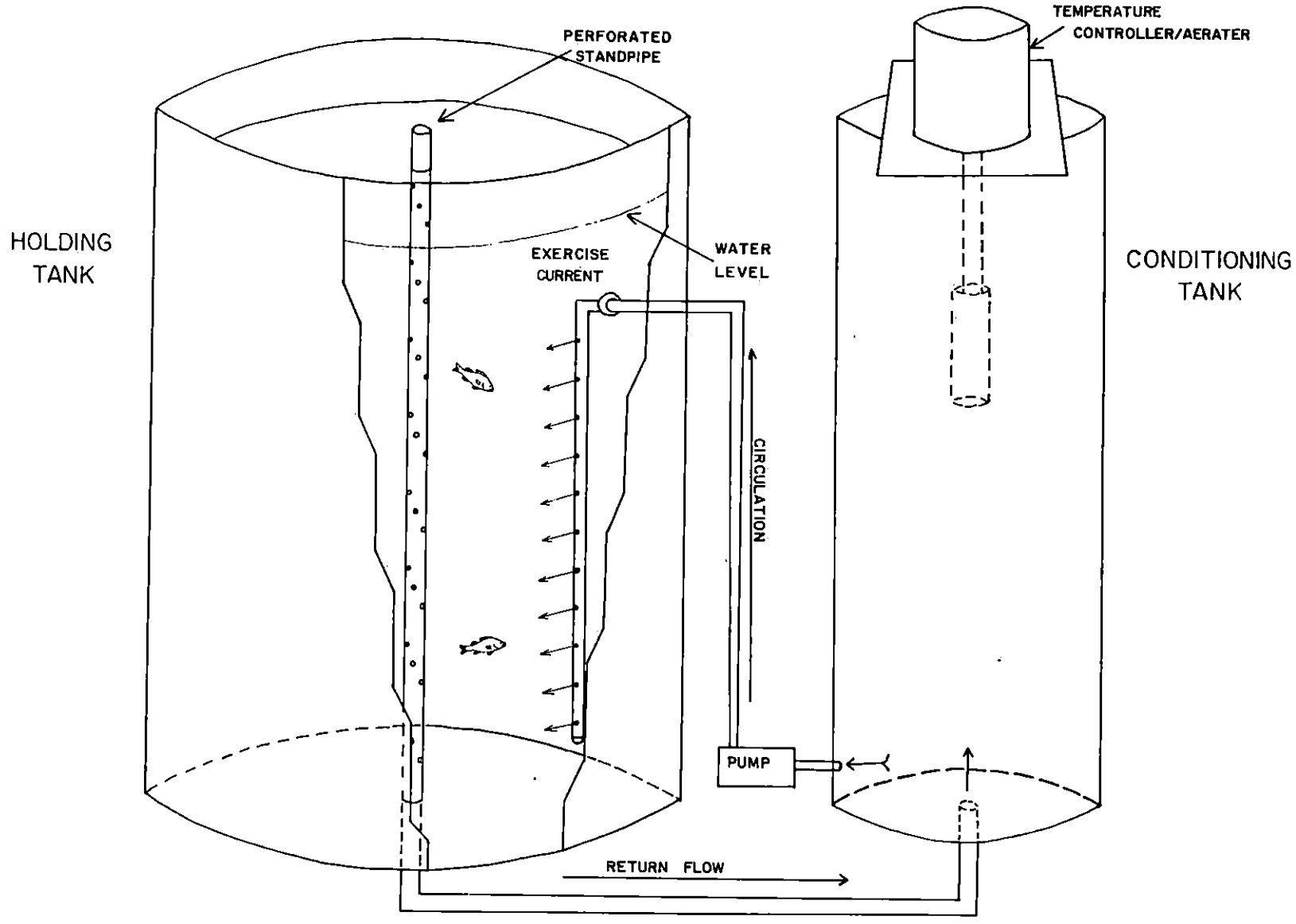
MATERIALS AND METHODS

LABORATORY EXPOSURE

Three groups of 200 yearling fathead minnows were kept in separate, 400-L fiberglass tanks (Fig. 1). Each tank was connected to a 100-L reservoir tank equipped with a cooling unit and aerator. The reservoir tank could be closed off from the holding tank, drained and refilled with fresh water to maintain high water quality in the tanks. This was done weekly throughout the experimental period. Photoperiod and temperature were altered to mimic seasonal cycles.

The fish were introduced into the holding tanks in September, 1982. In January , 1983, two of the tanks began receiving doses of an initial blend of three heavy metals (copper, cadmium and chromium) and two metaloids (arsenic and selenium). The relative concentrations were derived from the observed concentrations found in a fly-ash slurry entering a settling basin at a small coal- fired power plant in Virginia (Cherry et al., 1982b). The average values were: copper - 1.3 mg/L, chromium - 0.7 mg/L, cadmium - 0.03 mg/L, arsenic - 2.4 mg/L and selenium - 0.5 mg/L. Initial exposure levels were 0 (control), 5% and 10% of the fly-ash slurry content. A 10% slurry was roughly equivalent to the LC01 level in a

Figure 1. Circular holding tanks used for acclimating fish for avoidance experiments of heavy metal blends in the laboratory at Va. Tech. using dechlorinated tap water and at Glen Lyn using raw New River water.


HOLDING TANK
PERFORATED STANDPIPE
EXERCISE CURRENT
WATER LEVEL
CIRCULATION
PUMP
RETURN FLOW
TEMPERATURE CONTROLLER/AERATER
CONDITIONING TANK

flow-through bioassay. After six weeks, both treated groups experienced mortalities, and significant numbers of fish were displaying equilibrium loss, abnormal color and no response to food.

The exposure schedule was reduced to 0 (control), 1% and 2% of the fly-ash slurry content and cadmium was removed altogether because it was below reliable detection limits (Table 1). Exposure levels were maintained by dosing the reservoir tank each week when the water was renewed. Water samples for metals analysis were taken immediately before water renewal, preserved at pH-2 with redistilled HNO_3, and refrigerated prior to analysis. During the first season of dosing and periodically thereafter, water samples were also taken 24 hrs after water renewal to ensure that metals levels did not go wide fluctuations after dosing. In addition, several water-quality characteristics were routinely monitored (Table 1).

LABORATORY AVOIDANCE TESTING

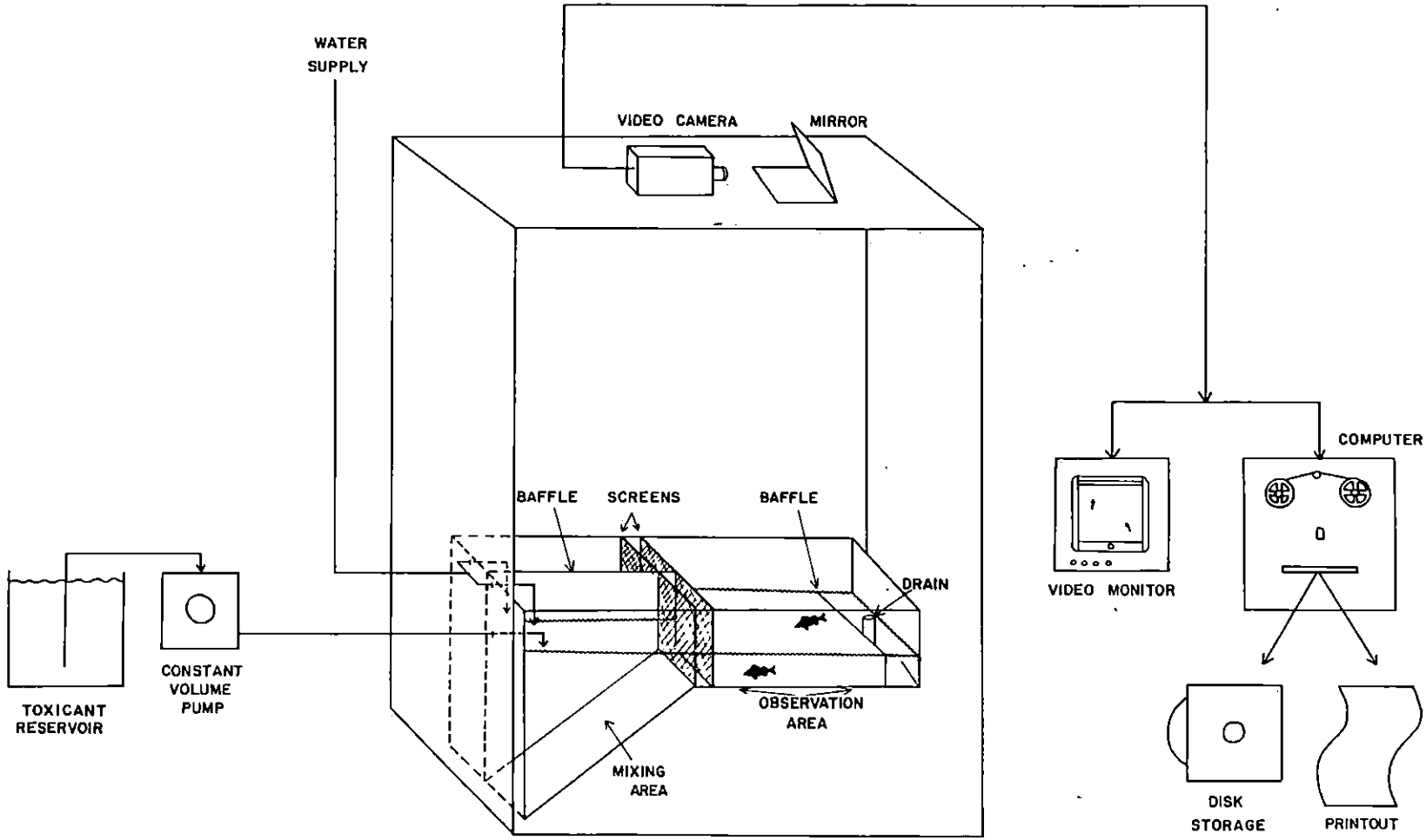
Avoidance response to the blend of the metals was determined in a steep gradient, laminar flow chamber (Fig. 2) previously described by Hartwell et al., (1985). Control fish were tested in 1983 during spring (6 months holding), summer (9 months holding) and fall (12 months holding) of 1983 and winter (15 months holding) of 1984. Low exposure (1%) fish

Table 1. Water quality parameters of holding water for fathead minnows during 15 months holding. All values are in mg/L except pH. Numbers in parentheses are standard deviations. Metals, n=5; non-metals, n=20 (Low dose, n=10). Nitrate, phoshpate etc. are expressed as mg/L of the ion; hardness and alkalinity as mg/L $CaCO_3$.

Parameter	Control	Low Dose	High Dose
NO_3	4.19 (2.89)	2.26 (0.85)	6.13 (4.38)
NH_3	0.076 (0.032)	0.064 (0.036)	0.152 (0.133)
PO_4	0.310 (0.183)	0.177 (0.119)	0.466 (0.039)
SO_4	11.16 (3.77)	6.85 (3.34)	12.58 (4.19)
Cl	7.92 (1.65)	8.70 (2.73)	7.49 (2.33)
Hard.	89.0 (32.5)	85.6 (26.0)	79.0 (27.7)
Alk.	32.0 (7.69)	28.8 (5.34)	36.7 (4.53)
pH	7.5 (0.3)	7.3 (0.2)	7.6 (0.2)
Cu[1]	0.011 (0.006)	0.012 (0.003)	0.009 (0.004)
Cr[1]	BD[2]	BD	BD
Cd[1]	BD	BD	BD
As[1]	BD	BD	BD
Se[1]	BD	BD	BD
Cu[3]	0.011 (0.006)	0.034 (0.006)	0.031 (0.009)
Cr[3]	BD	0.004 (0.003)	0.006 (0.002)
As[3]	BD	0.018 (0.006)	0.025 (0.005)
Se[3]	BD	0.011 (0.005)	0.008 (0.006)

[1]Before dosing begun, n=5
[2]BD-below detection limit (Cu-0.002, Cr-0.002, As-0.005, Se-0.005 mg/L)
[3]After concentration adjustment; control and high dose, n=28; low dose, n=11.

Figure 2. Fish avoidance chamber with a video monitor computer interfaced system.


WATER
SUPPLY
VIDEO CAMERA
MIRROR
BAFFLE
SCREENS
BAFFLE
DRAIN
TOXICANT
RESERVOIR
CONSTANT
VOLUME
PUMP
OBSERVATION
AREA
MIXING
AREA
VIDEO MONITOR
COMPUTER
DISK
STORAGE
PRINTOUT

were tested in the summer of 1983 after 3-months exposure. High exposure (2%) fish were tested in summer and fall of 1983 and winter of 1984 after 3, 6 and 9-months of metals exposure, respectively.

Tests were conducted by placing a school of five fish in the test chamber and allowing them to calm for 30 to 60 min and exhibit random exploratory activity. A 10-min control period followed, with no metals solution input. The location of each fish was manually recorded at 30-sec intervals. Activity was measured during every other 30-sec interval by counting how often fish crossed from one quadrant to another. If one fish moved from the upstream left quadrant to the downstream left quadrant, it was counted as one crossing. If the entire school moved from one quadrant to another, it was counted as five crossings.

Following the control period, a metals-blend solution, made up of the same relative concentrations as the long-term exposure blend, was injected into one side of the chamber by a peristaltic pump. After 5-min of dosing, another 10-min test period was conducted, and observations were made for fish locations every 30-sec and activity levels every other 30-sec. This procedure was followed such that test concentration increased in a stepwise progression to levels which were roughly linear on a log scale. Control fish were tested

at target concentration levels equal to 0 (control), 0.3, 0.5, 0.7, 1.0, 1.5 and 2.2% of the concentration of the fly-ash basin. Low exposure fish were tested at target concentration levels of 0 (control) 0.4, 0.6, 1.0, 1.4, 2.0, 3.0, 4.6 and 6.6% of the influent concentration of the fly-ash basin. High exposure fish were tested at target concentration levels of 0 (control),0.4, 0.6, 1.0, 1.4, 2.0, 3.0 and 4.6% of the influent concentration of the fly-ash basin in summer trials. In fall trials, they were tested at target concentration levels of 0 (control), 0.6, 1.5, 3.0, 4.6, 7.0, 11.0 and 16% and in winter trials at target concentration levels of 0 (control), 0.6, 1.5, 3.0, 4.6, 7.0, 11.0, 16.0 and 23.0% of the fly ash basin influent concentration. The target concentrations varied seasonally in the high dose trials because the tests were run until a response was definite or to the limit of the dosing set-up capabilities. There were always minor concentration variations from replicate to replicate due to manually setting pump rates, age of tubing in the pump head etc., so measured concentrations varied from target levels by minor amounts.

Water samples were drawn at each test concentration, from the test area via sample ports located in the floor of the test area. Metals were analyzed by the furnace technique on a Perkin-Elmer Model 703 atomic absorption spectrophotometer equipped with a deuterium arc background lamp for As and Se

analyses. In tests in which fish from the long-term exposure groups were used, a background concentration of metals equivalent to their long-term exposure level was injected into the avoidance chamber water supply. Following each set of tests, the fish were discarded so novice fish were used in each replicat in each season. All tests were replicated at least five times except the summer control group. Due to an accident in an adjacent laboratory, all the low dose (1% exposure) fish perished, and the control fish holding tank was damaged. Tests were suspended until the fall season, before the summer control group tests were completed.

The large number of samples necessitated a method for reducing the number of analyses. Thus, for the laboratory and field avoidance trials, chromium was analyzed in all samples because this analysis was the most precise. Copper, As and Se were measured at low, median and high test concentrations in each replicate. Least square regression equations were calculated for Cr versus each other element in each experimental cell, e.g. all spring season control fish. Based upon these regression slopes, the concentrations of Cu, As and Se were estimated from the measured Cr levels. Because all the elements were mixed into one solution for the avoidance trials, the relative concentrations did not change within one experimental cell. Correlation coefficients for

Cr versus each other metal for each experimental cell are shown in Appendix 1.

FIELD EXPOSURE

Two groups of 250 yearling, fathead minnows were kept in separate 400-L fiberglass tanks as described above. The tanks were located in a laboratory trailer on the premises of Appalachian Power Company's Glen Lyn, steam-generating plant. Fish were introduced into the holding tanks in February, 1984. Following two months of acclimation, one group was dosed with a blend of the four metals equal to the high-dose concentration in the laboratory exposure portion of the research. The other group served as controls.

The exposure level was maintained in the dosed group by renewing 20% of the water on a weekly basis and adding the appropriate amount of metals to the water by the same procedure as in the laboratory exposure. Water samples for metals analysis were taken weekly, preserved in the trailer at pH 2 with redistilled HNO_3 and refrigerated prior to analysis. Holding water samples and subsequent avoidance test samples required wet digestion with redistilled HNO_3 according to Environmental Protection Agency methods (EPA, 1979a)to attain 100% recovery in the analyses. In addition, water samples were filtered (0.45-um) for determination of dissolved

metals. Several water quality characteristics were routinely monitored (Table 2) including total organic carbon (TOC) and turbidity. The water supply was raw, New River water which was pumped from the intake-well in the power plant screen-house located upstream of the plant. Photoperiod was natural daylight and temperature was controlled to mimic seasonal cycles. Fish were fed twice a day with automatic feeders.

Avoidance responses to the metals blend was determined for both groups in a modified artificial stream supplied with New River water and in a natural stream setting in Adair Run, a small second-order tributary to the New River. Control fish were tested beginning in April in the artificial stream and during July and August in the artificial stream and in Adair Run. Metals acclimated fish were tested in the artificial stream in July (3-months metals acclimation) and in Adair Run beginning in September (5-months metals acclimation). Adair Run tests were delayed until September due to low water in the latter half of August.

ARTIFICIAL STREAM AVOIDANCE TESTING

The artificial stream was constructed of a galvanized hatchery trough (20 cm deep X 39 cm wide X 4.6 m long). The inside was coated with white epoxy paint. New River water

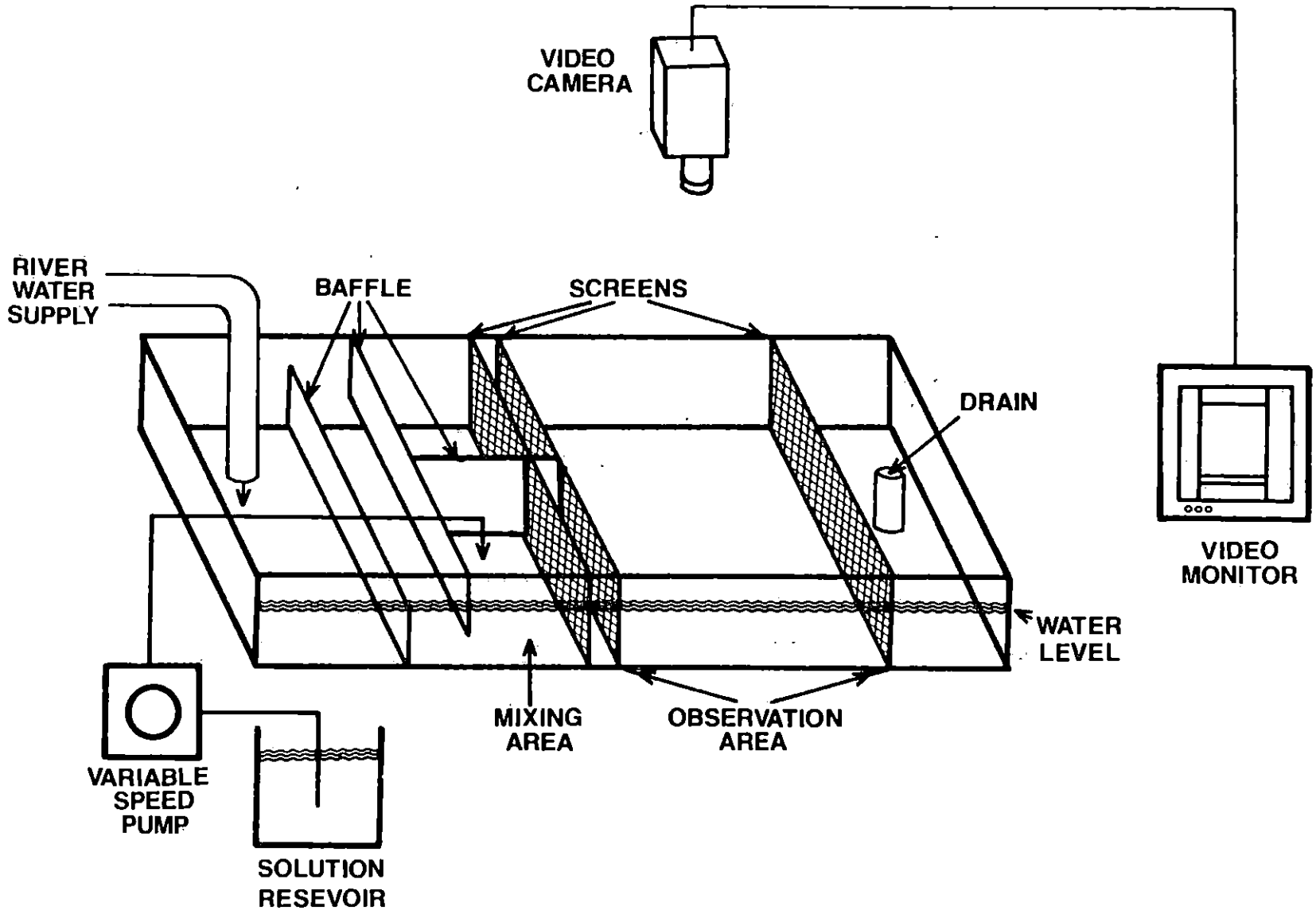
Table 2. Water quality parameters of holding water for fathead minnows during 7 months holding in New River water. All values are in mg/L except pH and turbidity (JTU). Numbers in parentheses are standard deviations. Nitrate, phosphate etc. are expressed as mg/L of the ion; hardness and alkalinity as mg/L $CaCO_3$.

Parameter	Control	Exposed
NO_3	41.2 (18.2)	50.9 (14.0)
PO_4	1.64 (1.23)	1.87 (1.32)
SO_4	13.9 (4.3)	15.2 (4.9)
Cl	4.6 (1.4)	4.9 (1.0)
Hard.	76.7 (8.3)	84.3 (8.0)
Alk.	26.5 (9.8)	19.9 (7.6)
pH	7.3 (0.4)	7.1 (0.4)
Turb.	1.4 (0.5)	1.3 (0.3)
TOC	6.1 (1.5)	5.2 (0.7)
Cu	0.012 (0.004)	0.040 (0.013)
Cr	0.003 (0.002)	0.011 (0.002)
As	BD[1]	0.053 (0.013)
Se	BD[1]	0.008 (0.003)

metals exposed fish n=16, control fish n=21, non-metals n=21
[1]BD=below detection limit (Cu-0.002, Cr-0.002, As-0.005, Se-0.005-mg/L)

was pumped from the screen-house intake well into one end of the trough and flowed through a series of Plexiglas baffles to eliminate turbulence (Fig. 3). Downstream of these, a glass baffle bisected the trough and screens smoothed the flow profile. A concentrated solution of metals was pumped into the mixing zone upstream of the screens through a perforated 'T' manifold to dose one half of the trough. Water flowed through the observation area in nearly laminar fashion with a boundary down the middle, offering the fish a dual choice situation. Dye testing demonstrated excellent separation of the two parcels of water all the way through the observation area. The observation area measured 39-cm X 39-cm and depth was maintained at 5-cm by the downstream standpipe. Flow rate was regulated at 20-L/min. The upstream ends and sides were shielded by a black-plastic canopy to prevent the operator from disturbing the fish. The top and downstream ends were open for indirect sunlight. A video camera was mounted on the canopy frame directly above the observation area and fed into a video monitor located in the laboratory trailer for remote observation of fish behavior. Sample ports located in the floor of each side of the observation area led to stopcocks outside the canopy for water sampling during testing without disturbing the fish. During tests with long-term metals acclimated fish, a background concentration of the metals blend equal to their exposure

Figure 3. Fish avoidance chamber within an artificial stream for testing response of fathead minnows to metal blend solutions in raw New River water. Canopy not illustrated.


VIDEO CAMERA
RIVER WATER SUPPLY
BAFFLE
SCREENS
DRAIN
VIDEO MONITOR
WATER LEVEL
MIXING AREA
OBSERVATION AREA
VARIABLE SPEED PUMP
SOLUTION RESEVOIR

level was injected into the water entering the artificial stream.

IN-STREAM AVOIDANCE TESTING

The avoidance trials in the natural stream setting were conducted in a second order tributary to the New River called Adair Run. It received the effluent from the power plant's fly ash pond ≈75-m above its confluence with the New River (Fig. 4). Avoidance tests were conducted in shallow pools ≈1-km above the New River confluence. The stream basin above the test area has no known urban of agricultural runoff point sources. In the test area, the stream bottom was flat slab rock with patchy areas of gravel and cobble.

Avoidance trials were conducted in a nylon mesh enclosure placed directly in the stream (Fig 5). The enclosure was constructed of a PVC frame 50-cm wide X 44-cm long at the base with 45° sloping sides. A nylon net (3.8-mm mesh) was stretched over the frame and secured at the four upright corners. The enclosure was surrounded on three sides by a black plastic canopy but was open on the upstream side. An observation slit was cut in the downstream canopy wall so an observer could stand downstream of the enclosure and watch the fish from above without disturbing them.

Figure 4. Map of field laboratory area showing Appalachian Power Co. generating station, field lab, screen house, fly ash basin and Adair Run field site.

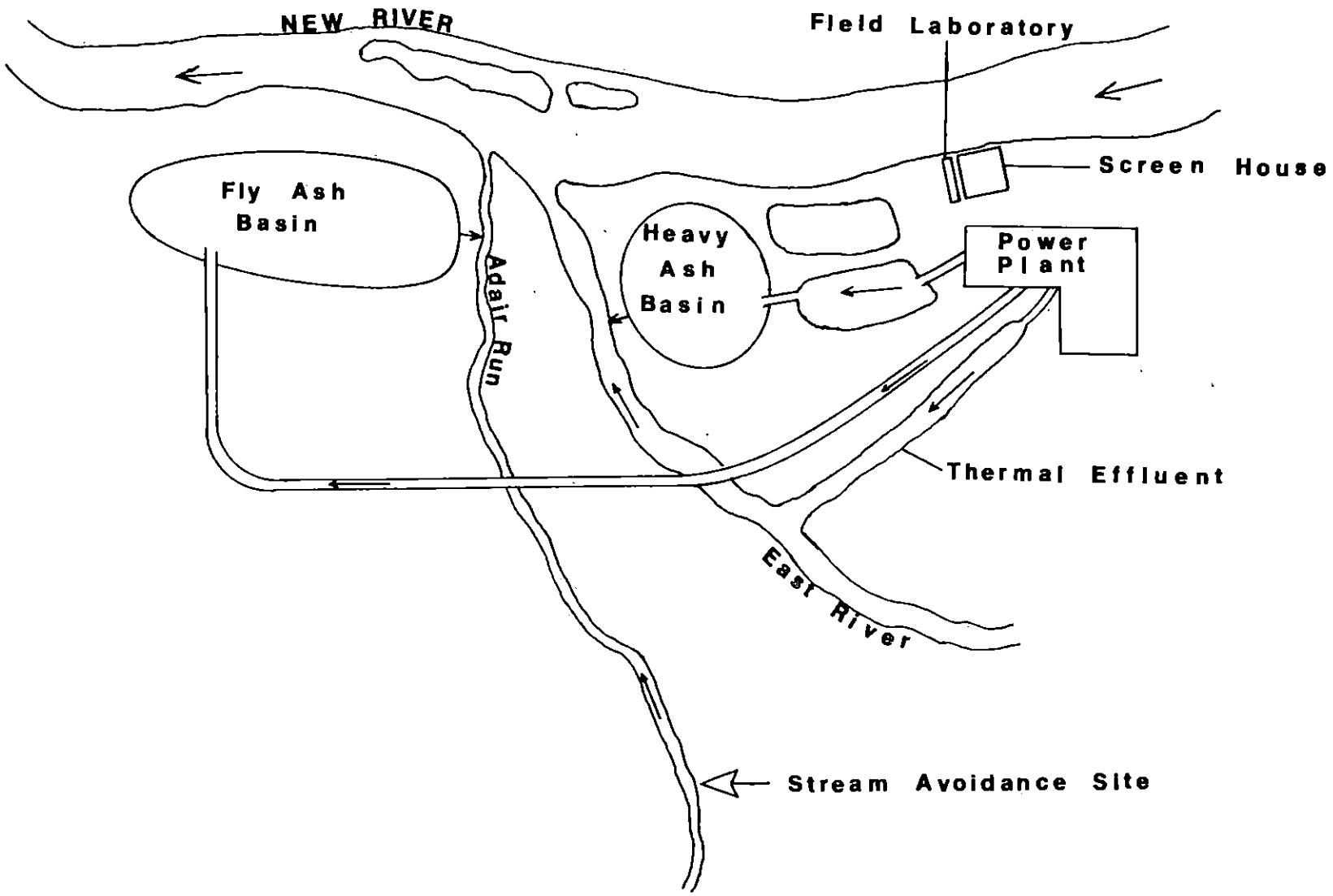
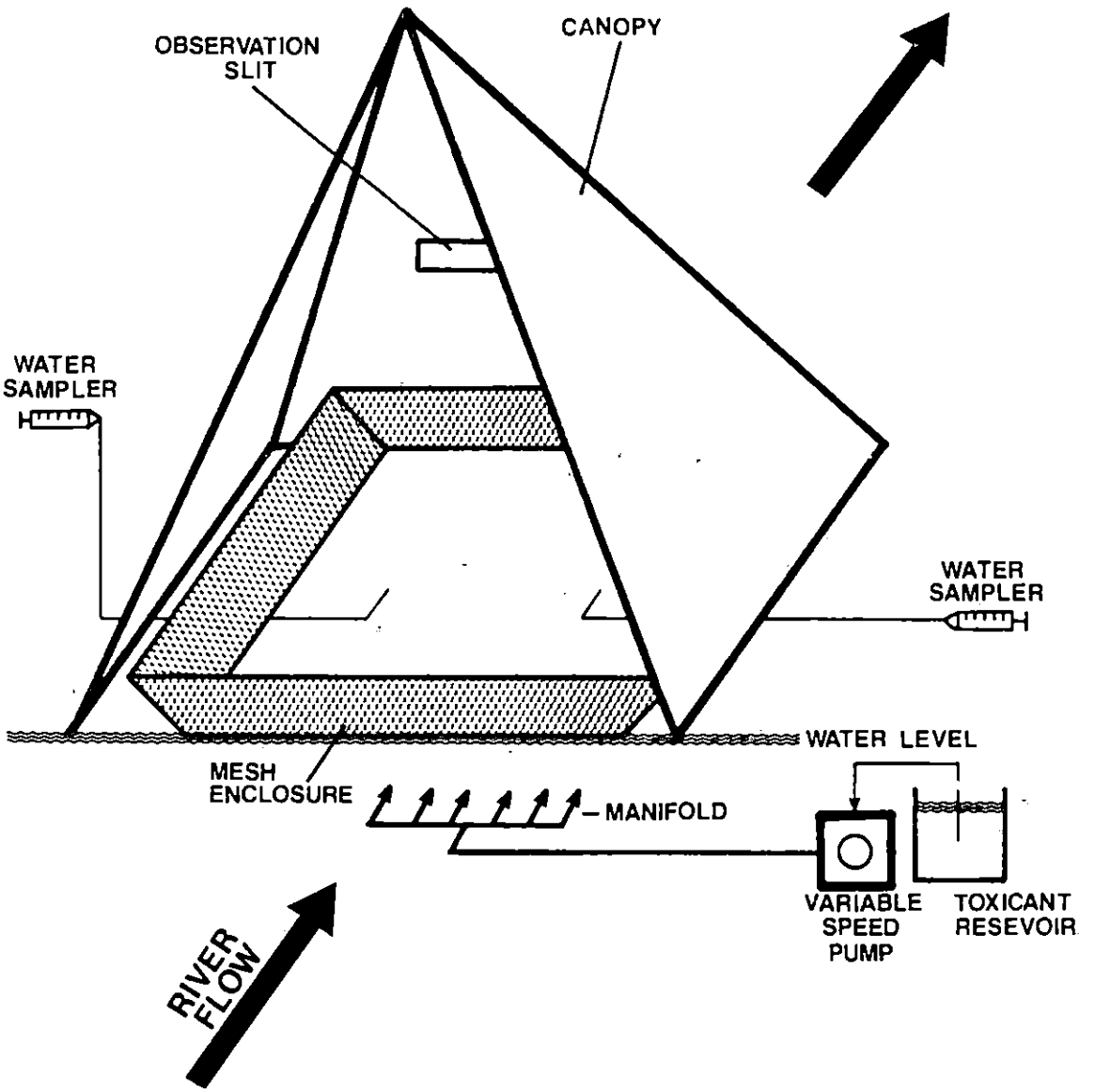

NEW RIVER
Field Laboratory
Screen House
Fly Ash Basin
Adair Run
Heavy Ash Basin
Power Plant
Thermal Effluent
East River
Stream Avoidance Site

Figure 5. Portable avoidance chamber for testing response of fathead minnows to metal blend solutions in a natural stream setting.


OBSERVATION SLIT
CANOPY
WATER SAMPLER
WATER SAMPLER
WATER LEVEL
MESH ENCLOSURE
MANIFOLD
VARIABLE SPEED PUMP
TOXICANT RESEVOIR
RIVER FLOW

A battery-powered, variable-speed pump injected the metals blend into a manifold located upstream of the enclosure which was positioned such that one half of the enclosure was dosed. No attempt was made to control flow rate, turbulence, or any aspect of stream flow through the enclosure. However, meticulous dye- testing prior to each replicate was necessary to position the enclosure such that the stream flowed straight through the enclosure and the metals solution did not meander over both sides. At times, this proved quite difficult and the exact test location varied by several meters from day to day so that suitable flow patterns and flow rates could be located. Dye tests were also conducted following each replicate because flow rates and patterns could vary over a matter of hours if it had rained during the previous 24-hrs. Flow rate was calculated prior to each replicate based upon water velocity through a known volume (the enclosure area X depth). Water depth varied from 2 to 5-cm between replicates, and flow volume through the enclosure varied between 26 and 120-L/min, (mean flow rate=58-L/min). Each side of the enclosure had a sampling tube affixed in the center. Water samples were taken directly from the two sides of the test area by drawing water through a silicone tube with a 50-cc syringe. In tests using long term metals acclimated fish, a background concentration of the metals blend equal to their exposure level was introduced into the stream above the test area with a mariote bottle. Again,

placement was guided by dye tests and flow rate was based upon measured stream-flow upstream of the enclosure.

Experiments were conducted in the stream enclosure and the artificial stream using the same procedures as in the laboratory so experimental procedure was not a factor in laboratory versus field comparisons. Because activity was not measured in these tests, fish location was recorded as either dosed side or undosed side instead of quadrants. Control fish were tested at target concentration levels equal to 0 (control), 0.5, 0.7, 1.0, 1.5 and 2.2% of the influent concentration of the fly-ash basin. The long term metals acclimated fish were tested at target concentration of 0 (control), 2.5, 3.5, 5.0, 7.0, 10.0, 14.0 and 20.0% of the fly ash basin influent concentration, above background (2% exposure) levels.

The fish usually showed a preference for one side or the other of the test area during the control periods, and in those cases, the dosing manifold was placed on that side at the beginning of the test. When an avoidance response was evident, the position of the manifold was switched to the opposite side at the end of the test series for another 10-min period to confirm the response.

Water samples were drawn from the test areas at each test concentration via the sampling tubes. In addition to metals analysis, water samples were drawn for analysis of nitrate (NO_3), sulfate (SO_4), phosphate (PO_4), chloride (Cl), hardness, alkalinity, pH, turbidity and total organic carbon (TOC). Following each test, the fish were discarded so each replicate in each season used novice fish. All tests were replicated at least eight times.

BIOASSAY METHODS

Acute toxicity bioassays were conducted on four groups of fish; 1, laboratory control, 2 high metals acclimated fish, 3 field control and 4, field metals acclimated fish. These tests were conducted after all avoidance experiments were concluded. The bioassay test fish were the fish remaining after all avoidance tested fish had been removed. In the case of the laboratory, high metals acclimated fish, a group different from those used in the avoidance trials were used. Not enough fish were left after avoidance trials and preliminary range finding bioassays to conduct an adequate bioassay. The second group of fish were originally slated to be used to repeat the three month exposure to the high metals blend, without an initial Cd spike. This experiment was abandoned due to lack of As supplies which lasted six weeks at the end of the three month exposure. However, they

were maintained for a full 10 months (May 24, 1984 - March 25, 1985) under exposure for the purposes of the bioassay. Water quality characteristics of their holding water are shown in Table 3. These values compare favorably with the previous laboratory values and the Glen Lyn holding values (Tables 1 and 2). The mean metals dose was 1.8% of the concentration of the fly-ash basin influent. The percentage of metals in the dissolved state (0.45-um) were ≈100% in all cases (n=4). Preliminary, 96-hr static tests were conducted in 4-L, glass aquaria. Ten fish were introduced into aerated aquaria in the morning, and dosing was initiated at noon by introduction of a small volume of concentrated test blend solution. Test solutions were made from reagent grade chemical salts, $CuCl_2$ $2H_2O$ (cupric chloride), $K_2Cr_2O_7$ (potassium dichromate), Na_2AsO_2 (sodium arsenite), and Na_2SeO_3 (sodium selenite). Elemental concentrations were determined on a Perkin-Elmer (Norwich, Conn.) model 460 atomic absorption spectrophotometer according to standard techniques (U.S. EPA, 1979a). Fish were not for fed 24-hr before or during the tests. The 96-hr LC50 was calculated by a Spearman-Karber technique (Finney, 1971), which determines the 50% toxicity level when there are no partial kills above and below the 50% level as required by standard probit analysis.

Based upon the static test results, test concentration of the metal blend were selected for the flow-through bioassays.

Table 3. Water quality parameters of holding water for fathead minnows during 12 months holding. All values are in mg/L except pH and turbidity (JTU). Numbers in parentheses are standard deviations. Metals concentrations shown are after dosing began. The only element present in detectable levels prior to dosing was copper at 0.014 mg/L, (std. dev.=0.002). Nitrate phosphate etc. are expressed as mg/L of the ion; hardness and alkalinity as mg/L $CaCO_3$.

Parameter	mg/L
NO_3	18.56 (17.71)
PO_4	1.29 (1.36)
SO_4	15.47 (3.28)
Cl	7.83 (1.40)
Hard.	77.5 (9.6)
Alk.	23.3 (3.4)
pH	7.6 (0.01)
Cu	0.026 (0.012)
Cr	0.011 (0.003)
As	0.051 (0.008)
Se	0.008 (0.002)

metals n=10, non-metals n=14

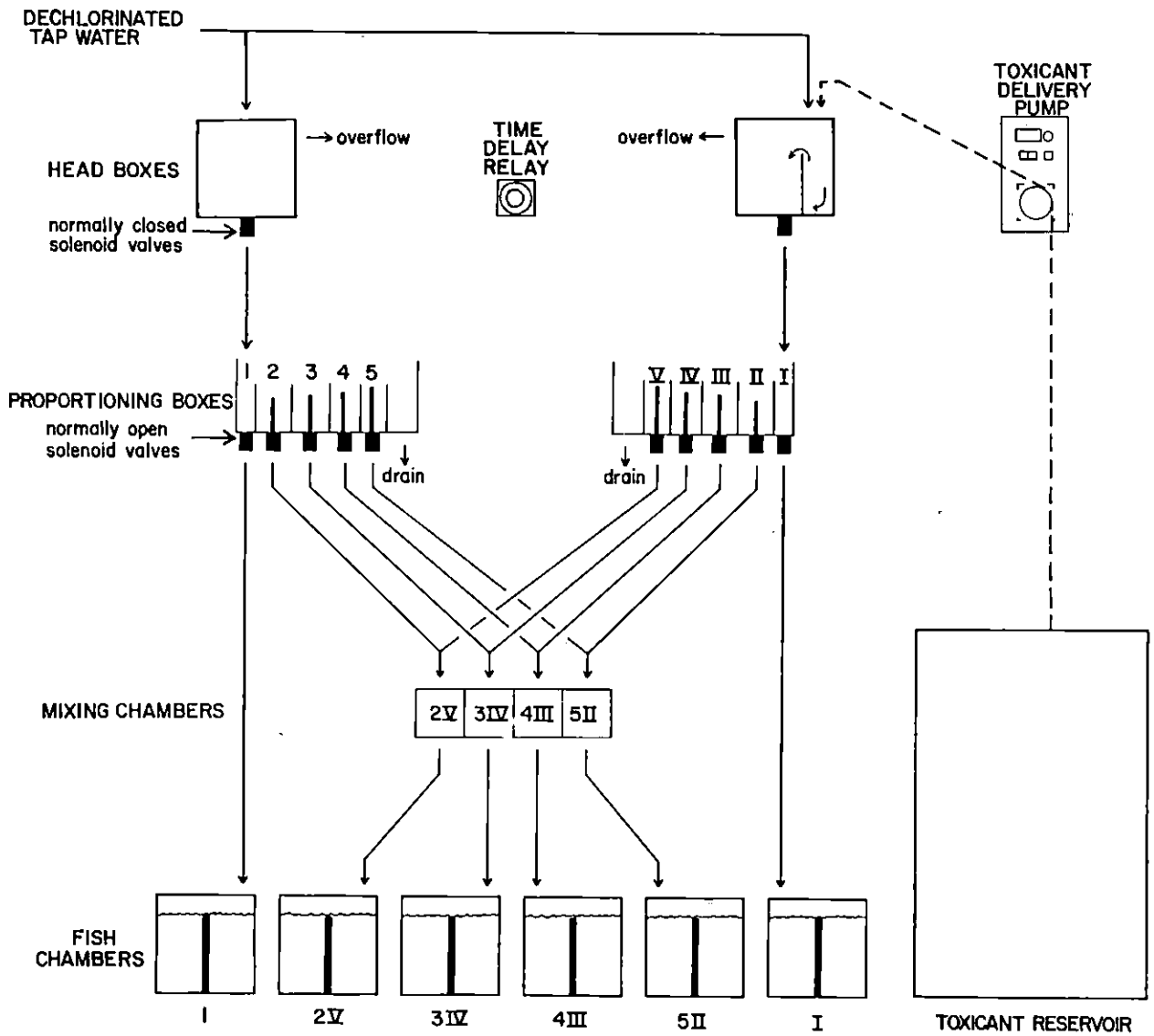
The 96-hr flow-through bioassays were conducted in a solenoid activated serial diluter (Fig. 6), originally described by Hendricks et al. (1977).

The diluter had two head boxes, each supplied with dechlorinated Blacksburg City water at controlled flow rates. The metals solutions were pumped into one head box at a controlled rate. The flow rates and solution concentrations varied according to target test concentrations. A timer relay controlled the solenoid valves leading from the head boxes. Five proportioning boxes were filled with test solutions or dilution water. The contents of those boxes were released through solenoid valves into mixing chambers where the desired concentrations of the solution were developed. The mixtures then flowed by gravity into the fish chambers.

As with the static tests, laboratory acclimated and field acclimated fish were introduced into the chambers and allowed several hours to calm prior to introduction of the test solutions. Fish were not fed 24-hr prior to or during the tests. Water was sampled at 24-hr intervals from each chamber and analyzed for metals content. The reported values for each replicate test are averages of the daily measurements.

In addition to the four metal toxicity bioassays using fish acclimated to the laboratory for long periods, a bioassay was conducted at the

Figure 6. Solenoid actuated flow-through diluter for determining laboratory LC50's to metal blends.


DECHLORINATED TAP WATER
TOXICANT DELIVERY PUMP
HEAD BOXES
overflow
TIME DELAY RELAY
overflow
normally closed solenoid valves
PROPORTIONING BOXES
1 2 3 4 5
V IV III II I
normally open solenoid valves
drain
drain
MIXING CHAMBERS
2V 3IV 4III 5II
FISH CHAMBERS
I
2V
3IV
4III
5II
I
TOXICANT RESERVOIR

conducted at the beginning of the experiments, using all five of the original elements, in the same relative proportions as found in the fly-ash basin influent, (Cu-1.3mg/L, Cr-0.7mg/L, Cd-0.03mg/L, As-2.4mg/L and Se-0.5mg/L). Cadmium was added to the stock solutions as $CdCl_2 2.5H_2O$. This preliminary bioassay was conducted using yearling fathead minnows which had been acclimated to laboratory conditions for one month, prior to testing.

STATISTICAL EVALUATION

All statistical tests were conducted using the Statistical Analysis System (SAS, 1982) on the Virginia Polytechnic Institute and State University IBM 3081 system. Optimum statistical analysis methods were developed using data from the laboratory control-fish avoidance data (Hartwell et al., 1985). All other laboratory and field data were subsequently analyzed using these methods. The total number of fish counts on the dosed side was summed for each 10-min period. With five fish and 20 counting periods, the total possible was 100. Quadratic regressions of proportion of fish on the dosed side vs test concentrations and the arcsin transformationof proportion vs test concentration were calculated to test for deviations from normality. The proportion of fish on the dosed side was used as the dependent variable in least square regressions against test concen-

tration, log transformation of concentration, quadratic regression of concentration, and concentration squared. Each of the above regressions was calculated with and without control periods, against test proportion minus control period proportions, and with test proportions corrected for control period proportions according to the equation

$$Pc=((P-C)/100-C) \times 100$$

where Pc is the corrected value, P is the test proportion, and C is the control proportion. Each data set was evaluated with and without replicate as a covariate. Quadratic regressions were calculated using replicates designated as dummy variables having the values 1 or 0 to test for parallelism and coincidence of replicate lines. Proportions of fish on the dosed side were also tested at each concentration using the Hochberg GT2 test (Hochberg, 1974) which is similar to Duncan's pairwise test (Steel and Tori, 1960) but is less sensitive to unequal replication in test cells.

The regression lines representing the avoidance response, were pooled within each season, and were tested for differences between seasons by analysis of variance.

Fish activity, as measured by quadrant crossings per 10-min test period, was used as the dependent variable in least square linear regressions against test concentration, quadratic regression of concentration, and concentration square.

Dummy variables were again used for individual replicates to test for parallelism and coincidence. Analysis of variance of avoidance response over seasons or over exposure level and for laboratory vs field control trials were run. For the field data, measured water quality parameters were employed as covariates in the analysis of variance comparisons of avoidance lines between test systems (artificial streams and Adair Run). Bioassay data was analyzed using standard probit analysis (SAS 1982).

RESULTS

STATISTICAL EVALUATION

The avoidance response of control fatheads to the metals blend in each laboratory trial season are shown in Fig. 7. Regression statistics for each line are shown in Table 4. The arcsin transformation of the data resulted in virtually identical results and examination of residuals from both methods did not indicate departures from homogenity of variances. Correlation coefficients (r^2) for linear, quadratic, and $\log_{10}$ transformed regressions are shown in Table 5 for each combination of covariate inclusion and control period correction. Correlation in the $\log_{10}$-transformed and quadratic methods was consistently at or above the levels for simple linear regression. Within each type of regression model, inclusion of replicate as a covariable also improved the performance of the models. The best overall correlation technique was the quadratic regression with replicate included as a covariable. The major exceptions to this were for tests from the summer season (#2 in Table 4) and for some of the control-corrected values. It should be noted, however, that only two replicates were conducted in that season for a total of only 10 data points. This small number of data points was more sensitive to model changes than data from

Figure 7. Avoidance response of fathead minnows to a blend of four metals following 6, 9, 12 and 15 months of laboratory holding in uncontaminated water. Vertical bars show 95% confidence limits of quadratic regression line.

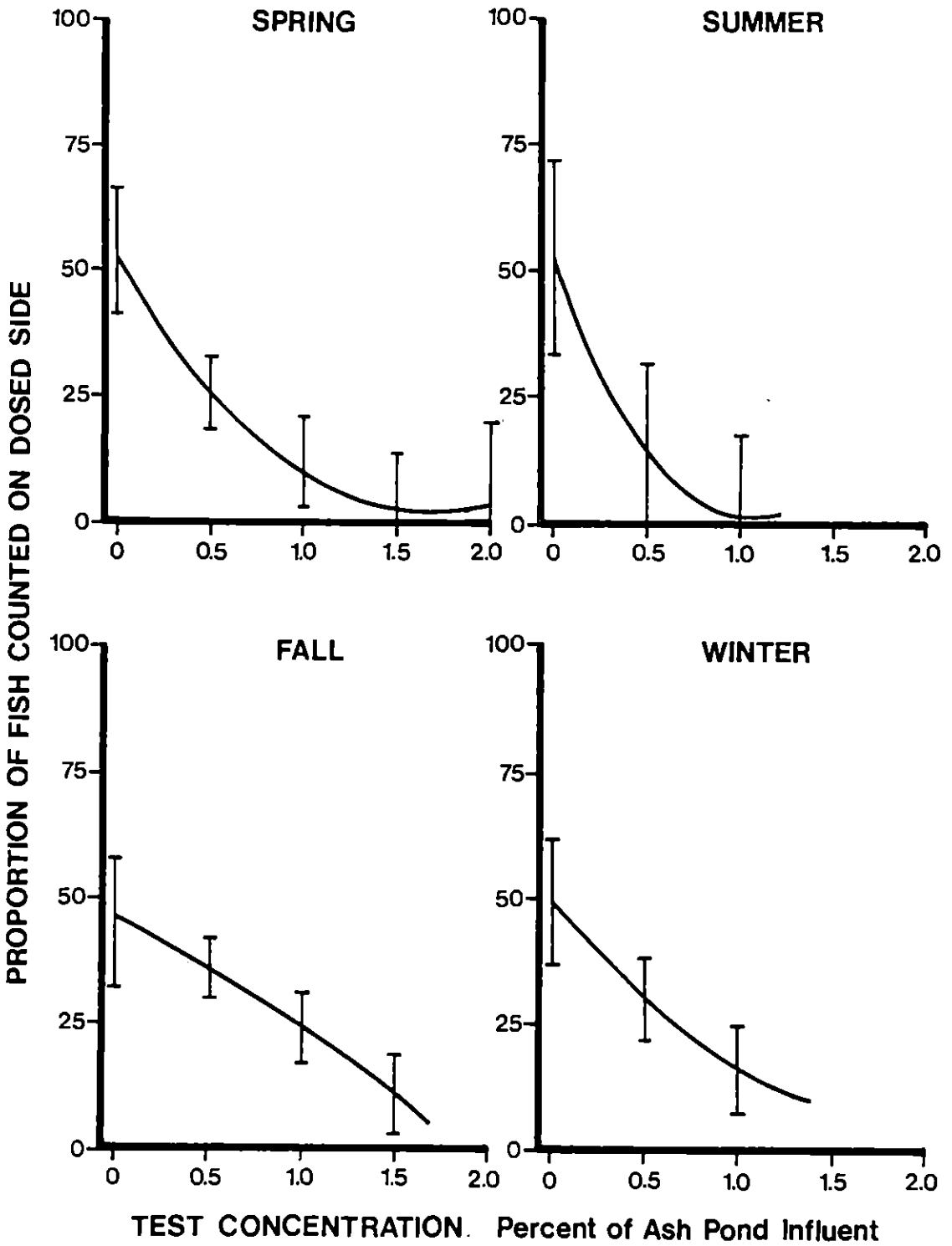

SPRING
SUMMER
FALL
WINTER
100
75
50
25
0
0
0.5
1.0
1.5
2.0
PROPORTION OF FISH COUNTED ON DOSED SIDE
TEST CONCENTRATION. Percent of Ash Pond Influent

Table 4. Slopes for multiple quadratic regressions of fathead minnow residence on exposed side of avoidance apparatus vs measured concentration. Slopes for linear and quadratic concentration terms and overall correlation coefficient (r^2) are shown.

		Regression Slope		
Exposure	Season	Concentration	Concentration^2	r^2
Control	Spring	-60.9[1]	18.2[1]	55.3
Control	Summer	-99.3[1]	47.6[1]	73.3
Control	Fall	-17.2[1]	-3.7[1]	54.2
Control	Winter	-42.9[1]	10.3[1]	47.9
Low Dose	Summer	-13.7	2.0	9.5
High Dose	Summer	-0.8[1]	1.8[1]	36.1
High Dose	Fall	-5.7[1]	0.3[1]	28.6
High Dose	Winter	0.6	0.0	17.6

[1]Significant at the 0.01 level

Table 5. Correlation coefficients (r^2) for regression of fish counted on the dosed side vs test concentration, concentration+ concentration squared, and log_{10} test concentration over four seasons for control fish. Each method contains results from different combinations of using or ignoring control periods as correction factors for replicate as a covariable[1].

		W/O Covariate			W/ Covariate		
Method	Season	Linear	Log_{10}	Quad.	Linear	Log_{10}	Quad
With control period counts[1]	1	49.5	71.5	79.7	49.6	71.8	81.5
	2	57.0	60.9	63.2	59.1	63.3	66.0
	3	55.8	49.6	55.8	63.6	57.0	63.6
	4	36.6	45.3	45.7	45.8	47.7	48.5
Without control period counts	1	23.7	40.9	63.5	24.7	42.0	63.5
	2	43.4	55.9	63.5	48.7	61.2	69.6
	3	45.3	43.9	45.4	59.6	57.5	59.4
	4	22.4	27.8	27.1	25.7	32.3	32.1
Test counts minus control period counts	1	3.8	9.5	26.4	57.7	63.9	69.2
	2	42.2	54.4	61.8	49.6	61.9	70.0
	3	37.3	36.1	37.3	59.5	57.7	59.5
	4	23.2	27.5	26.3	24.1	29.0	28.0
Test counts corrected for control period counts	1	0.0	2.8	14.6	76.1	78.8	80.6
	2	41.8	53.9	61.0	50.6	62.8	70.9
	3	34.7	33.4	34.7	60.3	58.5	60.3
	4	23.3	26.9	25.6	23.5	27.4	26.1

[1]Control period test concentration set to 0.1% for log_{10} transformation

other seasons. Correction for control periods did not consistently improve correlation. Quadratic regression statistics and F-test values for parallelism and coincidence are shown in Table 6. Within seasons, the individual replicate lines were statistically indistinguishable. Concentration was highly significant ($P<0.01$) in all cases. Individually, the concentration square term was significant in only one case, but the quadratic combination was significant in all cases. The tests for parallelism and coincidence were calculated sequentially such that if the lines were parallel, the interaction term was dropped before the model was run to test for coincidence.

Results of the Hochberg GT2 tests are shown in Table 7. The test identified significantly different groups in only two of the four seasons.

Activity, as measured by quadrant crossings, is shown in Figure 8. The trend of activity was generally positive with increasing concentration, but the response was highly variable. Regression statistics for each line are given in Table 8. Unlike regression results from fish counts versus concentration, a significant slope resulted in only one season. Correlation coefficients (r^2) for linear, $\log_{10}$ transformed, and quadratic regressions are given in Table 9. Correlation coefficients were generally low, and no particular model

Table 6. F-values for multiple quadratic regressions of fathead minnow residence on exposed side of avoidance apparatus vs measured concentration. Significant F-values in the parallelism column indicate that the replicate lines were not parallel. Significant F values in the coincidence column indicate that replicate lines were not coincident (i.e., had different intercepts).

		F values for	
Exposure	Season	Parallelism	Coincidence
Control	Spring	1.71	1.59
Control	Summer	0.08	0.50
Control	Fall	0.25	2.73
Control	Winter	0.56	2.13
Low Dose	Summer	2.26	5.63[1]
High Dose	Summer	0.81	2.48
High Dose	Fall	3.31[2]	[3]
High Dose	Winter	2.44	3.52[2]

[1]Significant at the 0.01 level
[2]Significant at the 0.05 level
[3]If lines are not parallel, by definition they cannot be coincident.

Table 7. Hochberg GT2 test for comparison of proportion of fish counted on dosed side at different dose concentrations.

Season	Dose	Proportion	Grouping[1]	n
	0.0	64.0	A	5
	0.3	34.0	B	5
	0.5	15.8	B C	5
1	1.5	15.5	B C	2
	1.0	7.5	B C	4
	0.7	6.4	C	5
	0.3	48.0	A	2
	0.0	47.0	A	2
	0.5	21.0	A	2
2	1.0	7.0	A	1
	1.5	2.5	A	2
	0.7	1.0	A	1
	0.0	44.4	A	5
	0.3	39.8	A	5
	0.5	28.0	A B	4
3	0.7	25.8	A B	5
	1.0	22.5	A B	4
	1.5	5.0	B	5
	0.0	46.4	A	5
	0.3	45.0	A	3
	0.7	25.2	A	5
4	0.5	19.0	A	3
	1.0	17.8	A	5
	1.5	12.5	A	2

[1]Proportions with the same letter grouping are not significantly different.

Figure 8. Linear regression lines and data points for fathead minnow activity response to metals blend as measured by quadrant crossings.

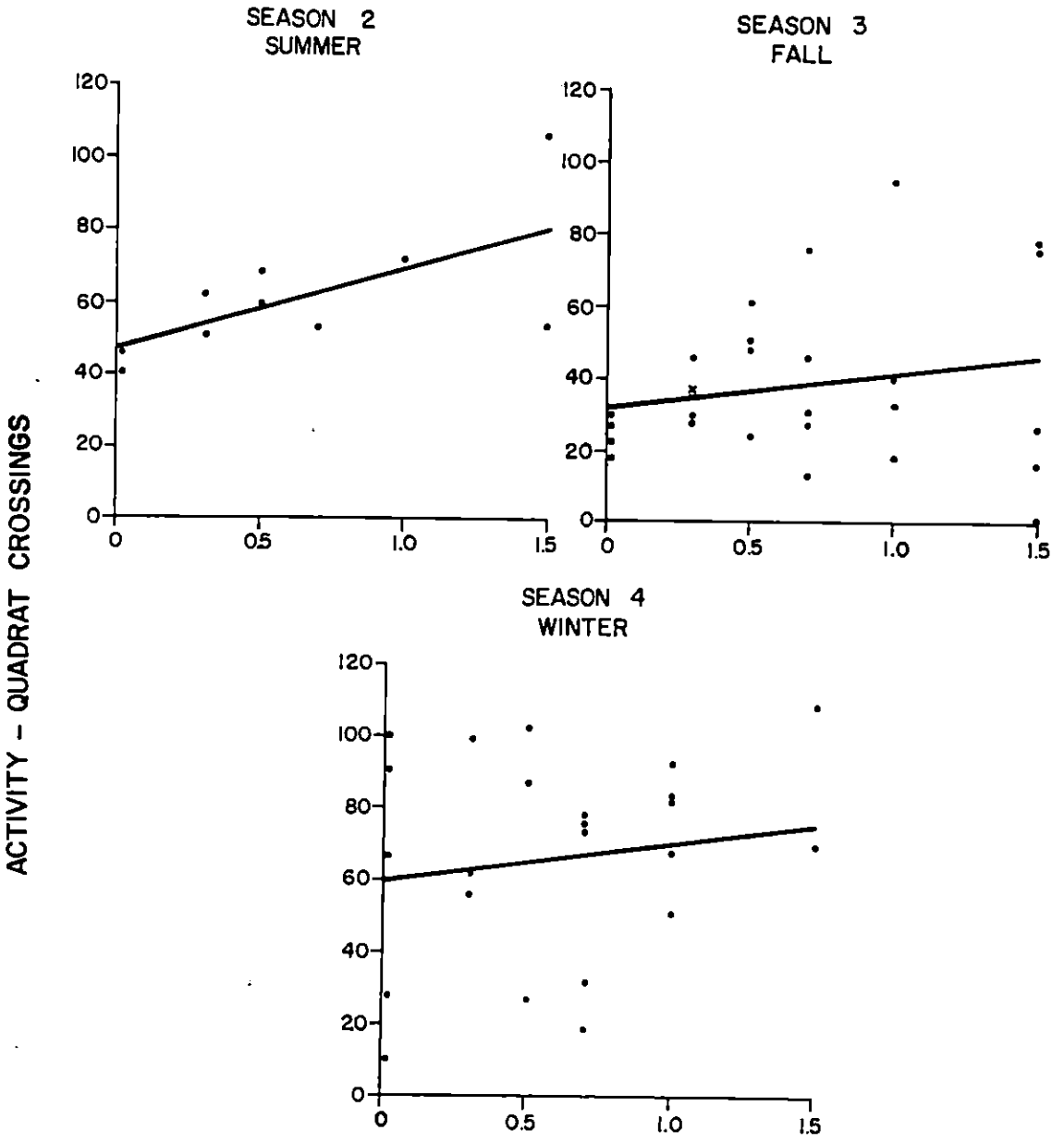

SEASON 2
SUMMER
SEASON 3
FALL
SEASON 4
WINTER
ACTIVITY - QUADRAT CROSSINGS
TEST CONCENTRATION (PERCENT OF ASH POND INFLUENT)
0
20
40
60
80
100
120
0.5
1.0
1.5

Table 8. Slopes for multiple quadratic regressions of fathead minnow activity vs measured concentration. Slopes for linear and quadratic concentration terms and overall correlation coefficient (r^2) are shown.

		Regression Slope		
Exposure	Season	Concentration	Concentration^2	r^2
Control	Spring	Not Measured		
Control	Summer	15.9	9.1	44.8
Control	Fall	2.9	-11.3	6.6
Control	Winter	-12.1	25.4	7.4
Low Dose	Summer	4.2	-1.5	13.4
High Dose	Summer	6.6	-0.8	0.3
High Dose	Fall	-5.6	0.3	7.6
High Dose	Winter	2.0	-0.1	9.4

Table 9. Correlation coefficients (r^2) for regression of fish activity vs test concentration, concentration+concentration squared, and log test concentration over three seasons for control fish. Each block contains results from different combinations of using or ignoring control periods as correction factors and replicate as a covariable[1].

		W/O Covariate			W/ Covariate		
	Season	Linear	Log_{10}	Quad.	Linear	Log_{10}	Quad.
With control period activity	2	43.6	42.3	43.9	55.9	53.8	56.2
	3	4.2	6.7	8.2	24.5	27.3	29.3
	4	4.7	3.0	6.4	9.78	7.8	11.7
Without control period activity	2	27.3	24.2	28.2	44.2	40.6	44.9
	3	0.3	0.6	1.4	27.5	27.9	29.1
	4	4.8	2.1	10.7	4.9	2.2	11.2
Test counts minus control period activity	2	31.1	27.6	32.1	39.0	35.0	39.7
	3	0.3	0.5	1.3	26.2	26.6	27.8
	4	2.5	1.7	3.1	52.9	52.7	57.2

[1]Control test concentration set to 0.1% for log_{10} transformations

stood out as being consistently better than others. Inclusion of replicate as a covariate improved correlation, but the improvement was highly variable between seasons.

Quadratic regression statistics and F-test values for parallelism and coincidence are given in Table 10. Activity responses between replicates were parallel in season 2 and 4, but not in season 3. The lines were also coincident in season 2 and 4 and, of course, not coincident in season 3. Neither concentration nor concentration-square terms were significantly correlated with activity in any season.

LABORATORY AVOIDANCE

The experiments clearly demonstrate that fathead minnows are sensitive to metal blends. Control fish strongly avoided low concentrations of the metal blend (Fig 7). Analysis of variance between seasons indicated that no significant differences existed between responses over the 12-month period ($F=0.39$, $df=4,83$). Also, regressions with replicate as a dummy variable indicate that all replicate lines are parallel and coincident within each season (Table 4). The pooled, mean concentration at which residence in the dosed side was half that of the control period was 0.63% of the fly ash basin influent concentration (std. dev.=0.23), (Table 11). The low exposure (1%) group did not respond to levels of the blend

Table 10. F-values for multiple quadratic regressions of fathead minnow activity vs measured concentration. Significant F values in the parallelism column indicate that the replicate lines were not parallel. Significant F values in the coincidence column indicate that replicate lines were not coincident (i.e., had different intercepts).

		F values for	
Exposure	Season	Parallelism	Coincidence
Control	Summer	2.81	1.69
Control	Fall	4.99[1]	6.73[1]
Control	Winter	0.54	1.98

[1]significicant at the 0.01 level

Table 11. Avoidance test levels expressed as a percentage of the fly ash basin influent concentration and elemental concentrations at those levels expressed in ug/L. Where no response was evident, the highest levels tested are shown.

Exposure History	Avoidance Test	%Influent Concentration	Concentration ug/L Cu	Cr	As	Se
Lab. Control	Laboratory	0.63[1]	8	4	15	3
Lab. Exposed Low Dose 3 Months	Laboratory	5.00NR[2]	65	35	120	25
Lab. Exposed High Dose 3 Months	Laboratory	5.00[3]	65	35	120	25
6 Months	Laboratory	14.00NR	182	98	336	70
9 Months	Laboratory	20.00NR	260	140	480	100
Field Control Spring	Artif. Stream	1.45	19	10	35	7
Summer	Artif. Stream	0.70	9	5	17	4
Summer	Adair Run	1.50	20	11	36	8
Field Exposed Summer	Artif. Stream	30.00NR	390	210	720	150
	Adair Run	60.00NR	780	420	1440	300

[1]pooled data
[2]no response
[3]50% attraction

elevated five times higher than acclimation levels (Fig. 9), (Table 11). While the individual replicate lines are statistically parallel, they are not all coincident, and there is no significant regression with test concentration (Table 4). Variability is very high as indicated by an r^2 value below 10%.

The high exposure (2%) fish had variable responses, depending on the length of metals acclimation (Fig 10). After three months exposure, they were attracted to metals levels elevated to three times their acclimation level (Table 11). All replicate lines were parallel and coincident (Table 4). The quadratic regression F value is highly significant but correlation is not as high as control test correlations.

After six months acclimation the overall response was reversed (Fig 10), with the fish displaying a mild avoidance response at high concentration levels. The quadratic regression F value is significant (Table 4) only at the α=0.05 level and correlation is lower than in the previous season. In addition, the replicate lines are not all parallel (Table 4). This indicates that the responses were not consistent from group to group at a given concentration. After nine months acclimation the fish were indifferent to low test concentrations and mildly attracted to high concentrations (Fig 10). The regression F value is significant at α=0.05

Figure 9. Avoidance response of fathead minnows to a blend of four metals following 3 months exposure to a concentration equal to 1% of the concentrations found in a fly ash basin influent. Test concentrations are expressed as test concentration plus background exposure. Vertical bars show 95% confidence limits of quadratic regression.

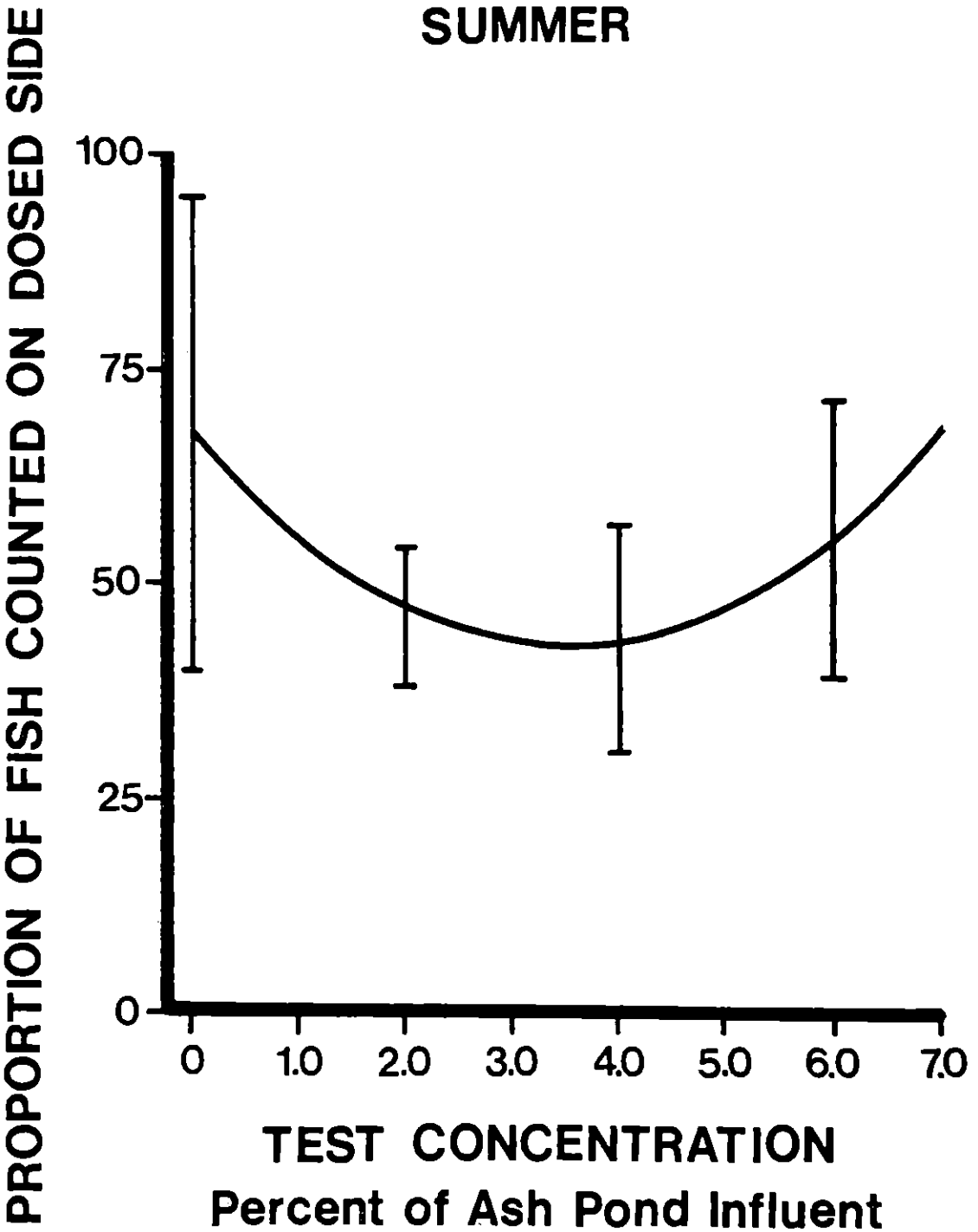
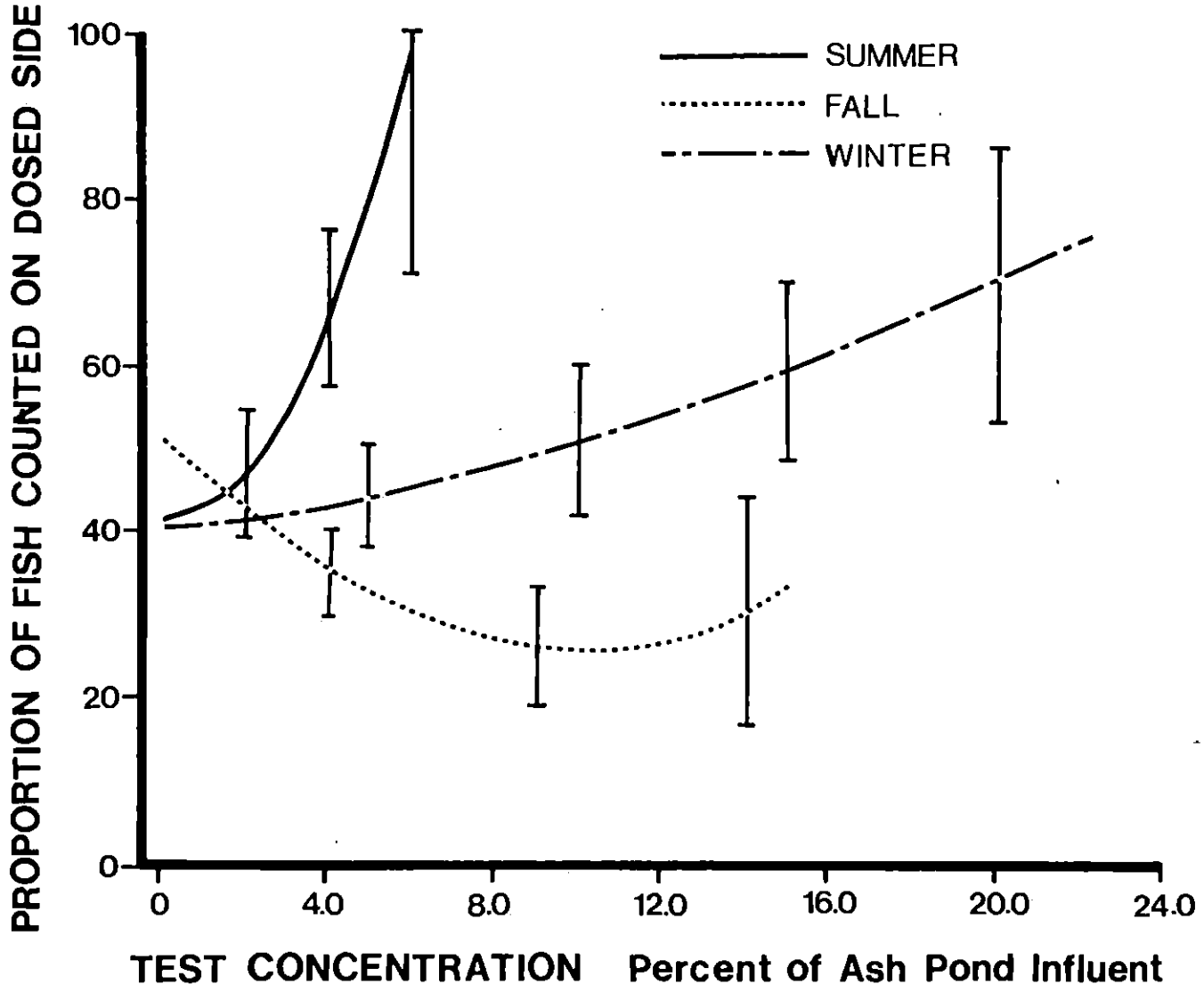

SUMMER
PROPORTION OF FISH COUNTED ON DOSED SIDE
100
75
50
25
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
TEST CONCENTRATION
Percent of Ash Pond Influent

Figure 10. Avoidance response of fathead minnows to a blend of four metals following 3 (summer), 6 (fall) and 9 (winter) months of exposure to a concentration equal to 2% of the concentrations found in a fly ash basin influent. Test concentrations are expressed as test concentration plus background exposure. Vertical bars show 95% limits of quadratic regression.


PROPORTION OF FISH COUNTED ON DOSED SIDE
100
80
60
40
20
0
SUMMER
FALL
WINTER
0
4.0
8.0
12.0
16.0
20.0
24.0
TEST CONCENTRATION Percent of Ash Pond Influent

and correlation is lower than both previous seasons (Table 4). The replicate lines are statistically parallel but not always coincident (Table 4). Again, this indicates that the responses were not consistent from group to group at a given concentration.

Activity of exposed fish did not correlate with test concentration in any season or exposure group (Table 8). As test concentration increased, overall activity increased in two cases, fell in two cases and remained essentially static in three cases. Correlation coefficients were very low in all but one experiment. None of the slopes of the lines were statistically significant because the variability was high.

FIELD AVOIDANCE

Holding water quality characteristics and metal concentrations during the experimental period are shown in Table 2. Most parameters were stable throughout the entire period. Nitrate, PO_4, and to a lesser extent SO_4 and Cl increased gradually from February to late May and early June, and then declined slightly. These values compare well with river characteristics during the test periods (Table 12). Nitrate, PO_4 and TOC were elevated in the holding tanks due to the presence of 250 fish and daily feeding. Likewise, alkalinity and pH were slightly depressed due to acidic metabolic waste

Table 12. Water quality parameters of the New River and Adair Run during field avoidance testing of fathead minnows to heavy metal blends. All values are in mg/L except pH and turbidity (JTU). Numbers in parentheses are standard deviations. Nitrate, phosphate etc., are expressed as mg/L of th ion; hardness and alkalinity as mg/L $CaCO_3$.

Parameter	New R. Control Spring (n=5)	New R. Control Summer (n=5)	New R. Exposed Summer (n=8)
NO_3	5.4 (2.6)	6.2 (3.6)	5.6 (2.4)
PO_4	0.04 (0.08)	0.00 (0.00)	0.00 (0.00)
SO_4	10.4 (1.9)	17.4 (9.6)	13.6 (4.2)
Cl	2.7 (0.7)	4.4 (2.2)	2.8 (0.7)
Hard.	78.0 (4.5)	70.0 (14.1)	67.5 (12.8)
Alk.	50.0 (0.6)	53.2 (1.8)	42.8 (7.6)
pH	7.8 (0.4)	7.9 (0.1)	7.7 (0.4)
Turb.	5.3 (1.5)	3.2 (1.1)	6.1 (2.4)
TOC	1.6(0.1)	1.9 (0.4)	2.4 (0.3)

continued...

Table 12.(cont.) Water quality parameters of the New River and Adair Run during field avoidance testing of fathead minnows to heavy metal blends. All values are in mg/L except pH and turbidity (JTU). Numbers in parentheses are standard deviations. Nitrate, phosphate etc., are expressed as mg/L of the ion; hardness and alkalinity are expressed as mg/L $CaCO_3$.

Parameter	Adair Run Control Summer (n=7)	Adair Run Exposed Summer (n=8)
NO_3	1.7 (0.6)	5.8 (6.4)
PO_4	0.00 (0.00)	0.00 (0.00)
SO_4	18.2 (5.4)	26.0 (2.7)
Cl	5.5 (0.5)	5.2 (0.9)
Hard.	100.3 (18.0)	141.3 (8.3)
Alk.	76.3 (9.6)	117.5 (8.5)
pH	7.8 (0.1)	7.8 (0.2)
Turb.	10.2 (3.2)	2.6 (0.3)
TOC	2.8 (0.4)	2.4 (0.3)

products from the fish. Copper was slightly elevated in the holding tanks probably due to leaching from the heat-exchange coils on the water coolers.

The long-term exposure group was acclimated to an average blend concentration equal to 1.8% of the fly ash basin influent concentration (target concentration = 2.0%) above background levels (control). The percentage of dissolved metals relative to total metals was greater than 90% in all cases.

Normal background concentration of Cu was 0.003-mg/L (S.D. = 0.001) and 0.004-mg/L (S.D. = 0.002) in the New River and Adair Run, respectively, throughout the entire test period. Chromium was undetectable during spring trials in the New River and Adair Run, but averaged 0.003-mg/L (S.D. = 0.002) in the New River in the summer. However, during both spring and summer artificial stream trials, background concentrations of Cr and to a lesser extent Cu in the New River increased to high levels (480-ug/L Cr) for short periods of time. This was not known until water samples were subsequently analyzed. The upstream source of the pollution was unknown, but the data in some trials show a rapid increase and slow decline of background levels during the tests. The problem did not result from a malfunctioning pump or incorrect test solution mixture, as these were monitored. It is

noteworthy that the fishes' behavior was severely affected. The records show erratic and agitated movement and no response to the test solution. As a result, 3 of 8, spring control replicates and 4 of 9, summer control replicates were discarded. Also, because of changed flow patterns and eddy formation in Adair Run, 1 of the 8 control trials was discarded. The crude measure of stream flow in both systems consistently overestimated dilution volume so measured test concentrations were higher than target levels. They were not high enough to compromise the experimental design however.

The avoidance experiments clearly demonstrate that unexposed fish avoid metals solutions in the field (Table 11). Control fish avoided the metals blend at relatively low levels in both seasons and in both test systems (Figs. 11 and 12). The percentage of fly ash basin influent concentration where residence was one half of the control period was 1.45% (95% CI=0.40%) and 0.70% (95% CI=0.15%) for spring and summer artificial stream trials respectively, and 1.50% (95% CI=0.50%) in the Adair Run trials. Mean control period residence varied between 68 an 70% over all three locations. Individual replicate lines within each test system were parallel but not always coincident (Table 13). The pooled slope of the summer artificial stream trials is significantly steeper than the spring artificial stream and summer Adair Run trials ($F=3.22$; 2,58 df, and $F=3.91$; 2,62 df respectively). The

Figure 11. Avoidance response of fathead minnows in an artificial stream to a blend of four metals. Zero exposure fish are control fish maintained in unaltered New River water for 3 (spring) and 6 (summer) months. High exposure fish were exposed in New River water to a metal blend concentration equal to 2% of concentrations found in a fly ash basin influent. Test concentrations are expressed as test concentration plus background exposure. Vertical bars show 95% confidence limits of quadratic regression.

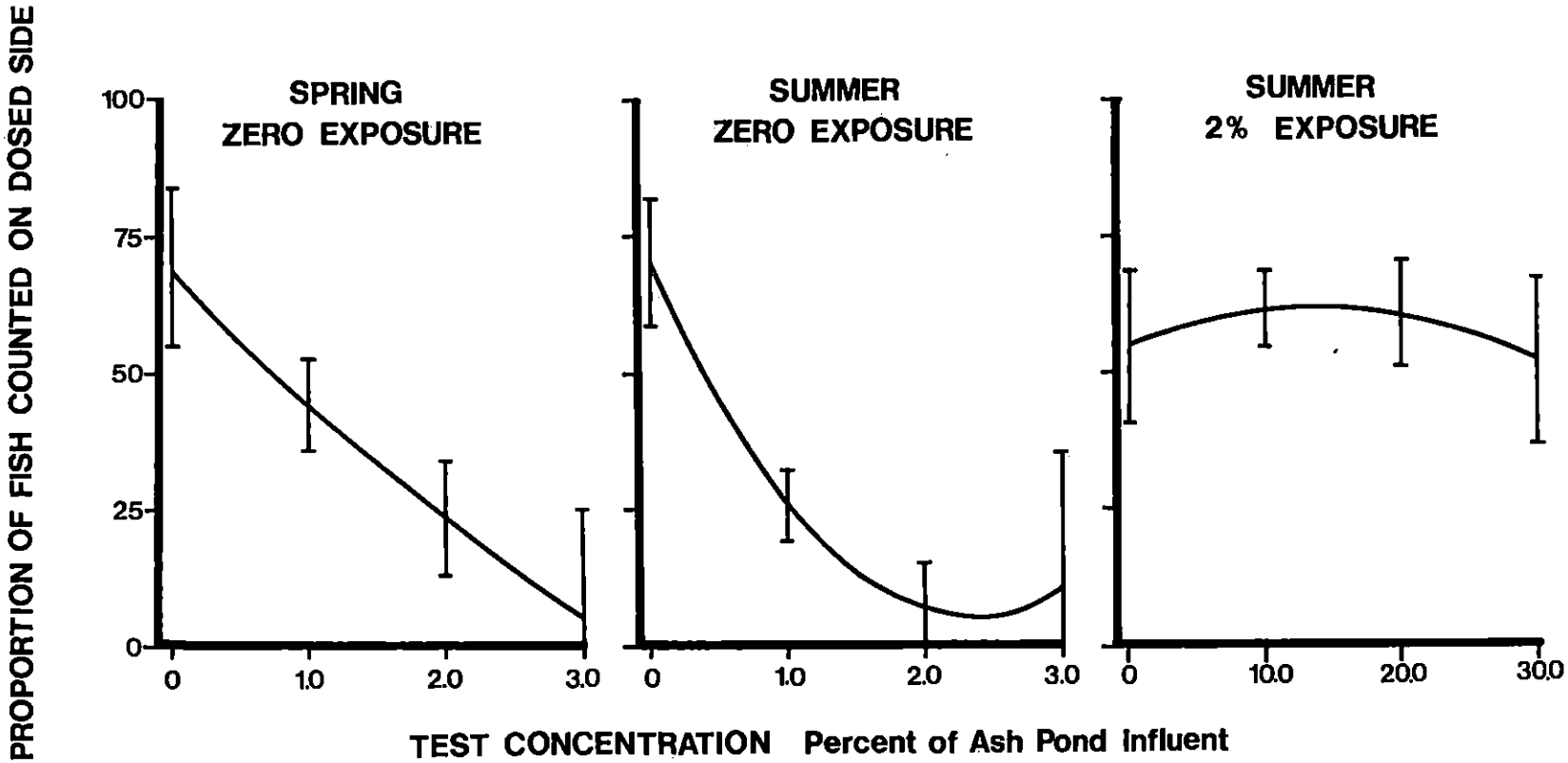

PROPORTION OF FISH COUNTED ON DOSED SIDE
100
75
50
25
0
SPRING
ZERO EXPOSURE
SUMMER
ZERO EXPOSURE
SUMMER
2% EXPOSURE
0
1.0
2.0
3.0
0
1.0
2.0
3.0
0
10.0
20.0
30.0
TEST CONCENTRATION Percent of Ash Pond Influent

Figure 12. Avoidance response of fathead minnows in Adair Run to a blend of four metals. Zero exposure fish are control fish maintained in unaltered New River water for 3 months. High exposure fish were exposed in New River water to a metal blend concentration equal to 2% of concentrations found in a fly ash basin influent for 5 months. Test concentrations are expressed as test concentrations plus background exposure. Vertical bars show 95% confidence limits of quadratic regression.

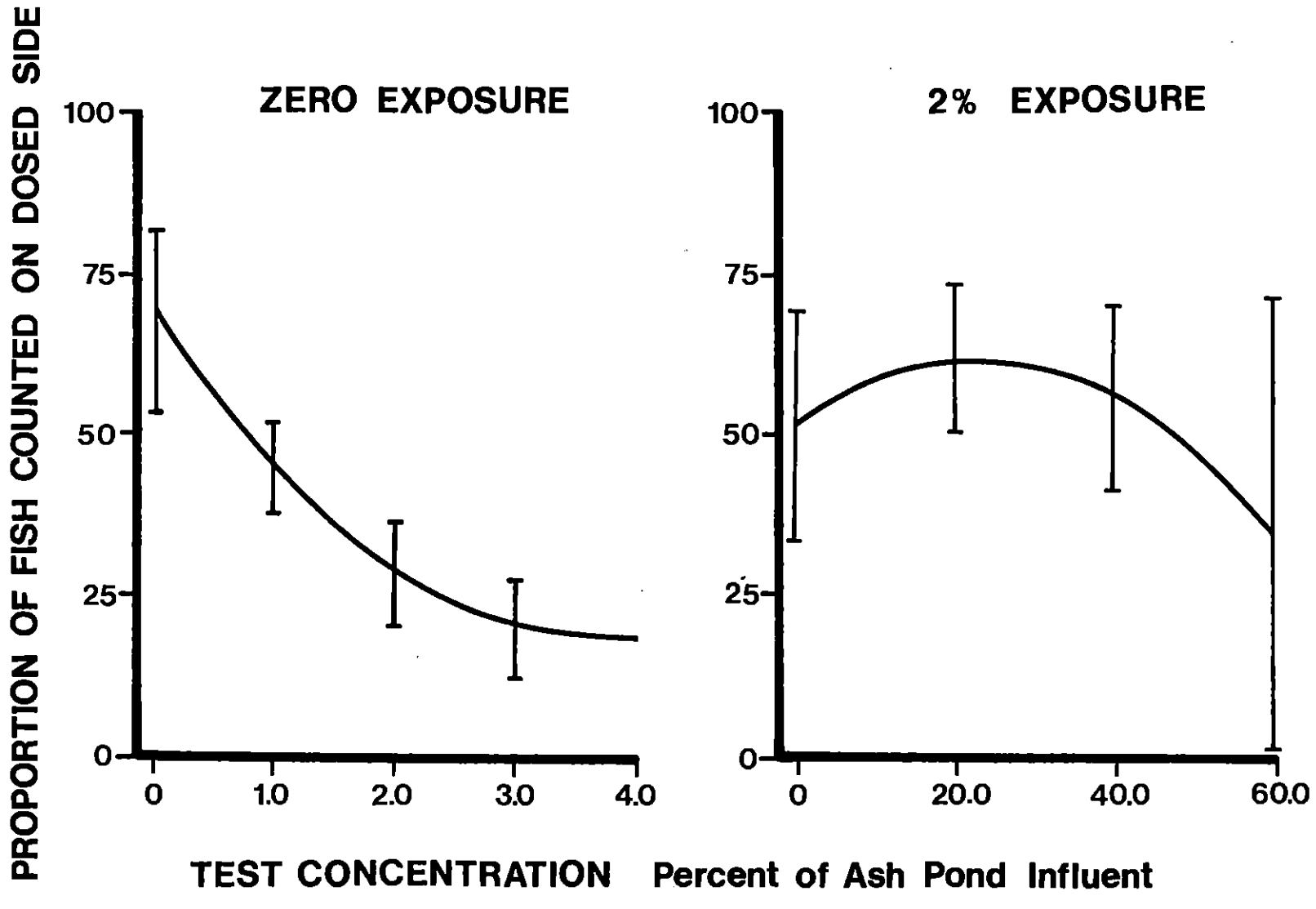

ZERO EXPOSURE
2% EXPOSURE
PROPORTION OF FISH COUNTED ON DOSED SIDE
100
75
50
25
0
0
1.0
2.0
3.0
4.0
0
20.0
40.0
60.0
TEST CONCENTRATION Percent of Ash Pond Influent

Table 13. F-values and slopes for multiple quadratic regressions of fathead minnow residence on exposed side of avoidance apparatus vs measured concentration. Significant F-values in the parallelism column indicate that the replicate lines were not parallel. Significant F-values in the coincidence column indicate that replicate lines were not coincident (i.e., had different intercepts). Slopes for linear and quadratic concentration terms and overall correlation coefficient (r^2) are also shown.

		F values		Slopes		
Test Group	Test System	Parallelism	Coincidence	Conc.	Conc.^{2}	r^2
Spring Control	Artificial Stream	0.85	3.51[1]	-26.21[1]	1.63	45.7
Summer Control	Artificial Stream	2.48	0.45	-75.32[2]	16.88[1]	73.5
Summer Exposed	Artificial Stream	3.56[2]	[3]	1.03	-0.04	6.2
Summer Control	Adair Run	1.95	3.54[2]	-27.30[2]	3.65	44.3
Summer Exposed	Adair Run	2.60[1]	[3]	0.86	-0.02	2.8

[1]Significant at the 0.05 level
[2]Significant at the 0.01 level
[3]If lines are not parallel, by definition they cannot be coincident.

spring, artificial-stream and summer, Adair Run slopes were not significantly different ($F=0.66$; 2,68 df).

P-values for significance of test system (artificial stream or Adair Run) ,with and without inclusion of water quality parameters as a covariate in the analysis of variance model of residence vs metals concentration between test systems are, shown in Table 14. Addition of alkalinity and hardness in the model explains a significant portion of the difference in avoidance response between the spring and summer artificial stream trials. Addition of total organic carbon, turbidity and hardness explains a significant proportion of the difference between summer, artificial- stream and Adair Run trials. Only the addition of hardness explains a significant portion of the difference in avoidance response between all three.

The acclimated fish did not respond to the metals blend in either test system (Figs. 11 and 12). The slopes of residence vs metals concentration are not significantly different from zero (Table 13). Correlation coefficients are extremely low and individual replicate lines are not parallel (Table 14).

Table 14. P-values for significance of test systems (artificial stream, spring or summer and Adair Run) with and without inclusion of water quality parameters as covariates in analysis of variance model of fish residence vs metals concentration for fathead minnows.

	Test System Comparison		
	All Locations	Artificial Stream vs Artificial Stream	Adair Run vs Artificial Stream
P value W/O Covariate	0.0309	0.0334	0.0130
P value W/ Covariate			
TOC	0.0034	0.0074	0.4538
Turb.	0.0362	0.0001	0.4345
NO_3	0.0408	0.0303	0.0337
Alk.	0.0001	0.8271	0.0001
Hard.	0.1470	0.1527	0.0606
Cl	0.0328	0.0387	0.0477
SO_4	0.0340	0.0115	0.0120

LONG TERM EXPOSURE AND TOXICITY

Ninety six hour LC50 values for all four groups of fish are shown in Table 15. The laboratory exposed group was 1.25 times more resistant to the metals blend than the laboratory control fish. The field exposed group was 1.41 times more resistant to the metals blend than the field control group. The two control groups were equally resistant to the metals blend, as were the two acclimated groups. The 96-hr LC50 value for the five metal blend experiment was only 80% of the concentration of those elements in the fly-ash basin, (Table 16), or approximately one half of the 96-hr LC50 of the four metal blend.

Table 15. Fathead minnow 96-hr LC50 and Fiducial limits for a blend of four metals, expressed as % concentration of the fly ash basin influent concentration, and calculated metals levels (mg/L) at the 96-hr LC50 level.

Exposure Group	96-hr LC50	Fiducial Limits	Cu	Cr	As	Se
Laboratory Control	156.8%	130.7-203.0%	2.038	1.098	3.763	0.784
Laboratory Exposed	195.3%	132.9-299.3%	2.539	1.367	4.687	0.977
Field Control	147.0%	123.3-186.2%	1.911	1.029	3.524	0.735
Field Exposed	207.8%	167.6-251.4%	2.701	1.455	4.987	1.039

Table 16. Fathead minnow 96-hr LC50 and Fiducial limits for a blend of five metals, expressed as % concentration of the fly ash basin influent concentration, and calculated metals levels (mg/L) at the 96-hr LC50 level.

96-hr LC50	Fiducial Limits	Cu	Cr	Cd	As	Se
80.0%	71.2-94.7%	1.04	0.56	0.02	1.92	0.40

Discussion

DISCUSSION

STATISTICAL TECHNIQUES

Use of parametric statistical procedures for analyzing this type of data has been debated relative to the validity of grouping individual observations of fish counts or residence time as independent observations (McInerney, 1964). However, if sufficient time between observations is allowed, such that the location of a fish cannot be predicted based upon its previous location, the observations can be considered as independent (Casterlin and Reynolds, 1977; Fritz and Garside, 1974). The data clearly show that the fish were quite active, with several quadrant crossings during each 30-sec interval. Actual activity was twice as high as indicated in Fig. 8 because activity was measured only during every other interval. Thus, the 30 sec intervals are adequate to ensure independence of observations. Also, the normalizing transformation did not improve performance of the regression model, indicating that the distribution of the data is consistent with parametric procedures.

The alternative is to use different groups of fish at each concentration level. This would greatly increase the time and complexity of a single experiment. It would also bias

the results in favor of lower avoidance thresholds since fish respond to the absolute value of a stimulus as well as to the steepness of the gradient (Collins, 1952; Ishio, 1964). In regard to schools of fish, the problem of recording total residence time of each fish has not been solved. Individual responsiveness has its own variability, as would be expected, and measurement of the "school" eliminates one component of variability. Also, measurements of a single school of fish through a series of increasing concentrations are more environmentally realistic for the case of fish moving upstream toward an effluent of within a lake or estuary. Downstream movement would, of course, be more accurately modeled by single, large gradient tests as the fish would encounter the plume abruptly.

The statistical models vary considerably in their ability to account for the observed variability. Of all the regression models examined here, quadratic regressions of exposure vs response have the highest correlation coefficients regardless of other model factors such as covariates or corrections for control periods (Table 5). In fact, correction for control period counts by simple subtraction may have severe impact on model efficiency. The effect is counterbalanced by using replicate as a covariable. This indicates that the replicate to replicate variation may be important, and thus, an examination of variation should be performed to ensure that the

desired level of precision and power can be achieved with the chosen number of replicates (Myers, 1979). If control period values are simply ignored (Table 5), the correlation coefficients decrease markedly relative to most other models and the presence or absence of replicate as a covariable has little effect. Higher variability of response at low stimulus levels is a common phenomenon (Campbell and Masterson, 1969), and removal of control points that are close to 50% in all replicates has the effect of raising variability.

Quadratic and $\log_{10}$ transformed models are only slightly different relative to correlation values. Both techniques have the effect of linearizing the data lines. This is essential when testing a series of increasing concentrations. Once avoidance levels have been reached, a plot of the data will reveal a flat tail as concentration increases, since the response obviously cannot drop below zero. Thus, the response lines are curved, not linear. Avoidance experiments should not be terminated as soon as avoidance levels are reached however. Several substances are known to be avoided at low levels but preferred at higher levels. A recent review of the literature is provided by Beitinger and Freeman (1983).

Inclusion of replicate as a covariable improved correlation in all cases. The increase was usually relatively slight.

Regressions using replicates as dummy variables indicated that the regression lines were statistically parallel and coincident (Table 5). Thus the small increase in correlation coefficients may have been due to the inclusion of more variables in the regression model and a reduction in error term degrees-of-freedom and are, therefore, artifacts. The equation for correction by control counts is designed to eliminate bias resulting from the preference of the test animal for one side of the test chamber prior to introduction of the test material. In the present case, where there was no strong bias, the procedure may drive the lines apart, and inclusion of a covariate (at the cost of error degrees-of-freedom) must be used. The field data was analyzed using this correction but the results were not improved. Here again, the control period data are consistently biased and the procedure only drives the lines apart. Also, this research was designed to evaluate not only the nature of the response to the metals in different test situations but also the degree of response. Elimination of a bias for location would remove a comparative aspect of the research design. Thus, only quadratic regressions without corrections for bias were used in all cases.

The correlation of activity and test concentration was highly variable and quite poor in two of three seasons. Inclusion of replicate in the model improved correlation only where

replicate lines were statistically different. Regression of activity on concentration was only significant in season #2 where only two replicates were performed and data points were limited. This does not indicate that activity cannot reveal information on the effect of chemicals on organisms. Stimulation or reduction of activity can occur (Dandy, 1972; J.R.E. Jones, 1948).

The results of the Hochberg GT2 test were quite different from the regressions (Table 7). Significantly different groups were identified in only two of the four seasons. Lack of grouping in season #2 was undoubtedly due to low replication. Reasons for the lack of grouping in season #4 are less clear. In both cases, the test clearly did not equal the sensitivity of the regression models.

LABORATORY AVOIDANCE

The consistency of the control fish response in the laboratory tests over four seasons indicated that behavioral tests are an effective method for testing for effects of metals . That is not to say that seasonal parameters, i.e. temperature, will not interact with behavioral responses (Kleerekoper and Waxman, 1973) or developmental stage is irrelevant (McInerney, 1964; McCauley and Huggins, 1979) but that the fundamental mechanism by which fish detect and re-

spond to metals can be assumed to be static in the absence of prior exposure. Another aspect of the control fish behavior in the laboratory is the lack of threshold in the response, in spite of the low concentrations used in these tests. Declines in residence on the dosed side begin at the lowest levels and continue throughout the tests. Similar results have been reported for copper (Giattina, 1982) and zinc (Sprague, 1968).

At an arbitrary cutoff point of half the control period residence, the average avoidance level was 0.63% of the fly ash basin influent concentration for these four elements (table 11), or 0.4% of the 96-hr LC50 value (Table 15), which is more than two orders of magnitude below the LC50 This is equivalent to a total dose of 30.6-ug/L, composed of 8.1-ug/L Cu, 4.4-ug/L Cr, 15.0-ug/L As, and 3.1-ug/L Se. These values compare favorably with literature values of metals tested individually. Copper has been most frequently studied in this regard. Fish avoid levels from 0.1-ug/L to 74-ug/L Cu (Giattina et al.,1982; Kleerekoper et al., 1973; Folmar, 1976; Black and Birge, 1980). Zinc is avoided by largemouth bass (Micropterus salmoides) at least as low as 7-mg/L, and 0.5-mg/L by rainbow trout , but is not avoided by bluegill sunfish between 11 and 43-mg/L (Black and Birge, 1980). However, Sprague (1968) reported that rainbow trout avoided Zn as low as 5.6-ug/L. Whether this difference is due to

methodology or background water characteristics cannot be determined. Sprague (1964) also reported that Cu and Zn were synergistic in eliciting an avoidance response. Rainbow trout avoid nickel at 23.9 ug/L (Giattina et al., 1982b) and Cd at 52 ug/L but were attracted to mercury at 0.2-ug/L (Black and Birge, 1980). Thus, the behavioral synergistic interaction of metals blends will be highly complex. It will be influenced by metals composition, absolute concentration, and the test species.

The response of both groups acclimated to the blend for 3 months in the laboratory was in marked contrast to the controls. Fish acclimated to the low dose of the metals blend were completely unresponsive to elevated metals levels. Fish acclimated to the high dose demonstrated a time dependent response. The response was reversed from initial strong attraction (summer) to mild avoidance (fall) to indifference (winter). The low dose fish were indifferent to elevated levels after only 3 months of exposure. This strongly suggests that there is a long-term sensory acclimation process to metals, and the rate and/or degree of acclimation is dose dependent.

Behavioral acclimation to the high levels of metals may result in fluctuating responses until stable acclimation is reached. Fluctuations in thermal resistance and metabolic

overcompensation during thermal acclimation have been documented in fish (Hanson and Stanley, 1970; Allen and Strawn, 1971). What influence the initial high dose levels (5 and 10% fly ash slurry), including Cd, may have had on the response sensitivity cannot be evaluated with the present data. However, the nature of the effect would presumably be the same in both groups and differ only by degree. Other researchers investigating long-term exposure to metals have observed variations in response over time in behavioral and metabolic parameters. Bengtsson (1974) reported a hyperactivity response in the minnow, Phoxinus phoxinus, followed by hypoactivity as Zn concentration and exposure duration increased. Scarfe et al., (1982) demonstrated that selected behavioral traits were altered by Cu exposure that were both species specific and reversed three weeks following cessation of Cu exposure. Several authors, using different methods and fish species, have demonstrated that Cu is ignored or preferred at high concentrations, yet avoided at low concentrations (Giattina et al.,1982; Black and Birge, 1980; Summerfelt and Lewis, 1967). Acclimation of fish to low levels of Cu for extended duration may also affect other behavioral traits such as feeding orientation, and overall activity (Drummond et al. 1973; Scarfe et al., 1982; Steele, 1983).

Acclimation in the respiratory rate of fish exposed to metals may return to normal or below normal levels over time (O'Hara, 1971), and apparent acclimation or compensation has been observed for ventilation rate and blood characteristics (McKim et al., 1970; Lewis and Lewis, 1971; Morgan and Kuhn, 1974; Brenner et al., 1976). The ultimate acclimation time may be quite long. McKim et al. (1976) and Benoit et al. (1976) reported that up to 20 weeks of exposure to a single metal was required to achieve a steady state body burden.

Fish in a river may be able to avoid heavy metal laden effluents successfully, but, in more restricted water bodies such as lakes or well mixed estuaries, constant low level exposure may not be avoidable. I have demonstrated that acclimation to metals in the laboratory may lead to potentially harmful behavioral changes i.e., attraction to or loss of response. In the present case, the fish did not avoid levels of metals roughly equivalent to their 96-hr LC10 level. Based upon the field results, the avoidance threshold may be considerably higher. Further questions that need to be addressed are: what are the rates of behavioral acclimation, and what are the concentration thresholds for that acclimation.

There was no difference in activity during testing between any of the exposure groups. Sprague (1968), Fava and Tsai

(1976) and Lubinski et al. (1980) have all reported that activity is not as sensitive an indicator of fish response as residence time. In this case, there appears to be no effect on overall excitability of the fish.

FIELD AVOIDANCE

The difference between the response levels of the control fish illustrates the importance of variation in background water quality in behavioral tests. In this case, the fish had significantly different response thresholds to the metals, depending on where they were tested. Fish tested in New River water in spring required a concentration twice that of fish tested in New River water in the summer, before avoidance occured (Table 11). Conversely, the avoidance concentration in Adair Run is equivalent to the avoidance level found in the New River in the spring season. The laboratory study demonstrates that this is not a seasonal effect inherent in the fish. The most obvious similarity in water quality parameters in the New River in spring and Adair Run in the summer are elevated turbidity and hardness relative to the New River summer values (Table 12). Spring rains and snowmelt increase the amount of material carried by the New River. Adair Run has a small drainage basin and a much steeper slope that the New River with many riffles and water falls, so the water velocity is higher and the stream's load

is high into the summer months. Whether the increased sediment load affects the metals directly i.e. sorption onto clay particles (EPA, 1979b) thereby lowering their availability to the fishes' sensory systems or directly affects the fishes' ability to detect the metals cannot be determined. Other chemical interactions are possible such as chelation by humic substances, precipitation, inorganic complexation etc. Determination of the chemical state of the metals was beyond the scope of this study. However, it is a reasonable hypothesis that the chemical state of the metals may affect how well the fish can sense them. Also, the chemical matrix in which fish are presented the metals may affect what the fish will choose to respond to. Collins (1952) has demonstrated behavioral competitive interactions between physical and chemical characteristics of water in migrating Alosids. Sprague et al. (1965) reported that Atlantic salmon avoid Cu and Zn blends at a level of 0.02X of their incipient lethal level in laboratory tests but based on counting fence returns, over ten times that level is required to repulse them in the wild.

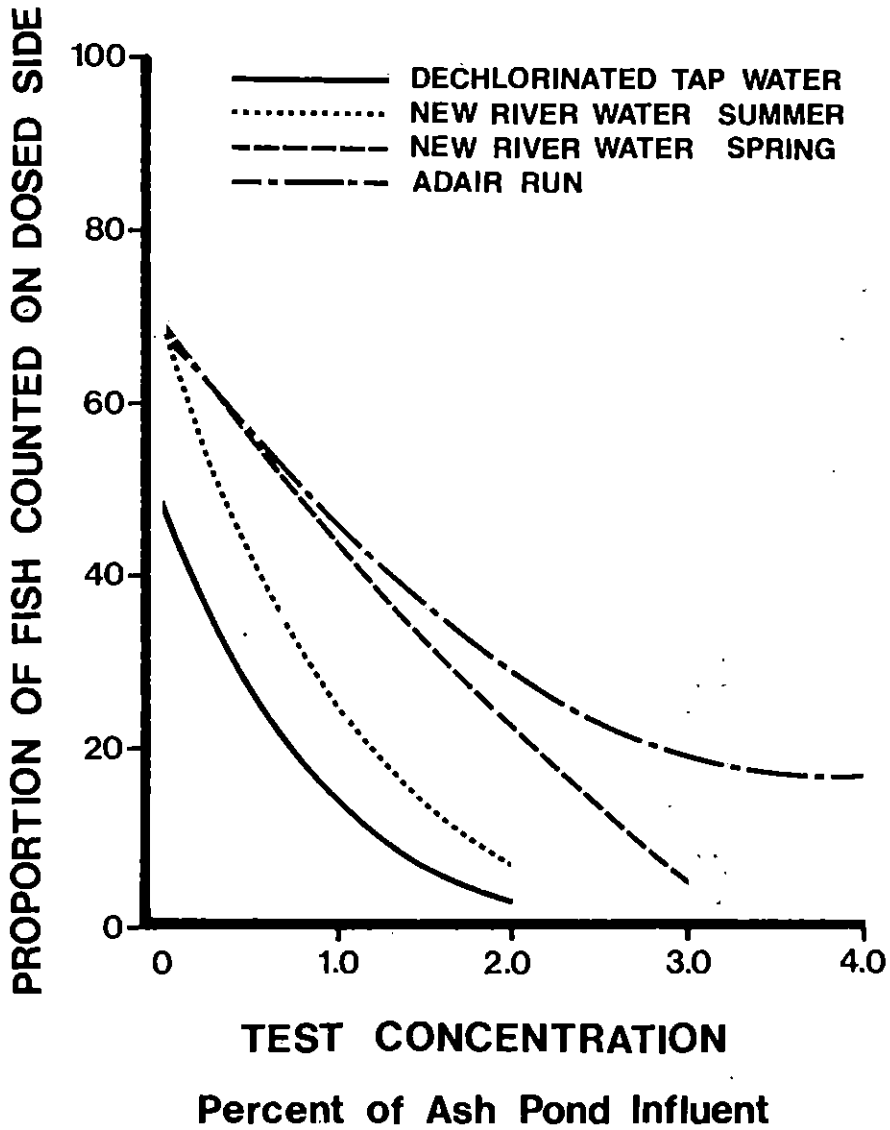
Comparisons between only two test systems are of limited usefulness however, since any consistent difference in a water quality parameter between them is therefore statistically confounded with the response to metals in those systems. The only parameter which significantly affects the analysis of

all three test systems together is hardness. Hardness has long been recognized as an important parameter in metals toxicity to fish (Black et al., 1973). The mechanisms by which this occurs are still debated. One view holds that metals are subject to complexation and/or precipitation in hard water (Pickering and Henderson, 1966). Another hypothesis is that calcium reduces membrane permeability to metals (Lloyd, 1960). Chapman and McCrady (1977) have shown that the toxicity of copper ions varies with pH as well as with degree of carbonate complexation. How these toxicological interactions relate to behavioral responses would be speculative at the present time. This is especially so in light of the fact that while it is known that metals can harm the olfactory tissue in fish (Hara et al. 1976; Bodammer, 1981). it is not definitely known what sensory system fish detect metals with. Bodznick (1978) has shown that the presence of calcium (Ca) ions evoke neurological responses in the olfactory tissue of sockeye salmon (Oncorhynchus nerka), however. Whether the olfactory tissue is sensitive to specific ions or to cations in general is not known.

The physical characteristics of the environment are also important in determining what level of metals will be avoided. In the present tests, the fish usually preferred one side of the avoidance enclosure or the other during the control period. This varied from one side to the other depending upon

replicate. The slope of the response line from the summer artificial stream trials is as steep as the slope from the laboratory control trials. However, due to preference for one side of the chamber, the fish were still present in the dosed side at concentrations above those avoided in the laboratory. The concentration (as % fly ash basin influent concentration) where the summer artificial stream fish were avoiding the metals 50% of the time was 0.7%, which is essentially the same as the laboratory result and is 0.5% of the 96-hr LC50 for these fish. However, at that concentration, the laboratory fish occupied the dosed side of the chamber 25% of the time. The field tested fish did not occupy the dosed side at that level until the concentration was up to 1% (Fig. 13). Even though the nature of response was the same, the actual concentration of metals which drove the fish to the other side was higher. This may also be partly responsible for the delayed reaction in Adair Run where turbulence, shadows, proximity to the stream bank and wild animals in the stream may influence where the fish preferred to be. No predators were seen during testing, but insects, small crayfish and other fish were usually observed around the enclosure during Adair Run trials. Casterlin and Reynolds (1978) have demonstrated that fish distributions may be influenced by the interactions of cover, substrate type and color, conspecifics, turbulence and depth. The Adair Run and artificial stream spring trial fish avoided concentrations

Figure 13. Avoidance responses of unexposed fathead minnows from all three test systems in their respective water types. The line labled dechlorinated tap water represents the pooled responses of the four laboratory control groups. Lines are quadratic regressions of residence vs concentration.


PROPORTION OF FISH COUNTED ON DOSED SIDE
100
80
60
40
20
0
DECHLORINATED TAP WATER
NEW RIVER WATER SUMMER
NEW RIVER WATER SPRING
ADAIR RUN
0
1.0
2.0
3.0
4.0
TEST CONCENTRATION
Percent of Ash Pond Influent

of about 1% of the 96-hr LC50, but did not reach 25% occupancy of the dosed side until concentrations reached 2.4 and 1.9% respectively (Fig. 13). Thus, as much as 25% of a population of fathead minnows may not avoid metals concentrations which, if they remain long enough, may result in a complete loss of response to vastly higher levels. The response of the fish which had been acclimated to the metals blend for 3 months was completely different from that of the control fish. The acclimated fish did not respond to elevated metals levels in either the artificial stream or in Adair Run (Figs. 11 and 12). Long term acclimation to very low levels of metals has a profound effect on behavioral responses to those metals. The exposure level of 1.8% of the fly ash basin influent concentration is within the range of 50% avoidance by the control fish in the spring artificial stream and Adair Run control trials. The implication is that under certain seasonal conditions, fish may not avoid low levels of metals, and become acclimated to them such that when they encounter much higher and possibly harmful levels they will fail to avoid these levels. Metals acclimated fish tested in the artificial stream showed no avoidance at concentrations as high as 30% of the fly ash basin influent concentration or Cu-390ug/L, Cr-210ug/L, As-720ug/L and Se-150ug/L. The Adair Run trials went as high as 60% of the influent concentration. These levels are 14.4 and 28.8% of the 96-hr LC50 for these fish. At this concentration, copper alone would be acutely

toxic to tolerant fathead minnow populations (Benson and Birge, 1985). In a situation where Cr and Cu slugs are present in the New River for example, these fish may not have responded to the contamination as the control fish did. This raises the questions of how often and how long an exposure is necessary to begin altering the defensive behavior of fish, and what are the mechanisms of these changes. Bodammer (1981) has suggested that Cu exposure has a neuro-toxic effect in fish. Exposure to Se resulting in alkali disease or 'blind staggers' is well documented neurological disorder in mammals (Venugopal and Lucky, 1978).

The results of the five metal blend toxicity bioassay are of particular intrest in this regard. The fish were much more sensitive to the five metal blend than the four metal blend. The decrease in the LC50 value is of much greater magnitude than the difference between control and acclimated fish. This indicates that cadmium, although present only at low levels, is the most important element that contributed to toxicity. These results may be due to direct cadmium toxicity and/or synergistic interactions with the other elements. It also has implications for the interpretation of behavioral data and its use in hazard assessment. It demonstrates that the element which elicits the strongest behavioral response may not be the element which is primary environmental concern from a toxicological point of view.

LABORATORY VS FIELD BEHAVIOR

The response of fish acclimated to the metals blend for 3 months in the field is in marked contrast to that of laboratory acclimated and tested fish (Table 11). In the laboratory tests, fatheads acclimated to the high concentration for 3 months were attracted to elevated levels of the metal blend. Acclimation for longer periods and to lower levels resulted in loss of responsiveness in the laboratory. Laughlin et al. (1978) found that the field distributions of blue crabs (Callinectes sapidus) did not correlate with laboratory results. The reason(s) for this may be partly due to developmental and social influences as well as chemical influences (Reynolds and Casterlin, 1978). Sprague et al. (1965) noted considerable differences between laboratory metals avoidance by Atlantic salmon and their field distributions. Cairns et al. (1981) found that laboratory avoidance and field distributions of fish relative to thermal and chlorine discharges were in close agreement however. Laboratory testing used thermally acclimated fish, but not chlorine exposed fish.

In the present case, the difference between laboratory and field tests with metals acclimated fish is a matter of the time course of the change in behavior. Predictive responses based upon short term laboratory exposure may be erroneous,

depending on the exposure level. Predictive results based upon unexposed fish may be in closer agreement, but will likely overestimate the responsiveness of fish to metals pollution in the wild.

The bioassays clearly demonstrate that when fatheads are exposed to metals, their tolerance to metal toxicity increases. This phenomenon has been observed in fathead minnows and a variety of other fish species (Dixon and Sprague, 1981; Roch and McCarter, 1984a; Benson and Birge, 1985). Increased levels of metallothionein as a result of exposure to metals has been demonstrated in fathead minnows (Benson and Birge, 1985) The rates of metallothionein production and loss requires up to 4 weeks for equilibrium to be reached in fish (McCarter and Roch, 1984) but changes in resistance as a result of changes in exposure level can be observed in as little as 7 days in fathead minnows (Benson and Birge, 1985).

Experimental field exposure of fish to metals is not as well studied as laboratory exposures. In the present case, the fish were acclimated for up to 9 months to the blend of metals in both laboratory and river water and, the resulting increase in tolerance is the same, when LC50s are determined in the lab. Similar results have been reported for fathead minnows using a blend of copper, cadmium and zinc (Benson and Birge, 1985). Conversely, when trout are exposed to a blend

of Zn, Cu and Cd in a ratio of 400:20:1, (154-ug/L total metals) in the field and also tested for resistance using river water, there is not an apparent increase in LC50 values, even though there is an increase in hepatic metallothionein (Roch and McCarter, 1984b). This is a result of the exposure matrix and not a species difference as Roch and McCarter (1984a) have previously shown that laboratory exposure to metals results in higher laboratory LC50s. As in the case of the field avoidance tests, the experimental acclimation matrix may have significant impact on the bioassay test results.

SUMMARY

Acclimation to ≈90-ug/L of the metals blend in the laboratory and the field, results in an increased resistance of 1.25-1.41 times that of unexposed fish to metals when tested in the laboratory, but the increased resistance does not match the degree of loss of behavioral responsiveness to the metals. Addition of Cd to the blend reduces the 96-hr LC50 of unacclimated fish to the blend by one half.

Prior exposure to metal blends has a profound affect on fish avoidance behavior. The effect is both dose dependent and time dependent. Unexposed fish avoid ≈29-ug/L total metals in the laboratory. This response is consistent over a 12

month period. Acclimation to ≈45-ug/L for three months results in a loss of responsiveness to elevated metals levels (≈245-ug/L)in the laboratory. Acclimation to ≈90-ug/L for three months results in a preference for elevated metals levels (≈245-ug/L) in the laboratory. Acclimation for six and nine months to ≈90-ug/L results in a loss of responsiveness to elevated metals levels (≈980-ug/L) in the laboratory.

Unexposed fish avoided ≈71-ug/L and ≈34-ug/L total metals in an artificial stream during spring and summer conditions respectively. Acclimated fish did not respond to elevated metals levels (≈1470-ug/L) in the artificial stream during summer conditions. Unexposed fish avoided ≈74-ug/L total metals in a natural stream during summer conditions. Acclimated fish were unresponsive to ≈2940-ug/L total metals in a natural stream during summer conditions.

The nature of the response of unexposed fish to the metals blend in the field trials was similar to that of fish in the laboratory trials but the degree of response was not. This was due to specific chemical differences between test systems and to the physical setting of the test systems.

Thus, laboratory derived avoidance data is representative of field derived data but will be inaccurate due to differences in the background chemical matrix and the physical setting,

and may be entirely erroneous depending upon the acclimation history of the fish. Fly ash basins and landfills must be operated not only with the percent removal of metals in mind, but also the degree of dilution of the effluents available over long time spans.

APPENDIX

The following scheme was used to calculate the precise avoidance concentrations.
1) Measure chromium in all samples. 2) Measure copper, arsenic and selenium on all samples from only one complete replicate and at zero, medium and high dose levels on all other samples from other replicates within one experimental cell. 3) Calculate regression lines for chromium vs each other element for each experimental cell, e.g. all spring season control fish. 4) Based upon the regression slopes, calculate the copper, arsenic and selenium concentrations from the measured chromium concentrations. 5) Using these calculated concentrations, determine the average test concentrations.

EXAMPLE

slope of Cr vs Cu = 1.86

measured Cr = 7.0 ug/l

concentration of Cu= 7 X 1.86 = 13.02 ug/l

slope of Cr vs As = 3.43

measured Cr = 7.0 ug/l

concentration of As = 7 X 3.43 = 24.01 ug/l

slope of Cr vs Se = 0.71

measured Cr = 7.0 ug/l

concentration of Se = 7 X 0.71 = 4.97 ug/l

THUS

[Cr] = 7.0 ug/l = 1.0% fly ash basin influent
[Cu] = 13.02 ug/l = 1.0% " " " "
[As] = 24.01 ug/l = 1.0% " " " "
[Se] = 4.97 ug/l = 0.9% " " " "
+____
3.9%

{3.9%}/4 = 0.98% of the fly ash basin influent concentration for that test level

The procedure worked very well, and r^2 values for chromium vs copper, arsenic and selenium are shown in the following table.

Correlation coefficients for Cr vs Cu, As, and Se.

		Cu	As	Se
Laboratory Exposure	Season			
Control	Spring	89.0	85.8	86.8
"	Summer	94.5	99.1	94.1
"	Fall	97.6	96.1	97.1
"	Winter	98.3	96.1	84.5
Low dose	Summer	86.0	94.7	95.2
High dose	Summer	86.9	91.4	98.2
"	Fall	98.6	96.1	98.1
"	Winter	99.0	99.0	99.0
Field Exposure	Test Season/System			
Control	Spring	88.5	93.8	89.9
"	Summer	85.1	79.0	84.4
"	Adair Run	95.9	92.5	92.9
High dose	Summer	98.0	99.0	99.5
"	Adair Run	92.2	96.9	98.5

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