THESIS

HEAVY METAL TOXICITY IN TWO SPECIES OF AQUATIC INSECTS

Submitted by

R. Barry Nehring

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY R. BARRY NEHRING ENTITLED HEAVY METAL TOXICITY IN TWO SPECIES OF AQUATIC INSECTS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Adviser

Head of Department

ABSTRACT

HEAVY METAL TOXICITY IN TWO SPECIES OF AQUATIC INSECTS

A study was conducted at Fort Collins and Creede, Colorado to evaluate the effects of lead, zinc, copper, and silver on a mayfly (Ephemerella grandis) and a stonefly (Pteronarcys californica). E. grandis was very tolerant of zinc with the incipient (threshold) lethal level above 10 mg/liter. Lead and copper were more toxic to this mayfly with TL_{50} values of 3.5 and 0.18 mg/liter, respectively. Silver was extremely toxic to E. grandis with 100% mortality at exposure levels of 0.01 mg/liter. The stonefly (P. californica) was very tolerant of lead, zinc, and copper with incipient lethal levels greater than 19.2, 13.6, and 10.1 mg/liter, respectively. Silver was also very toxic to the stonefly naiads with a TL_{50} value of 8.8 μg/liter and an incipient lethal level of less than 5 μg/liter.

The feasibility of using aquatic insects as biological monitors of heavy metal pollution was also investigated. In general, these insects were more tolerant of heavy metals than fish tested under similar conditions. Both species of insects concentrated heavy metals in relative proportion to the occurrence of the metals in the water. Both species accumulated the heavy metals by some predictable

concentration factor. The results of this study indicate aquatic insects can be used as biological monitors of heavy metal pollution, especially in heavy metal fish-kill investigations. The aquatic insects offer many advantages in documenting average exposure levels in heavy metal fish-kills, and at the same time avoid many of the shortcomings of water sample analysis for heavy metal content.

R. Barry Nehring
Department of Fishery and
Wildlife Biology
Colorado State University
Fort Collins, Colorado 80521
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TABLE OF CONTENTS

Table of Contents cont.

LIST OF FIGURES

INTRODUCTION

Background Information

Heavy metal pollution has been a chronic problem in Colorado trout streams for over three-quarters of a century. Colorado's last great silver strike occurred at Creede in 1889. Leadville boomed in the 1860's. As the mills in these great mining towns concentrated the gold and silver ores, other heavy metal by-products were generally dumped into nearby streams, where they undoubtedly wrought havoc with wild trout populations.

Heavy metal pollution problems still exist in Colorado. A massive fish-kill occurred on the Rio Grande in 1963, due to the washout of a zinc mill tailings pond (Tanner, 1963). I personally investigated another zinc fish-kill on the Rio Grande in 1971.

Documentation of evidence in a heavy metal fish-kill is an extremely difficult task for the aquatic biologist. But it must be done to obtain a conviction in a court-of-law when stream standards are violated. Present tactics usually involve collection and analysis of many water samples taken from the stream where the fish-kill occurred. The water samples are acidified to keep the metals in solution; however, acidified samples may drop in heavy metal concentration in a few hours. The metal is adsorbed onto the container or precipitated out.

Heavy metal fish-kills are often caused by the discharge of a slug of waste materials. Fish mortality may lag behind first exposure to the toxicant by several days. By the time the fish-kill is observed and reported, the slug of effluent causing the kill can be many miles downstream and diluted beyond detection. Unless the discharge is still continuing, water samples at the site of the kill may reveal nothing. These inadequacies can be decimated in the courts by a skilled defense attorney. Clearly, a new method of monitoring and documentation of heavy metal pollution in fish-kill investigations must be found.

Aquatic insects may provide a new method. Aquatic invertebrates have been known to concentrate various water-borne pollutants through biological magnification over a very short period of time. Welkes and Weiss (1971) found the accumulation of DDT in the dragonfly nymph, Tetragoneuria, to increase with higher levels of DDT and longer exposure times. Johnson, et al, (1971) documented the biological magnification of DDT and aldrin in a number of freshwater invertebrates. The mosquito larva, Culex pipiens, accumulated DDT to levels 133,000X that measured in the water during 48 hours of exposure. A mayfly, Hexagenia bilineata, demonstrated biological magnification factors on DDT of 9400, 16,700, and 32,600 over 1, 2, and 3 day exposures, respectively. Similar results occurred with Siphlonurus sp. (Ephemeroptera), Ischnura verticalis and Libellula sp. (Odonata), and Chironomus sp. (Diptera). Kawatski and Schmulbach (1971) demonstrated similar concentration factors in ostracods exposed to dieldrin.

The accumulation of heavy metals by aquatic organisms has not been as thoroughly investigated; however, evidence is mounting that this is indeed the case. Brooks and Rumsby (1965), using marine bivalves, demonstrated concentration factors for cadmium more than two million times those levels occurring in the local marine environment. Scallops, mussels, and oysters showed concentration factors for metals ranging from 330 to 18, 700 for silver, 3, 000 to 13, 700 for copper, 3, 300 to 5, 300 for lead, and 9, 100 to 110, 000 for zinc. Kormondy (1965) demonstrated the rapid uptake of zinc-65 by the dragonfly nymph, Plathemis lydia. Equilibrium levels were reached in 24-48 hours. Total uptake of the isotope also appeared to be directly proportional to the isotope concentration in the medium. Warnick and Bell (1969) found significant uptake of heavy metals by aquatic insects exposed to various heavy metals. Goettl, Sinley and Davies (1971) found heavy uptake of lead, zinc, and copper by mayflies (Ephemeroptera), stoneflies (Plecoptera), caddisflies (Trichoptera), and true flies (Diptera) in four streams in Colorado suffering from chronic heavy metal pollution.

If aquatic insects could accumulate and hold concentrations of heavy metals in relative proportion to occurrence of the metal in the water, they might be very useful in detecting heavy metal pollution. In essence, the aquatic insects might serve as biological monitors of heavy metal pollution in lieu of water samples.

Preliminary Tests and Experimental Design

Aguatic insects used as biological monitors in heavy metal fish-kill investigations must satisfy three prerequisites. First, the organism should be less adversely affected by the pollutant than the fish. However, if the heavy metal pollution does not involve a fish-kill, this prerequisite is unnecessary. Second, it would be helpful if the insect were capable of concentrating the pollutant by a known factor over a short period of time. This would simplify detection and analysis procedures, especially when the pollutant is toxic to trout in extremely minute amounts. Third, the insect must concentrate the pollutant in relative proportion to the level found in the stream.

A stonefly, Pteronarcys californica, and a mayfly, Ephemerella grandis, were chosen as test insects because they are both herbivorous (to simplify feeding) and they are both very abundant in the Rio Grande at Creede, Colorado where the tests were to be conducted.

In preliminary tests during the summer of 1971, I used static bioassays to test the effects of lead, zinc, copper, and silver on these aquatic insects. Lead, zinc, and copper were selected because they are the most common heavy metal pollutants found in Colorado trout streams. Silver was chosen as it may be a serious problem in the future if it is widely dispersed in the Colorado Rockies as a weather modification agent.

The static tests revealed no short-term deleterious effects with lead, zinc and copper. The naiads did concentrate all three metals. Some positive correlation did exist between the amount of metal accumulated and that present in the surrounding medium. In the static bioassays, the metals plated out on the aquaria or precipitated as carbonates of lead, zinc, and copper in a few hours; thus, I felt it pointless to calculate concentration factors for each of these metals.

Silver was extremely toxic to the naiads (100% mortality at 5 μg/liter) in a few days. Nonetheless, silver was included in the study since reliable data were not available on the toxicity of silver to trout.

With the above three prerequisites apparently satisfied I set forth the following objectives:

1. Determine the incipient lethal levels (minimum concentration causing mortality) of lead, zinc, copper and silver to a mayfly, Ephemerella grandis, and a stonefly, Pteronarcys californica.
2. Determine TL_{50} values (levels causing 50% mortality) of lead, zinc, copper and silver for Ephemerella grandis and and Pteronarcys californica.
3. Determine the amount of lead, zinc, copper and silver in the insects at the time of death.
4. Correlate the uptake of lead, zinc, copper and silver in the tissues of Ephemerella grandis and Pteronarcys californica as exposure time and concentration increase.

With these data I felt I could effectively test the feasibility of using aquatic insects as biological monitors of heavy metal pollution.

METHODS AND MATERIALS

Collection of Specimens

The insects, Ephemerella grandis and Pteronarcys californica, used in this study were collected from the Rio Grande River immediately above the river's confluence with Willow Creek at Creede, Colorado. Willow Creek suffers from severe heavy metal pollution. The primary pollutant is zinc (1-10 mg/liter) with traces of lead, copper, and silver present at times (Goettl, Sinley, and Davies, 1971, 1972). Collection of insects from the Rio Grande above its confluence with Willow Creek precluded exposure to high concentrations of heavy metals prior to testing. Water from the Rio Grande above Willow Creek has been analyzed quarterly since 1970 for cadmium, chromium, copper, iron, lead, nickel, manganese, molybdenum, silver and zinc. Of these 10 metals, only zinc has been consistently present in detectable amounts (0.0-0.6 mg/liter) by direct atomic absorption analysis (Goettl, Sinley, and Davies, 1971, 1972).

Since P. californica has a three or four year life cycle, naiads are available year around; thus I was able to conduct tests during the winter months. I collected over 500 P. californica naiads from the Rio Grande in 1971. They were transported to Fort Collins and held

in a 100-gallon aquarium containing rocks, sticks, and branches for shelter and food. These naiads were used in acute bioassays with lead, copper, zinc, and silver from December 1971 through March 1972.

Predaceous stonefly naiads (Claassenia sabulosa) were also brought back to Fort Collins and used in preliminary tests to evaluate the performance to the test equipment. This test information was subsequently compared with data gathered on P. californica.

The mayfly, Ephemerella grandis, was tested in acute bioassays on lead, zinc, copper, and silver at Creede in May and June 1972. The site of collection of the mayfly naiads was the same as that described above for the stoneflies.

Feeding and Holding Procedures

The holding tank for the stonefly naiads was supplied with the same water source as the bioassay test equipment. This permitted continuous acclimation of the naiads to the same water source used in the bioassay, thus eliminating the need for lengthy acclimation periods prior to testing. Atomic absorption analysis of the water supply for lead, zinc, copper, and silver content was 0-3 ug/l, 20 ug/l, 5 ug/l, and less than 0.05 ug/l, respectively (personal communication --- John Goettl). One gallon of fresh water per minute was supplied to the holding aquarium continuously. Three large airstones permitted continuous aeration and circulation of the water. My attempt at

holding these stream insects was successful. Mortalities were less than 10% over the seven month holding period.

Feeding proved to be no problem as the naiads fed well on willow leaves gathered in September 1971 prior to a hard freeze. The willow branches were stored in a freezer and put in the aquarium as the naiads stripped the branches from the previous feeding. With my supply of leaves exhausted in January 1972, I fed the naiads cottonwood leaves for the last two months.

Testing Procedures

I conducted three types of tests on Pteronarcys californica and Ephemerella grandis. First, I carried out individual acute tests with lead, zinc, copper and silver on both species. These tests lasted for 10 days and if no mortality occurred in that time the test was terminated. All tests started at high levels (10-20 mg/liter for lead, zinc, copper; 1 mg/liter for silver). If these tests produced no mortality no further efforts were made since toxicants at these concentrations in the wild would kill all trout in the stream in a few days. I base this statement on TL_{50} values for lead, zinc, copper and silver on rainbow trout (Salmo gairdneri) determined at the Colorado Division of Wildlife Research Center in Fort Collins (Goettl, Sinley and Davies, 1971, 1972). If 100% mortality resulted in all concentrations I conducted a new test at lower concentrations.

The second type of test was a sub-chronic on both P. californica and E. grandis. These insects were put in a livebox and exposed to Willow Creek for 400 hours (about 16 days) to follow the build-up of zinc in the naiads with increasing exposure time. I cropped the naiads at 24 hour intervals for the first 96 hours and at irregular intervals thereafter.

The third type of test was a two-month chronic on E. grandis and P. californica. Willow Creek water was pumped over to the Creede hatchery with a large pump (1 ft^3/sec) and diluted with various amounts of well water to produce four different concentrations and one control. Each concentration and the control was replicated. Each replicate contained 100 stoneflies and 100 mayflies. The stoneflies were held in the upper half of each trough and the mayflies in the lower half. A drip siphon was set up half way down the trough in each replicate to take a 12-hour composite sample of the toxicant. These tests were conducted to evaluate the effects of zinc on development and emergence of the naiads.

Test Apparatus for Individual Acute Tests

The test apparatus was a proportional diluter similar to the one described by Mount and Brungs (1967). This apparatus provided five concentrations and one control. The toxicant was mixed, diluted, and dispensed to the test containers by the diluter. The test containers

held 8 liters of toxicant. The diluter delivered 2 liters of toxicant every 90 seconds, giving a turnover of toxicant in each container every 6 minutes (Sprague, 1969).

Stock solutions were prepared by mixing the appropriate amount of reagent grade chemical with 20 liters of demineralized water. Reagent grade silver nitrate ($AgNO_3$) supplied the source of silver, lead nitrate ($Pb[NO_3]_2$) the source of lead, zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) and copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) the sources of zinc and copper, respectively.

Toxicant Water Metal Analysis

Metal analysis of daily water samples at Fort Collins were analyzed on a model 303 Perkin-Elmer Flame-Emission Atomic Absorption Spectrophotometer. Water samples were taken each morning in 5 ml Corning glass test tubes and acidified with two drops of concentrated analytical grade nitric acid. I analyzed these samples for metal analysis within 30 minutes of the time they were taken.

Daily water samples at Creede were analyzed for metal analysis on a model 290B Perkin-Elmer Flame-Emmission Atomic Absorption Spectrophotometer. Water samples on the mayfly acute tests were handled as described in the preceding paragraph. Twelve-hour composite samples were analyzed on the subchronic and chronic tests.

The atomic absorption unit was calibrated with primary standards made from 1000 mg/liter standards (Aztec Instruments, Inc.). The

model 290 B spectrophotometer lacked the sensitivity of the model 303, with minimum detection limits for silver at 0.15 mg/liter. For water samples below this level a Hach Kit was used for the analysis. The Hach Company has developed a low range colorimetric silver test with a range of 3 to 60 μg/liter. A check on this method of analysis against the model 303 spectrophotometer (5 μg/liter detection on silver) proved the Hach Kit both accurate and reproducible.

Water Quality Analysis

Water quality analysis was conducted on all containers in each acute test at least three times; at test initiation, midway through the test, and at test termination. Parameters measured included pH, dissolved oxygen, methyl orange alkalinity, hardness, temperature, and specific conductance. All parameters were measured according to Standard Methods (1971). A model 12 Corning pH meter gave pH readouts in 0.01 pH unit graduations. A Beckman model RB-2 Solu Bridge measured the conductance in micromhos/cm. Dissolved oxygen, alkalinity, and hardness were determined by the azide modification method, sulfuric acid titration to pH 4.5 (M.O.) end point, and EDTA titrimetric method, respectively. Water temperatures were below 10 C in all acute tests with variations less than 0.5 C. Dissolved oxygen remained at 100% saturation and the pH was at or near 7.0 in all tests. Conductivity, alkalinity, and hardness varied with the type of test and water source (Fort Collins or Creede). Water

quality analyses, other than the concentration of metal used in the tests, will not be referred to in the text.

Water quality analysis on the two month chronic bioassay on Willow Creek was performed weekly with the same parameters measured as described in the preceding paragraph.

Water quality analyses were not performed on the sub-chronic zinc accumulation tests as the highest concentration in the two month chronic bioassay (undiluted Willow Creek water) provided the same information.

Metal Accumulation Analysis---Symptoms of Stress

The stoneflies exhibited characteristic behavior patterns when stressed by exposure to a particular metal toxicant. Observations of these behavior patterns provided a method of detecting stress and the imminence of death resulting from exposure to heavy metals. The naiads "waggled" (swayed from side to side as if dancing) and pumped vertically as if trying to jump off the floor of the test containers. The next symptom was lo: of equilibrium and the naiad would flip onto its back. Death followed in a few hours in most cases.

Circadian rhythm was also interrupted. Under normal conditions the naiads remain hidden during the daylight hours; however, under stress from heavy metals they would openly expose themselves.

The mayfly naiads usually increased movement of the abdomenal gills with the first onset of stress. Loss of equilibrium followed with

the naiads flipped over onto their backs. Again, death followed in a few hours. Once on their backs, if no movement resulted when stimulated with a forceps, the naiads were considered dead and removed for analysis.

Effects of Age, Size, and Sex Differences

The stonefly, P. californica, has a 3 or 4 year life cycle. To minimize variability within a given test due to sex and size differences, certain precautions were taken. First, the stonefly naiads were measured prior to the initiation of all acute tests to insure that the mean length of the 10 naiads in each concentration was the same. Second, the naiads were sexed prior to test initiation and when possible naiads of one sex were used in the test. When the supply of naiads became limited, the same ratio of males to females was used in all concentrations within a given test.

Age and size variability with Ephemerella grandis is minimized by the life cycle of this mayfly. Ephemerella grandis is a univoltine (one generation per year) mayfly. The naiads mature, molt, emerge and mate in the span of 7 to 10 days on the Rio Grande at Creede, Colorado. Consequently the E. grandis naiads collected at a given location are essentially of the same age and size. For this reason it was not necessary to measure each naiad used in the test. Sex differentiation was not possible.

Digestion Procedures

Preparation for analysis involved several steps. The naiads were rinsed three times with distilled water to remove any adhering toxicant water, dried in an oven at 90 C for 48 hours, and removed to a desiccator for storage. Dried naiads were weighed on a Sartorius Analytical Balance to the nearest 0.0001 g and placed in a disposable plastic tube[1] for digestion.

The digestion procedure, described by Adrian (1971), was a wet digestion method for biological materials utilizing pressure. The digestive agent was a mixture of two parts concentrated nitric acid (HNO_3) to one part perchloric acid ($HClO_4$). I used 0.1 ml of the acid mixture for each 0.05 g dry weight up to 0.15 g. For weights from 0.15 - 0.30 g I used 0.5 ml of the acid mixture. The cap was then loosely replaced and the tube allowed to sit overnight and predigest. The loose cap allowed gases to escape as digestion proceeded, thereby preventing tube explosion. After predigestion the caps were tightened and the tubes placed in a constant temperature water bath at 70 C for 3 hours or until all solid materials were completely liquefied. Often some lipid material would not go into solution.

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[1] Test tubes are available in bulk lots from Falcon Plastics, 1950 Williams Drive, Oxnard, California 93030. The tubes (#2027, 13 x 100 mm style with screw caps) are sterile, have a 5 ml calibration line etched on them, and are completely free of metal contaminants. They are ideal for digestions of up to 0.3 g dry weight of aquatic insects. Cost is $0.05/tube.

Once digestion was complete, the tube caps were removed to allow trapped gases to escape. With the caps removed, 2 ml of distilled water were added to each tube and heating continued for 30 minutes to drive off all dissolved nitrogen (N_2) gas. The tubes were removed from the bath following expulsion of nitrogen gas, allowed to cool to ambient temperature, and diluted up to 5 ml volume. I then replaced the caps and shook the tubes vigorously for 5 seconds.

Atomic absorption analysis should be completed as soon as possible for metal analysis. This is imperative for silver! Silver plates out on the plastic test tube very rapidly, with losses as high as 80-90% in 24 hours. I completed all analyses for silver within 2 hours after digestion and dilution to 5 ml volume. If the samples are too concentrated for analysis, a 1:10 or 1:100 dilution must be made.

Digestion Procedure ---Safety Precautions

Proper safety precautions must be observed when working with nitric-perchloric acid mixtures. Pure concentrated perchloric acid should not be used as contact with some organic materials may result in instantaneous explosion (Blaedel and Meloche, 1963). Safety glasses, lab coat, plastic apron, and rubber gloves are an absolute necessity!

The pressure digestion should be done only in a fume hood and behind a protective shield. If the sample has not been sufficiently predigested prior to heating, the test tube will explode. Cap removal with

rubber gloved hands behind the protective shield is absolutely necessary. I have had tubes with just enough pressure built up explode in my hand when I picked up the tube to remove the cap.

Atomic Absorption Analysis --- Metal Accumulation

The same standards were used for metal accumulation analysis as in the toxicant water analysis. The standards, prepared from commercial stock solutions, were acidified with "Suprapur" nitric acid (EM Laboratories, Inc.) and diluted up to volume with distilled water. Six blank digestions in 5 ml test tubes containing 0.5 ml nitric-perchloric acid mixture and 4.5 ml distilled water were included with each set of digestions to cross check for contaminants. If contaminants were detected, that amount was subtracted from the sample analyses prior to calculation of the metal content in the samples.

The calculation equation yields the metal accumulation in the naiads in micrograms of metal per gram of tissue ($\mu g/g$).

$$\frac{\mu g \text{ metal}}{g \text{ tissue}} = \frac{\text{A. A. readout in } \mu g \text{ metal/ ml} \times \text{dilution factor in ml}}{\text{gram(s) tissue dry weight}}$$

The "A.A. readout" comes from the strip chart by comparison of the sample reading against the standards curve. The "dilution factor" is the volume of the sample in the test tube (5, 25, 125 ml, depending upon the number of times it was necessary to dilute the sample to obtain an accurate reading). The "gram(s) tissue dry weight" was the

dry weight of the naiad at the time of weighing and placing in the sample tube.

Detection limits on the Perkin-Elmer model 303 Spectrophotometer for lead, zinc, copper and silver in mg/liter were 0.05, 0.01, 0.001, and 0.005, respectively.

Detection limits on the Perkin-Elmer model 290B Spectrophotometer for lead, zinc, copper and silver in mg/liter were as follows: zinc, 0.10; lead, 1.0; copper, 0.10; and silver, 0.15-0.20.

Linear Regression Analysis

Linear regression analyses were performed on all tests. The primary purpose of these analyses was twofold. The first was to evaluate the relationship between metal accumulation by the naiads and metal concentration in the water. The second was to evaluate the accumulation of metals by the naiads as exposure time increased. The only regression statistic referred to in the text will be the correlation coefficient r, which is an indicator of the linearity of the regression line and the goodness of fit of the data to that regression line.

Use of the Appendices

See Appendix A for details on water quality data for all acute tests conducted. Mean levels of metal accumulation versus metal concentration will not be referred to for all concentrations in the acute tests discussed below. For details on metal accumulation versus metal concentration see Appendix B. Appendix C lists details on metal

accumulation by the naiad as exposure time increases. Appendix D lists regression equations ($y_i = a + bx_i$), standard errors (e_i), and correlation coefficients (r) for all tests conducted.

RESULTS

Results of the acute bioassays are presented alphabetically by metal and chronologically for a given metal; however, the tests were not conducted in this order. Silver acute bioassays one, two and three were the first bioassays conducted. Thereafter, no special sequence of testing existed except that the stonefly (P. californica) acute tests were conducted first and the mayfly (E. grandis) acute tests were last.

Copper Acute Bioassay One

The stonefly Pteronarcys californica was the test species. Six males and four females were used in each concentration. Mean length of the naiads in all concentrations was 36-38 mm. The range of copper levels was 0.74 to 13.9 mg/liter. Mortality was 100% in the highest concentration after 70 hours exposure. No mortality occurred in any other concentration. The test lasted 11 days.

All concentrations were checked at least twice daily for signs of stress and mortality. Naiads in the highest concentrations exhibited signs of stress (waggling from side to side) after 24 hours exposure. The circadian rhythm was also disrupted.

Linear regression analysis of mean copper accumulation by the naiads versus copper concentration in the water gave a correlation coefficient of 0.986 indicating a linear relationship. Mortality did not

span a long enough time period to effectively document increased copper accumulation with increasing exposure time.

Death of the naiads was not caused by the amount of copper accumulated, but rather the level of exposure. The naiads in the highest concentration (13.9 mg copper/liter) accumulated an average of 771 μg copper/g tissue. Mortality in this concentration was 100%. Copper accumulation by the naiads in the second concentration was 1106 μg copper/g tissue. The dose level here was 5.64 mg copper/liter. Despite the higher accumulation by the naiads in this concentration, no mortality occurred. Using the t- test statistical analysis on the accumulation data from the two concentrations gave a value for t = 2.033. The t value at $t_{.05(19)} = 2.093$. The t value at $t_{.1(19)} = 1.729$.

On the basis of the wide range in copper levels between the first and second concentrations I was unable to calculate either a TL_{50} value for copper on P. californica or an incipient lethal level for copper on this stonefly. Thus, it was necessary to test this species with copper again.

Copper Acute Bioassay Two

With the supply of naiads becoming limited, I used three males, three females, and four immature naiads per concentration in this test. The males averaged 33 mm in length, the females 30-33 mm, and all immatures 28 mm.

The range of copper concentrations was 5.51 to 18.5 mg/liter. Resultant mortality in the top concentration was 100% in 48 hours. Mortality in the second concentration (10.1 mg copper/liter) began after 52 hours exposure and was 38% after 64 hours. All naiads in this concentration exhibited severe signs of stress after 52 hours of exposure.

Malfunction of the toxicant delivery system precluded completion of the test. Thus, I was unable to calculate a TL_{50} value, nor could an incipient lethal level be determined. Due to the short duration of the test no data on copper accumulation over time could be determined. However the second concentration in copper test one averaged 5.64 mg copper/liter. The lowest concentration in copper test two averaged 5.51 mg/liter. Considering together the copper accumulation by the naiads from these two concentrations, a regression analysis of copper accumulation versus increasing exposure time is possible. The correlation coefficient for this regression is 0.993.

Regression analysis of copper accumulation by the naiads versus copper level in the water gave a correlation coefficient of 0.901.

In an attempt to determine a valid incipient lethal level and TL_{50} value for copper on P. californica, a third bioassay with copper was necessary.

Copper Acute Bioassay Three

This bioassay was the last test conducted at the Fort Collins Research Center. Nine adult female naiads were used per concentration.

The range of copper concentrations was 6.47 to 12.2 mg/liter over the 8 days of the test. The naiads showed minor signs of stress during the first 48 hours of the test and then recovered. Two naiads of the nine died in the highest concentration for a mortality of 22%. No mortality occurred in the second concentration which averaged 10.4 mg copper/liter.

Again, no incipient lethal levels or TL_{50} values could be determined; however, the results of all three copper bioassays indicate both the incipient lethal level and the TL_{50} value probably lie between 10.1 and 13.9 mg/liter.

This test provided additional evidence supporting the statement made under Copper Acute Bioassay One that "the level of exposure determines mortality and not the accumulation level". Levels of exposure in copper bioassay three ranged from 6.47-12.2 mg/liter. Mean accumulations in the four concentrations ranged from 1200-2540 ug/g, yet no mortality occurred except 22% in the highest concentration. In contrast, mean accumulation in the highest concentration from Copper Acute Bioassay One was 771 ug/g and mortality was 100% due to a mean exposure level of 13.9 mg/liter. Using the t-test statistic to compare mean accumulations in this test with mean accumulation in copper bioassay one-concentration one (13.9 mg/l) revealed t values of 2.454 or greater. Any t value greater than $t_{.05(18)} = 2.101$ is significant at $p = .05$ or less.

Regression analysis on copper accumulation by the naiads versus copper concentration in the water from copper test three gives a correlation coefficient of 0.994, indicating a very linear regression line.

Copper Acute Bioassay Four

The mayfly Ephemerella grandis was the test species in this bioassay. Total exposure time was 8 days, with 50 naiads per concentration. Mortality was 100% in the top four concentrations with 98% mortality in the fifth. No mortality occurred in the control except for an 8% loss due to cannibalism. Cannibalism occurred in this test and subsequent tests where E. grandis was the test organism.

Each concentration was checked twice daily for signs of stress and mortality. Indication of stress included hyperventilation of the abdomenal gills, loss of equilibrium, and naiads turned over on their backs.

The range of toxicant levels in this test was from 0.63 to 10.0 mg copper/liter. Regression analysis of the copper accumulation by the mayfly naiads versus copper in the water gave a correlation coefficient of 0.982.

Since mortality was virtually complete in all concentrations, estimates of incipient lethal levels or TL_{50} values for copper on E. grandis could not be determined. Thus, a second bioassay with copper was conducted on this mayfly.

I believe cannibalism was due to crowding of the naiads thereby increasing their aggressive behavior. These naiads exhibited

elaborate territorial displays when another naiad approached. The posterior portion of the abdomen was arched upward with the three anal cerci spread apart in a fan-like manner. The defending naiad would also stand higher. By standing higher and arching the abdomen upward the naiad was able to display horny protuberances on the head and prothorax and hooks on the dorsal surface of the abdomen to the approaching naiad.

By crowding 50 naiads into such a small space I may have put too much stress on the social structure thereby increasing aggressive behavior which manifested itself in cannibalism. The naiads attacked were always recent molts. After the molt the exoskeleton is very soft and pliable for 12-24 hours, making the molted naiads very easy prey for other naiads nearby. These cannibalistic tendencies may be operative in nature under natural conditions, but to my knowledge no one has documented this in the order Ephemeroptera.

Losses due to cannibalism occurred only in the control. I believe stress from exposure to copper upset the territorial behavior in the other concentrations, thereby preventing cannibalistic behavior and losses due to cannibalism.

Copper Acute Bioassay Five

Total exposure time in this test was 9 days. The range of copper concentration was from 0.08-1.06 mg/liter. Each concentration

contained 50 naiads. Mortality from concentration one through the control was 100%, 100%, 94%, 18%, 2% and 0%, respectively.

With only 2% mortality in the fifth concentration (0.08 mg copper/liter) the incipient lethal level for copper on E. grandis can be set at 0.08 mg/liter. The TL_{50} value for copper on E. grandis, determined by log-probit analysis as outlined by Sprague (1969), was 0.18 mg/liter.

Correlation of copper accumulated by the naiads with exposure time was impossible. Biological variability between naiads within a given concentration and small sample sizes for a given exposure time precluded any correlation on this basis. Regression analysis on copper accumulation by the naiads versus copper concentration in the water gave a correlation coefficient of 0.974.

Lead Acute Bioassay One

The stonefly P. californica was the test organism used in lead acute test one. Each of the six concentrations contained 10 naiads, six males and four females. Mean length of the naiads in all concentrations was 33 mm. The test lasted 11 days. The range of lead concentrations was 1.08-19.2 mg/liter. No mortality occurred in any concentration.

All concentrations were checked at least twice daily for signs of stress and mortality. All naiads fed well for the first seven days of the test. On the eighth day the naiads in the three highest concentrations

were not feeding as well as those in the two lowest concentrations and control. No other signs of stress (vertical pumping, "waggling" from side to side, or loss of equilibrium) were observed during the test.

Accumulation of lead by the naiads appeared to be by adsorption of the lead to the exoskeleton in the form of a white precipitate, possibly lead carbonate. The naiads in the highest concentration became grayish-white as the test proceeded.

Since no mortality occurred, it was not possible to calculate TL_{50} values for lead, nor determine incipient lethal levels for lead, and no data on accumulation of lead in P. californica over time could be determined.

Linear regression analysis of the mean accumulation of lead by the stonefly naiads versus dissolved lead in the water gave a correlation coefficient of 0.991.

Lead Acute Bioassay Two

The mayfly E. grandis was the test organism for this test. Each concentration contained 50 naiads. The test duration was 12 days. The range of concentrations of lead was 0.69-9.24 mg/liter.

Mortality occurred in all concentrations except the control. Mortality from the highest to lowest concentrations was as follows: 82%, 98%, 8%, 14%, and 6%. Losses due to cannibalism in concentrations three, four, five, and control were: 2%, 8%, 10%, and 8%. No cannibalism occurred in the two higher concentrations. Successful emergence

as adults occurred in all concentrations except the highest one. Numbers of emergers in concentrations two through the control were as follows: 1, 13, 13, 9, and 8.

The TL_{50} value for E. grandis exposed to lead in this test was about 3.5 mg/liter. Incipient lethal levels of lead on E. grandis was less than 0.7 mg/liter, based on 6% mortality in this test at an exposure level of 0.69 mg/liter.

Lead accumulation in the mayfly naiads was primarily through the adsorption of lead carbonate ($PbCO_3$) to the exoskeleton. Lead carbonate accumulation on the exoskeleton was visible 24 hours after test initiation in the highest concentration. After several days exposure time the naiads in the top two concentrations were rendered immobile, incased in a lead carbonate case resembling concrete or plaster of paris. It is my opinion that death of the naiads resulted from exhaustion, starvation, or inhibition of respiration, and not from the toxic effects of lead.

Regression analysis of mean lead accumulation by the naiads versus mean lead concentration in the water gave a correlation coefficient of 0.985.

Silver Acute Bioassay One

The stonefly Pteronarcys californica was the test species. Mean length of the naiads was 36-38 mm in all concentrations. Each concentration contained 10 female naiads. Total exposure time was 6 days

with the range of concentrations from 0.050-0.738 mg silver/liter. The test was terminated prematurely because several naiads escaped from the third concentration during the test.

Each concentration was checked at least twice daily for signs of stress and mortality. Mortality began to occur after 60 hours of exposure. Indications of stress, evident after 24 hours exposure, included "waggling" from side to side, vertical pumping, and disruption of circadian rhythm. All naiads were near death when the test was terminated. Mortality in the lowest concentration (0.050 mg silver/liter) was 60% at test termination, indicating the TL_{50} and incipient lethal level values lie below this point.

Regression analysis of silver accumulation by the naiads versus silver concentration in the water gave a correlation coefficient of 0.996.

Regression analysis of silver accumulation versus length of exposure time gave an r value of 0.968. This regression was taken from the highest concentration where mortalities occurred at five different times from 58 hours of exposure to 143 hours exposure.

Silver Acute Bioassay Two

The predaceous stonefly Claassenia sabulosa was the test species in this test. The purpose of this test was twofold. The first objective was to insure the proper operation of the toxicant delivery system prior to beginning more tests on P. californica. The second

objective was to test the tolerance of Claassenia sabulosa to silver in comparison to P. californica.

The range of silver concentrations in this test was 0.050-0.900 mg/liter. The test lasted for 73 hours. Mortality in concentrations one through the control was 77%, 100%, 85%, 100%, 86%, and 0% respectively. Those naiads surviving after 73 hours were near death also. As with P. californica, signs of stress ("waggling" from side to side, vertical pumping, loss of equilibrium, and disruption of circadian rhythm) indicated death was imminent.

Claassenia sabulosa was less tolerant of silver than P. californica as the first mortality occurred in 24 hours with this species versus 55 hours for P. californica. Mortality was virtually complete after 73 hours in this test versus 143 hours in silver test one with P. californica. These results parallel the findings of Sanders and Cope (1968), who found P. californica to be more tolerant of nine organic pesticides than C. sabulosa.

The correlation coefficient for regression analysis of silver accumulation by C. sabulosa versus silver concentration in the water was 0.950.

Silver Acute Bioassay Three

The stonefly P. californica was the test species. Mean length of the naiads was 40 mm, with three males and seven females used per concentration. Test duration was 15 days. The highest

concentration was 104 μg silver/liter, the lowest 6 μg silver/liter. Mortality in concentrations one through the control was 100%, 100%, 80%, 70%, 30%, and 0% respectively.

Males died first in all concentrations. This phenomenon is well documented in the class Insecta. Males are generally smaller than females and possess fewer fatty tissues. Many toxic substances are fat soluble and sequestered in fatty tissues. Thus, males are often less tolerant of toxic substances than females (personal communication--- Dr. Don Fronk, Department of Zoology and Entomology, Colorado State University).

Mortality commenced in concentrations one, two, and four after 69 hours of exposure. Signs of stress were apparent after 48 hours of exposure in the two highest concentrations.

Determination of the TL_{50} value for silver on mature (last instar) P. californica naiads by log-probit analysis resulted in a TL_{50} value of 8.8 μg/liter. Incipient lethal level for silver on mature naiads of these stoneflies lies below 5 μg/liter.

Mean silver accumulation in the lowest concentration was 7.30 μg silver/gram tissue and 11.32 μg silver/gram tissue in the highest concentration. With such a small range in accumulation levels between the lowest and highest concentrations, variability between naiads precluded any correlation between silver accumulation by the naiads and increasing exposure time. This was the reason for a correlation coefficient of 0.830 when regression analysis of mean

silver accumulation by the naiads versus silver concentration in the water was performed.

Silver Acute Bioassay Four

The purpose of this test was to compare the toxicity of silver to immature (early instar) P. californica naiads with the tolerance of the mature naiads. Immature naiads (no genitalia visible) averaging 24 mm in length were used in each concentration. The range of silver concentrations was from 4 to 67 μg/liter. Test duration was 7 days. Mortality in concentrations one through the control was 100%, 100%, 70%, 70%, 80%, and 0% respectively.

Each concentration was checked three times daily for signs of stress and mortality. Mortality occurred in concentrations one, two, and three after 48 hours of exposure. This indicated immature naiads were much more susceptible to silver than mature naiads. Silver levels in this test were approximately one-half the corresponding concentrations in silver test three and yet first mortality occurred after 48 hours in this test versus 69 hours in silver test three. Total exposure time here was 166 hours as compared to 363 hours in silver test three.

I was unable to determine a TL_{50} value for silver on these immature (early instar) naiads as mortality exceeded 50% in all test concentrations. The TL_{50} value was less than 4 ug silver/liter (the

mean level of exposure in the lowest concentration where 80% mortality occurred). Incipient lethal levels for silver on immature naiads may be less than 1.0 μg/liter.

The immature naiads accumulated greater amounts of silver despite exposure to lower levels of silver over a shorter period of time than the mature naiads. For example, silver accumulation by the naiads in the highest concentration (67 μg/liter) in this test was 20.84 μg/g tissue for 166 hours exposure. In contrast, 11.32 μg/g tissue was accumulated by the mature naiads in silver test three at an exposure level of 104 μg/liter for 363 hours. Variability between naiads within a given concentration precluded regression analysis of silver accumulation versus increasing exposure time.

Regression analysis of mean silver accumulation by the naiads versus mean silver concentration in the water gave a correlation coefficient of 0.909.

Silver Acute Bioassay Five

The mayfly Ephemerella grandis was the test organism in this bioassay. The range of silver concentrations was from 0.06 to 0.75 mg/liter with 50 naiads per concentration. Total exposure time was 7 days. Mortality commenced after 15 hours exposure in concentrations one, two and four. Complete mortality occurred in all concentrations.

E. grandis was much less tolerant of silver than P. californica. In the highest concentration (0.75 mg silver/liter) mortality was complete after 48 hours exposure. In contrast, P. californica naiads exposed to 0.738 mg silver/liter did not suffer lethal effects until after 58 hours exposure and 143 hours of exposure was necessary for 100% mortality. Similar comparisons can be drawn between all corresponding concentrations in silver bioassays one and five.

Despite mortality in all concentrations being spread over time, no correlations could be drawn between silver accumulation by the mayfly naiads with increasing exposure time due to biological variability between naiads within a given concentration. With 100% mortality in all concentrations it was impossible to draw any conclusion on TL_{50} values or incipient lethal levels of silver on E. grandis.

The correlation coefficient (r) for regression analysis of mean silver accumulation by the naiads versus mean silver concentration was 0.893.

Another bioassay was conducted on E. grandis with silver as the toxicant in an attempt to establish incipient lethal levels and a TL_{50} value for silver on this species of mayfly.

Silver Acute Bioassay Six

Again, 50 naiads were used per concentration. The range of silver levels in this test was 9 to 150 µg/liter. Total exposure time

was 10 days. First mortality occurred after 12 hours of exposure. Mortality in concentrations one through the control was 100%, 100%, 100%, 98%, 96%, and 0%, respectively.

In this test the mean level of silver in concentration four was 20 µg/liter. At this level mortality began to occur after 21 hours. Mortality was 98% after 241 hours. In contrast, P. californica naiads exposed to 22 µg silver/liter in silver test three did not begin dying until 167 hours of exposure and mortality only reached 80% at test termination (363 hours). Again, this points out that the mayfly E. grandis was less tolerant of silver than the stonefly P. californica.

Variability between naiads again precluded any correlation between silver accumulation and increasing exposure time. Correlation between mean silver accumulation by the naiads and level of silver exposure gave an r value of 0.666. Variability between naiads within a concentration contributed to the low r value; however, differences in exposure times between concentrations was the primary factor. Naiads in the lower concentrations lived longer and consequently accumulated silver levels similar to the naiads in the higher concentrations.

Determination of the TL_{50} value for silver by log-probit analysis (Sprague, 1969) was impossible since mortality exceeded 90% in all test concentrations.

The point of incipient lethal effect was impossible to determine. No attempt was made to further isolate the TL_{50} value or point of incipient lethal effect as this would have required elaborate analysis procedures for silver involving organic extraction with MIBK (methyl-isobutyl-ketone) solvent.

Zinc Acute Bioassay One

The stonefly Pteronarcys californica was the species used in zinc test one. The test lasted 14 days. Each concentration contained 10 females. The mean length of the naiads in all six concentrations was 41.8 mm. The range of toxicant concentrations was 0.77-13.6 mg/liter dissolved zinc. No mortality occurred in any of the six concentrations.

All concentrations were checked twice daily for mortality and signs of stress. The naiads in the two higher concentrations did not feed for the first 96 hours after initiation of the test. The naiads in the three lower concentrations fed during the first 96 hours but not as voraciously as the control naiads. After the first 8 days all naiads began feeding and at test termination all naiads were feeding very well and exhibited no signs of stress. Acclimation to the toxicant appeared to be complete.

I conducted a linear regression analysis on mean zinc accumulation by the naiads versus mean zinc levels in the water. The

correlation coefficient (r) was 0.779 for this test, indicating a mediocre correlation between zinc accumulation by the naiads and zinc dissolved in the water.

There were two reasons for this poor correlation. The first was that a sample size of 10 naiads per concentration was not large enough to minimize biological variability between naiads. Second, and more important, the naiads built up an equilibrium level of zinc over a period of time. Once the equilibrium level was reached, zinc was excreted almost as readily as it was taken up. Kormondy (1965) documented this phenomenon in a species of dragonfly. Thus, a plot of zinc in the water versus zinc accumulation in the naiads revealed a curvilinear regression line instead of a linear relationship and hence the lower correlation coefficient.

Since no mortality occurred during the test no TL_{50} values could be calculated, no incipient lethal levels were determined, and no data on accumulation versus time could be determined.

Zinc Acute Bioassay Two

The mayfly Ephemerella grandis was the test organism in zinc test two, with 50 individuals per concentration. The range of concentrations of dissolved zinc in this test was 0.60-9.2 mg/liter. As with P. californica in zinc test one, no mortality due to zinc poisoning was observed in this test.

Linear regression analysis of zinc accumulation by the naiads versus mean zinc levels in the water again gave a low correlation coefficient, $r = 0.694$. The sample size of 50 individuals per concentration was adequate enough to minimize biological variability. But E. grandis reached an equilibrium level of zinc very rapidly and again a plot of the x and y pairs from the regression analysis revealed a curvilinear relationship, thus, a low correlation coefficient.

Since no mortality occurred during the test no TL_{50} values could be calculated, no incipient lethal levels were determined, and no data on accumulation versus time could be determined. Mean levels of zinc accumulation by the naiads versus zinc dissolved in the water are listed in Appendix B.

Cannibalism was observed in this test. Losses due to cannibalism ran 4-6% in concentrations three, four, five and the control. One naiad was lost to cannibalism in the second concentration, none in the first.

Zinc Chronic Bioassay on Willow Creek

This experiment was set up with two objectives in mind. First, I wanted to test the effects of Willow Creek water on the growth, emergence, and survival of P. californica and E. grandis. My second objective was to determine zinc accumulation levels by these two species over a long period of time.

Prior to testing, both species of insects were collected from the Rio Grande and placed in the test trough for 30 days. Four concentrations and one control were used with all concentrations and the control replicated. Each replicate contained 100 naiads of each species. Sexually mature and immature P. californica naiads were used in the test. Each replicate contained 45 males, 30 females, and 25 immatures. The three to two ratio of males to females was the ratio we found to exist in the Rio Grande as we collected the test insects. The 25 immatures were used to test the growth effects and zinc accumulation levels at the end of the test.

During the 30 day acclimation period all P. californica naiads remained in their respective test compartments; however, after initiation of the test, the stoneflies in all concentrations except the control exhibited a peculiar behavior for one week. Willow Creek water began flowing into the test troughs at 1600 hours on May 12, 1972. The next morning virtually all stonefly naiads in the replicates receiving Willow Creek water had migrated to the troughs below containing the mayfly naiads. They did this by crawling over, under, and around the retaining screens in the troughs. In some cases it was necessary to crawl out of the water. I returned the naiads to their respective troughs and the next morning many naiads were found in the lower troughs again. This phenomenon, although diminished in magnitude, continued for a week.

Two explanations were possible: the naiads were trying to escape either the zinc, or the temperature fluctuations in Willow Creek. I ruled out an avoidance reaction to zinc because zinc was present at all times after test initiation, yet no avoidance efforts were made during the day.

Temperature sensitivity is a more logical explanation. The stonefly P. californica inhabits big rivers. Large rivers do not exhibit great diurnal temperature fluctuations. The naiads were exposed to daily temperature fluctuations of 15 C when Willow Creek was introduced into the troughs. Afternoon temperatures in Willow Creek were 15 C while temperatures at dawn were 0.2 C. The temperature in the Rio Grande did not fluctuate more than 4 C (4-7 C).

Willow Creek had no detectable effects on the emergence of the stonefly P. californica. Emergence of adults was actually greater in the concentrations receiving Willow Creek water than in the controls. Emergence in the controls was 52% versus 73-84% in the replicates receiving Willow Creek water. Emergence in the non-controls more closely coincided with the emergence of P. californica in the Rio Grande than did the controls.

I believe temperature was the reason behind the phenomenon. Emergence of the "willow fly" (P. californica) on the Rio Grande at Creede occurs about June 16th every year. Stream watchers and avid fly fishermen in the area say, "You can tell the day of the year by the

hatch of the willow fly." If this stonefly is to emerge at the same time year after year, photoperiod must play a primary role in the emergence process.

But temperature must have an effect as well. Willow Creek water was much warmer during the daylight hours than either the Rio Grande or the control water. Thus, all test naiads were under the same photoperiod but different temperatures. The naiads exposed to pure Willow Creek water (the warmest) were the first to emerge, beginning June 12, with a peak on June 16 and finished on the 18th of June. Naiads in the other three concentrations began emergence on June 13, peak emergence on June 19-20 and finished on June 25. The controls (the coldest water) began emergence on June 15, with peak emergence on June 22 and continued through June 30.

Growth and survival were the same in all concentrations, including the controls. The naiads fed on dead leaves, fresh green leaves, and dead wood.

Accumulation of zinc by the stonefly naiads versus mean concentration of zinc in the water was positively correlated with the correlation coefficient of 0.897.

The mayfly portion of the chronic bioassay was a complete failure except for the zinc accumulation-concentration regression analysis. Emergence of E. grandis in the Rio Grande was 100% complete by the 6th of July 1972. No emergence of the mayflies occurred in any concentration or the control, nor did the mayfly naiads grow. The

average dry weight of naiads on April 14, 1972 when the acclimation began was 0.01 g. This was the dry weight of naiads at termination of the test on July 6, 1972. The dry weight of naiads in the Rio Grande at the time of emergence was 0.03-0.05 g. This weight was three to five times greater than that of naiads in the chronic bioassay at the time of termination.

Many reasons for the failure of the mayflies to grow and emerge are possible. Improper or insufficient food and diet, less than ideal temperatures, and sub-lethal effects of zinc are a few of the plausible reasons.

Accumulation of zinc by the mayflies versus mean zinc levels in the water was analyzed by linear regression analysis. The relationship was curvilinear, again giving a low correlation coefficient, $r = 0.878$.

Sub-chronic Bioassays on Zinc Accumulation Over Time

The test objective was to document the accumulation of zinc in E. grandis and P. californica as exposure time increased. Both stonefly and mayfly naiads were collected from the Rio Grande and put in a livebox in Willow Creek.

Zinc levels in the creek were monitored with 24 hour composite samples collected at a drip station in the hatchery from the two month chronic bioassay in operation concurrently. Composite samples, taken from the chronic concentration receiving undiluted Willow Creek water,

gave a more accurate reading on the actual average zinc load in Willow Creek for a 24 hour period than might have been obtained on a grab sample analysis collected at one instant in the 24 hour period.

Regression analysis of the zinc accumulation in naiads versus increasing exposure time was positively correlated in each of the four tests. Test one on E. grandis revealed an r value of 0.975. The r value for P. californica in test one was 0.993. This test lasted 96 hours. Test two was conducted on E. grandis for a total exposure time of 404 hours. The r value was 0.905. Test three on P. californica lasted for 96 hours. The r value was 0.934. Test four was conducted on P. californica with a total run time of 400 hours. The r value was 0.910.

Examination of data in Appendix C on zinc accumulation versus exposuretime readily reveals a high correlation between zinc accumulation and exposure time up to 96 hours. At this point the naiads have reached an equilibrium level of zinc accumulation. Beyond 96 hours the only way to increase zinc accumulation is to increase zinc concentration in the water. As zinc concentration in the water rises and falls with time so does zinc accumulation level in the naiads rise and fall around the equilibrium accumulation level.

DISCUSSION

As was stated in the introduction, aquatic insects must satisfy three prerequisites if they are to be used as biological monitors of heavy metal pollution in fish-kill investigations. First, the insects must be more tolerant of a specific pollutant than the fish. Second, the insect must be capable of concentrating the metal pollutant by some predictable factor(s) over a short period of time (1-10 days). This will simplify detection and analysis procedures, especially when the pollutant is toxic to fish in extremely minute amounts (ppb or pptr). Third, and most important, the insect must concentrate the pollutant in relative proportion to the level found in the stream.

The insects must be more tolerant of the metal pollutant than the fish. The insect must be alive and holding a position in the stream. If the insect dies along with the fish, the insect will float downstream. Even if it were possible to determine the level of exposure the insect suffered, we cannot be sure of the location in the stream where the insect suffered this exposure. Unable to say that "X" level of this pollutant occurred at a given point in the stream, the biologist has nothing that will stand as evidence in a court-of-law.

The insect must concentrate the pollutant by some predictable factor over a short period of time. The whole hypothesis of monitoring heavy metal pollution with aquatic insects is predicated on this

statement. If the insect does not concentrate the metal pollutant by some predictable factor over a given time period, the hypothesis in essence "holds no water". Depending upon the degree of accuracy and precision desired in estimating (after the fact) the level of exposure, deriving the concentration factor could be a most difficult task.

The insect must concentrate the pollutant in proportion relative to the amount of pollutant found in the stream? This prerequisite is closely tied to the second premise above. It is not really possible to have one without the other; to use the old cliche, a horse and carriage effect.

Discussion of the Copper Bioassays

Comparison of the TL_{50} values for copper on these two aquatic insects with the TL_{50} values on fish indicates aquatic insects are much more tolerant of copper than are fish. The TL_{50} for copper to Ephemerella grandis is between 180-200 µg/liter. The TL_{50} value for copper to Pteronarcys californica lies between 10,100-13,900 µg/liter. In contrast, the TL_{50} value for copper to rainbow trout (Salmo gairdneri) is 22-25 µg/liter (Goettl, Sinley and Davies, 1971). Mount and Stephan (1969) set the 96-hour TL_{50} of copper for the fathead minnow (Pimephales promelas) in soft water at 75 µg/liter. McKim and Benoit (1971) found a 96-hour TL_{50} of 100 µg copper/liter to brook trout (Salvelinus fontinalis). Thus, the mayfly (E. grandis) is two to ten times more tolerant of copper as the fish cited above.

The stonefly (P. californica) is 100 times as tolerant as any of the fish studied.

Below exposure levels of 1 mg/liter, concentration factors for copper vary both with level of exposure and length of exposure. For a given time period the concentration factor decreases with increasing levels of exposure. For a given level of exposure, concentration factors increase with time. At exposure levels above 1 mg/liter concentration factors remain relatively constant for a given exposure time.

Concentration factors are easily calculated: the mean level of copper accumulation (μg/g = ppm) by the insects is divided by the mean level of exposure to copper (μg/ml = ppm). Copper acute bioassays one and three on the stonefly (P. californica) provide the data to test the concentration factor hypothesis. Concentration one in copper test one is not considered as 100% mortality occurred in this concentration making a valid analysis impossible in this case.

Copper Acute Bioassay One 264 hrs.			Copper Acute Bioassay Three 195 hrs.		
Mean Copper Accumulation	Mean Copper Exposure	Conc. Factor	Mean Copper Accumulation	Mean Copper Exposure	C.F.
1105.5 /	5.64	196.0	2539.85 /	12.22	207.8
472.46/	2.92	161.8	2095.78 /	10.49	199.8
274.90/	1.70	161.7	1766.74 /	8.13	217.3
156.78/	0.738	212.4	1198.59 /	6.47	185.3
Mean concentration factor 183			Mean concentration factor 202.6		

By dividing the mean accumulations in test one by the mean concentration factor in test three, exposure levels in test one can be estimated. The two tests have nothing in common except that the species of insect, water quality, and pollutant are the same. In essence, test three is the control situation and test one is the unknown we want to estimate.

Copper Acute Bioassay One

$\bar{X}$ Accumulation		C.F.(Cu Test#3)		Estimated Cu level (mg/l)	Actual Cu level (mg/l)
1105.5	/	202.6	=	5.46	5.64
472.46	/	202.6	=	2.33	2.92
274.90	/	202.6	=	1.36	1.70
156.78	/	202.6	=	0.774	0.738

Reversing the order and using the average concentration factor from copper test one to estimate exposure levels in copper test three gives similar results.

Copper Acute Bioassay Three

$\bar{X}$ Accumulation		C.F.(Cu Test#1)		Estimated Cu level (mg/l)	Actual Cu level (mg/l)
2539.85	/	183	=	13.88	12.22
2095.78	/	183	=	11.45	10.49
1776.74	/	183	=	9.65	8.13
1198.59	/	183	=	6.55	6.47

The estimated levels of exposure are remarkably similar to the observed values.

The concentration factor from copper acute bioassay five-concentration one can be used to estimate levels of copper exposure in copper acute bioassay four. These two tests were performed on the mayfly E. grandis. The concentration factor from copper test five-concentration one was 1650. The concentration factor used here is not an average for the following reason. Concentration factors seem to remain relatively constant above exposure levels of 1 mg/liter. Below this level concentration factors increase very rapidly with a small increment decrease in exposure level. Thus, the concentration factor from copper test five-concentration one is used since it is the only level of exposure above 1 mg/liter in this test.

Copper Acute Bioassay Four

$\bar{X}$ Accumulation		C.F. (Cu Test #5)		Estimated Cu level (mg/l)	Actual Cu level (mg/l)
9124.8	/	1650	=	5.53	10.0
5787.0	/	1650	=	3.51	4.82
3882.3	/	1650	=	2.35	2.51
1932.8	/	1650	=	1.17	1.22
1240.4	/	1650	=	0.752	0.63

Again, the lower four estimates are relatively close to the actual values. The highest value is off by a factor of two. The reason is the exposure time in this concentration was much less than it was in concentration one of copper test five, and as exposure time increases the concentration factor also increases. The actual factor (concentration

one-copper test four) was 912.5 versus 1650; thus, the estimated copper level (5.3 mg/liter) grossly underestimates the actual value of 10 mg/liter. However, this is not too serious. A trout population exposed to copper at 5 mg/liter or 10 mg/liter will be annihilated in a very few hours regardless of the actual level of exposure.

The correlation coefficients (r) for all five copper acute tests are above 0.90. Four of the five tests have r values in the 0.97-0.99 range. This indicates the naiads do concentrate copper in relative proportion to its occurrence in the water.

The three objectives necessary for using aquatic insects as biological monitors of copper pollution have been satisfied. Both species are more tolerant of copper than fish, concentration factors are reasonable predictors in estimating levels of copper exposure, and the naiads do concentrate copper in relative proportion to its occurrence in the water.

Discussion of Lead Bioassays

Since no mortality occurred at 19.22 mg lead/liter, the TL_{50} value for lead on the stonefly P. californica is above 20 mg/liter. The TL_{50} value for lead on the mayfly E. grandis is 3.5 mg/liter. In contrast, the TL_{50} value for lead on rainbow trout (Salmo gairdneri) lies in the .14-.29 mg/liter range (Goettl, et al, 1971). This indicates rainbow trout are at least ten times more susceptible to lead than these aquatic insects. Water quality data on the insect

bioassays and the rainbow trout bioassays are identical or very similar for all parameters cited in Appendix A.

Using the average concentration factor from lead acute bioassay one (P. californica) to calculate estimated exposure levels for that same test, the following data results.

Actual lead levels-mg/liter	Estimated lead levels-mg/liter
19.22	19.05
7.44	5.24
4.43	3.88
1.96	1.72
1.08	1.67

Results from lead acute bioassay two on E. grandis are similar

Actual lead levels-mg/liter	Estimated lead levels-mg/liter
9.24	8.87
4.90	6.20
2.31	2.69
1.34	1.23
0.69	0.48

Once again the estimated lead levels reasonably approximate the actual levels of exposure.

The correlation coefficients (r) on lead accumulation by the naiads versus lead concentration in the water for lead tests one and two are 0.991 and 0.985, respectively. This indicates the naiads do accumulate lead in relative proportion to its occurrence in the water.

With the three objectives sighted earlier satisfied I conclude these two species of insects can be used as biological monitors of lead pollution.

Discussion of Silver Bioassays

The TL_{50} value for silver on P. californica naiads vary with size and age. Mature (final instar) P. californica naiads have a TL_{50} value of 8.8 µg silver/liter. The TL_{50} value for silver to immature (early instar) P. californica naiads is less than 4 µg/liter. The TL_{50} values for silver on E. grandis naiads is probably less than 1 µg/liter. In contrast, TL_{50} values for silver on rainbow trout (S. gairdneri) lie in the 5-8 µg/liter range depending on water temperature and fish size (personal communication --- John Goettl, Wildlife Researcher, Colorado Division of Wildlife). Thus, these two species of aquatic insects are at least as sensitive to silver as rainbow trout. Rainbow trout also exhibit a chronic lethal effect and stunted growth at .160 µg silver/liter (parts per billion).

Using the concentration factor from silver acute bioassay three (concentration one), estimated levels of exposure to silver were calculated for silver acute bioassay one. These two tests were run on the stonefly P. californica.

Silver Acute Bioassay One

Estimated silver levels (mg/l)	Actual Silver levels (mg/l)
0.488	0.738
0.282	0.399
0.211	0.217
0.125	0.105
0.084	0.050

Once again, the estimated level of silver exposure closely approximates the actual silver concentration.

The concentration factor hypothesis could not be tested on the mayfly E. grandis because only two tests were run on this species and the overlap in concentrations between the two tests was not great enough to adequately test the hypothesis.

Correlation coefficients (r) for silver accumulation by the naiads versus silver concentration in the water are quite high in most cases. The r values for silver acute bioassays one through six are 0.996, 0.950, 0.830, 0.909, 0.893, and 0.666, respectively. The low r value for test six is due to differences in survival times between concentrations in this test and accidental loss of 40% of the samples from this test during the digestion process. Mortality occurred much faster in the highest concentration than in the lowest. This permitted the naiads in the lowest concentrations to accumulate silver for a longer time period, thereby building up levels of silver accumulation similar to the highest concentrations.

Silver Iodide as a Weather Modification Agent

Cooper and Jolly (1970) prepared an excellent review paper on the ecological effects of silver iodide as a weather modification agent. They conclude the use of silver iodide as a nucleating agent in weather modification poses no serious threats to the environment. I do not endorse this conclusion.

When we begin to talk of one part per billion (μg/liter) or parts per trillion (ng/liter) as TL_{50} values for silver to aquatic insects and fish the possible ecological implications become obvious. Cooper and Jolly base their conclusion primarily on the insoluble characteristics of silver iodide and its propensity to adsorb or bind to almost any material. The Handbook of Chemistry and Physics (Weast, 1966) lists the solubility of silver iodide in cold water as $2.8X10^{-7.25}$ g/100 cc, or about 3 μg/liter. This approaches the TL_{50} values of silver determined for the aquatic insects used in this investigation as well as for rainbow trout.

In addition, the insolubility of silver iodide does not necessarily preclude accumulation of silver by benthic invertebrates. These organisms could accumulate silver through the food chain by consuming dead leaves and detritus which contain silver. Fish in turn would pick up silver from the stream invertebrates. With aquatic insects demonstrating concentration factors as high as 1000 (P. californica - Silver Acute Bioassay Four) the dangers of cloud-seeding with silver iodide can not be underestimated.

The map below (Fig. 1) is a schematic representation of Willow Creek at Creede, Colorado. Insect collections were made in the fall of 1969 and 1970 at the four stations indicated on the map. The numbers in parentheses in the following data (Goettl and Sinley, 1970) indicate number of genera and orders, respectively.

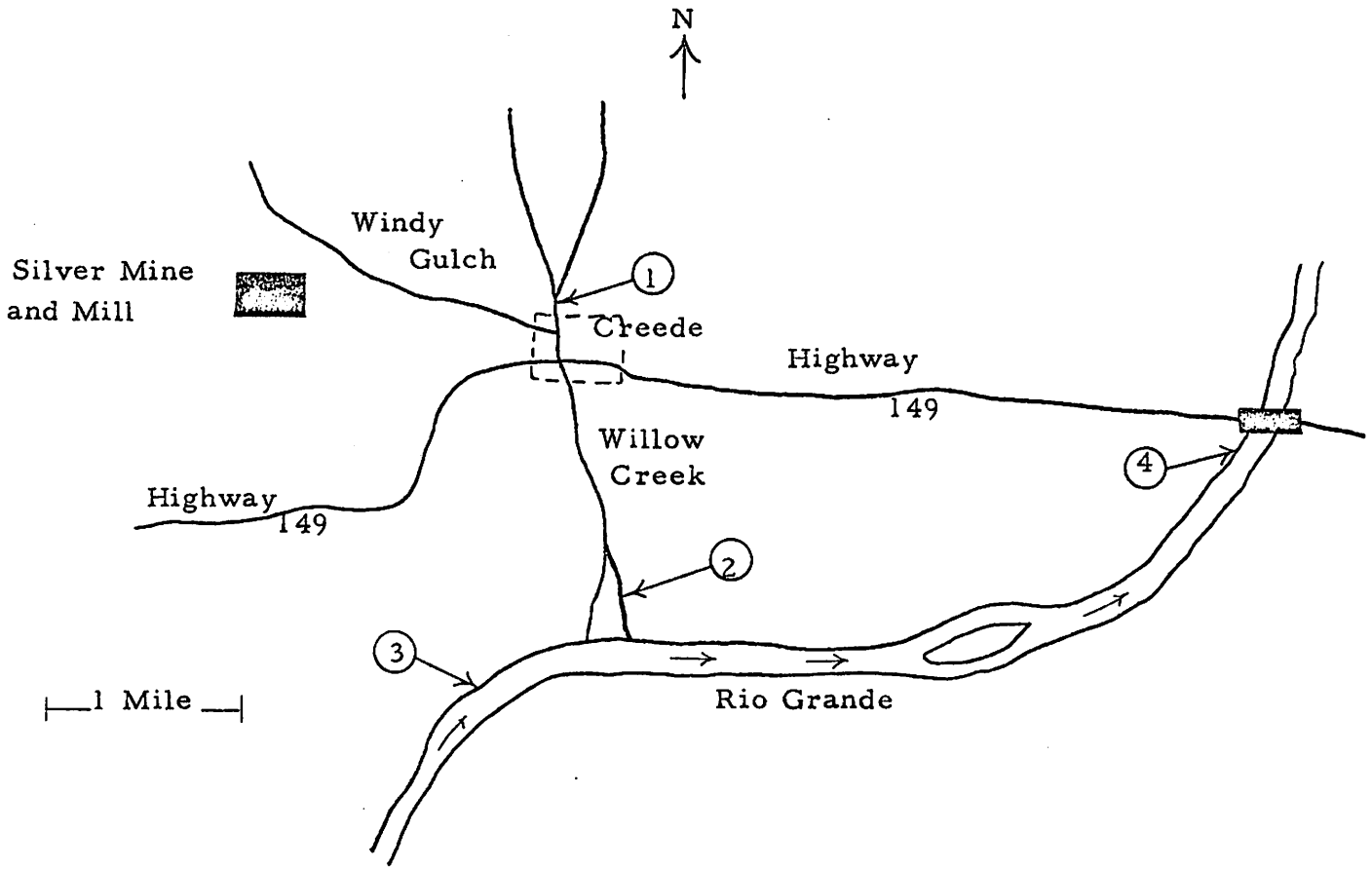

Figure 1. Map of the confluence of the Rio Grande and Willow Creek at Creede, Colorado.

Number of organisms/ $3ft^2$ / station

Date (month & year)	Station 1	Station 2	Station 3	Station 4
September 1969	1941 (10-5)	2 (2-1)	372 (17-5)	106 (13-4)
September 1970	250 (9-4)	3 (2-2)	7 (5-3)	14 (3-2)

The values at stations one, three and four in 1970 were lower than in 1969 due to severe floods in both Willow Creek and the Rio Grande at the time of sampling. It was impossible to sample the Rio Grande in the normal streambed in 1970 as the river was over its banks. Bottom-scouring floods often cause drastic reductions in populations of benthic invertebrates in streams (Hynes, 1970).

The remarkable statistics are the differences between stations one and two on Willow Creek in both 1969 and 1970. This phenomenon is not a coincidence. Intensive sampling revealed that Willow Creek from Creede to its confluence with the Rio Grande is virtually devoid of resident fish and aquatic insect populations. Occurrence of aquatic insects (Trichoptera and Diptera) at station two is in all likelihood the result of drift from areas in Willow Creek above station one. Trichopterans and Dipterans are insects that do drift. This phenomenon is compounded by the fact that the substrate in Willow Creek from Creede to the Rio Grande is much more fertile and varied than at station one upstream. Riffles, pools, rocky substrates and dense algal populations exist which normally would provide many and varied habitats for resident benthic invertebrate populations, yet none exist. Why?

Zinc pollution can be ruled out. Willow Creek at station one is chronically polluted with zinc from less than 1 to more than 5 mg/liter. Station two generally averages from less than 1 to 5 mg zinc/liter, at times as high as 10 mg/liter. The zinc acute bioassays showed no lethal effects or inhibitory effects on the molting processes of either the mayfly E. grandis or the stonefly P. californica. Yet when these aquatic insects were put in Willow Creek for two to six-week periods, considerable mortality always occurred, especially during the months of August and September when the flow in Willow Creek was at its minimum level.

I believe silver is the most probable toxic agent. During the summer of 1972 water running down Windy Gulch above Creede was tested for silver content. Silver levels ranged from 10-50 μg/liter over a period of several weeks. Water in Windy Gulch was waste water from a silver mining and milling operation. The gulch usually ran 1-2 ft^3/sec. In August and September this made up about 25% of the total flow in Willow Creek. Silver content of Willow Creek at station two was 0.72 μg/liter when checked in September, 1972. This was near the TL_{50} values of silver for both Ephemerella grandis and Pteronarcys californica. Was silver lethal to these naiads during the molting process? This was when mortality often occurred in Willow Creek and the silver acute bioassays. This could be the reason for elimination of resident benthic invertebrate populations from an

otherwise favorable habitat. Admittedly, more intensive investigation is needed before conclusions can be drawn, but the implication is most certainly there.

Cooper and Jolly (1970) feel that the bacteriocidal properties of silver could have deleterious effect on microorganisms responsible for the breakdown of detritus and recycling of nutrients in aquatic ecosystems. This may be where silver iodide will reap its effect on the aquatic ecosystem---not through direct lethal effects, but by eliminating the food web through elimination of the decomposers. Silver (as $AgNO_3$) has long been known in the medical profession as an effective bacteriostat.

Most certainly the use of silver iodide should be carefully scrutinized before it is widely applied in cloud seeding and other weather modification programs. Without proper analysis and close monitoring programs silver could become as ubiquitous and more deadly in aquatic ecosystems than either mercury or DDT.

Discussion of Zinc Bioassays

The TL_{50} values for zinc on E. grandis and P. californica are very high, above 10 mg/liter. In contrast, zinc is quite toxic to many species of fish. Sprague (1964) found a TL_{50} value of 1.92 mg zinc/liter on young Atlantic salmon. Brungs (1969) found a TL_{50} value of 9.2 mg zinc/liter of fathead minnows, but this is in hard water. In soft water the TL_{50} value would be lower. TL_{50} values for rainbow

trout lie in the 0.4-0.6 mg zinc/liter range in soft water. Thus, aquatic insects are more tolerant of zinc pollution than fish by at least a factor of ten.

Zinc acute bioassay two-concentration four provides a concentration factor for estimating zinc levels in Willow Creek Build-up Test Two. The concentration factor is 1950. This should provide a true test of the concentration factor hypothesis since the acute bioassay was a laboratory test while the build-up test in Willow Creek was a natural situation. This concentration factor (1950) is divided into mean levels of zinc accumulation by the mayfly naiads that were cropped off at 24 hour intervals. The data below compares the estimated levels of zinc in Willow Creek with known 24 hours composite samples for the same time period.

Hours Exposure	Mean Zinc Accumulation	Est. zinc exp. level (mg/liter)	Actual zinc exp. level (mg/liter)
49	1730.5	0.89	1.08
73	2025.2	1.04	1.14
97	1882.9	0.97	1.26
121	2114.2	1.08	1.28
145	2414.0	1.24	1.40
169	2291.5	1.18	1.37
193	2460.5	1.26	1.43
217	2432.0	1.28	1.24
241	2245.2	1.15	1.34
308	3160.1	1.62	1.73
404	3129.6	1.60	1.72

This proves (under the conditions in this study) the concentration factor can be used to estimate levels of zinc exposure. Graphical interpretation of mean zinc accumulation and zinc exposure levels with increasing time further elucidates the correlation between zinc accumulation and zinc concentration in the water (Fig. 2).

Correlation coefficients (r) determined from linear regression analysis of zinc accumulation by the naiads versus zinc exposure levels for zinc acute bioassays one and two are 0.7791 and 0.6940, respectively. The reason for the low correlation coefficients is easily understood if the data are plotted. The accumulation of zinc versus exposure level is a curvilinear relationship, not a linear one. But the accumulation-concentration relationship is predictable.

On the basis of the above evidence, aquatic insects can be used as biological monitors of zinc pollution. All three prerequisites have been satisfied.

Preliminary data collected in 1971 provide additional evidence that aquatic insects can be used as biological monitors of heavy metal pollution. Cast exoskeletons of the stonefly Claassenia sabulosa were collected from rocks near the island in the Rio Grande at Creede, Colorado (refer to Figure 1). These exoskeletons were separated into two groups: (1) those from the north side of the island, and (2) those from the south side of the island.

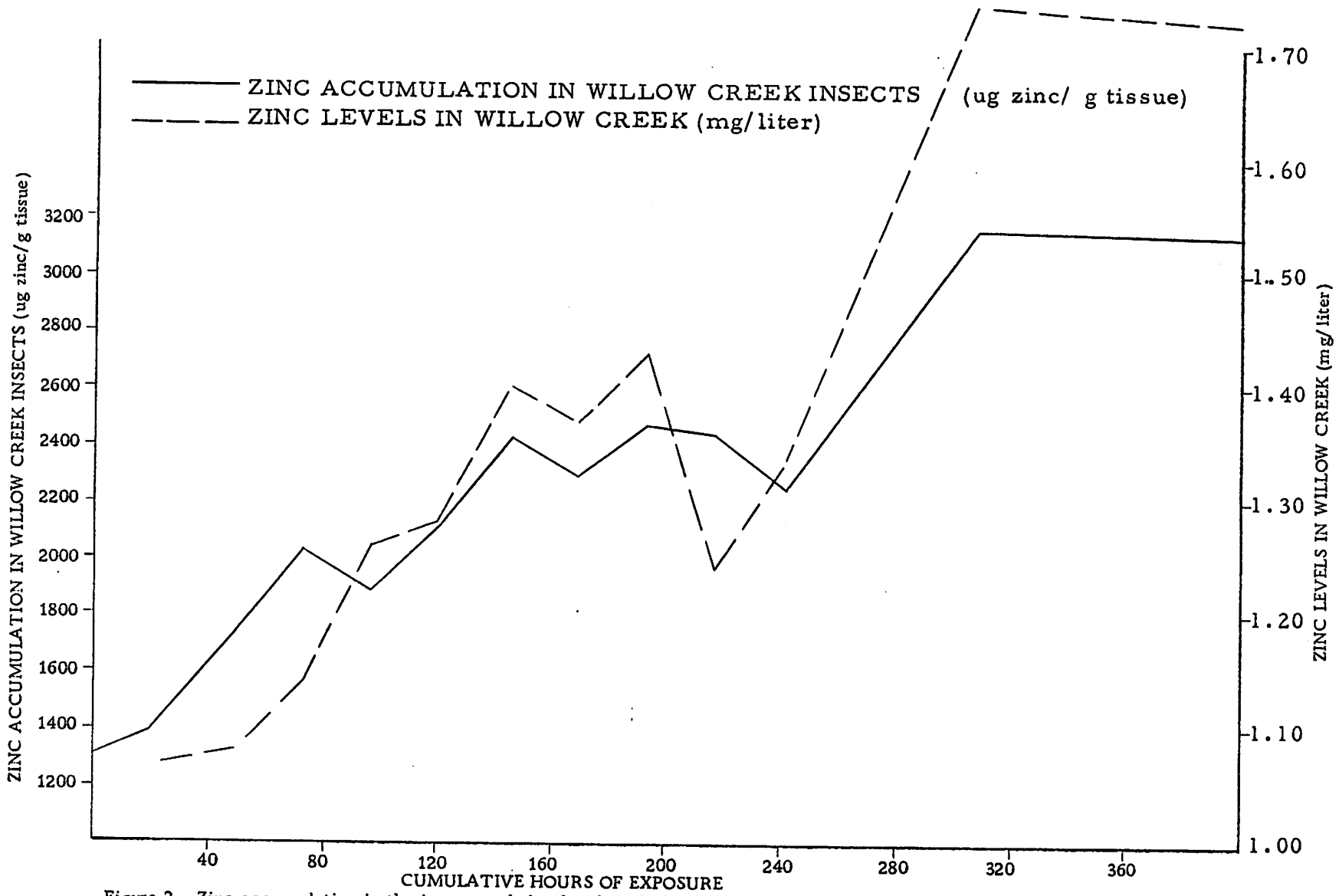

Figure 2. Zinc accumulation in the insects and zinc levels in Willow Creek over time.

The water from Willow Creek does not mix with the Rio Grande until it has passed the island in the river. If this is indeed true, then stoneflies (or cast exoskeletons) collected from the north side of the island should show a significantly higher accumulation of zinc or other heavy metals than stoneflies or cast exoskeletons collected from the south side of the island. Such is the case. Exoskeletons collected from the north side of the island show mean accumulations of zinc, copper, and lead of 1260, 38.78, and 1117 ug metal/ g tissue, respectively. Exoskeletons from the south side of the island show mean accumulations of zinc, copper, and lead of 941.3, 28.07, and 854.0 ug metal/ g tissue, respectively. A t-test analysis of these three pairs of data reveal statistically significant p values of .05, .05, and .10 for zinc, copper, and lead respectively.

Heavy Metal Toxicity to Aquatic Insects

Aquatic insects appeared most susceptible to heavy metals during the molting process. Copper and silver mortalities occurred primarily during the molt of the mayfly naiads. A six-week chronic test on 60 immature stonefly (P. californica) naiads conducted in Willow Creek in 1970 resulted in 30%-40% mortality. All mortalities occurred either just as the molting process began, during the molt, or within 24 hours after the molt. Many mortalities occurred just as the thoracic split appeared. Jensen and Gaufin (1964) found the stonefly naiads P. californica and Acroneuria pacifica most susceptible to organic

insecticides during the molting process. Either the molting process was inhibited or mortality occurred during the molt. Recent molts were easy to determine as it took 12-24 hours for the new exoskeleton to develop normal coloration and harden. Prior to this the exoskeleton was very soft and light colored, often white.

The mode of action of silver and other toxic heavy metals is probably through inhibition of certain metabolic processes. Evidence is accumulating that silver forms mercaptides with protein complexes by inactivating -SH groups which are very important constituents of several enzyme systems. Christensen (1971) found silver inhibited the enzymes GOT (glutamic oxalacetic transaminase) and LDH (lactic dehydrogenase) in the blood of white suckers (Catostomus commersoni). Admittedly, experimental results on white suckers do not necessarily apply to aquatic insects. Nonetheless, biochemical processes are quite similar throughout the animal kingdom and the toxic mode of action of heavy metals may be quite similar in widely divergent groups of animals. For example, silver has been shown to inhibit many enzyme systems in cattle (personal communication---Dr. Duane Anderson, Pharmacology Department, University of Wyoming). Cooper and Jolly (1970) concur with the theory that binding of -SH groups is the mechanism by which silver inhibits enzyme activity.

CONCLUSION

The feasibility of using aquatic insect naiads as monitors of heavy metal pollution has been explored. With the exception of silver, the two species studied fulfill the prerequisites necessary if aquatic insects are to be used effectively as biological monitors of heavy metal pollution in fish-kill investigations. The naiads are more tolerant of heavy metals than fish. They do concentrate heavy metals in some predictable and repeatable manner (concentration factor) over a short period of time. And the naiads do concentrate heavy metal pollutants in relative proportion to the level of occurrence of the metals in the water.

Certain precautions are necessary in using aquatic insects as biological monitors of heavy metal pollution. First, the same species of insect must be used in the pollution monitoring program to obtain reliable data. Concentration factors for a specific metal and specific level of exposure change from one species of insect to another. Base levels of biologically important metals (zinc and copper) also differ between different species. For example, baseline zinc levels in the stonefly P. californica are about 360-370 μg zinc/g dry weight. Baseline zinc levels in the mayfly E. grandis are about 1300 μg/g.

If the species has a two or three year life cycle additional precautions are necessary. Naiads of a similar size and age must be used as immature naiads exhibit higher concentrations factors over shorter

periods of time than mature naiads exposed to similar concentrations. Even disparities in sex ratios could ruin the data. For example, male P. californica naiads concentrate copper more rapidly and to higher levels than female naiads exposed to identical levels of copper for the same period of time.

In general, the larger the body size of the naiad, the more tolerant it is of a given pollutant. This held true in all tests in this study with lead, zinc, copper, and silver. Research on the effects of organic pesticides on many species of aquatic insects supports this generalization (Jensen and Gaufin, 1964a, 1964b; Carlson, 1966; Sanders and Cope, 1968).

Will other species or orders of aquatic insects work as well as E. grandis and P. californica as biological monitors of heavy metal pollution? The answer in all likelihood is yes, perhaps even better. Mayflies and stoneflies are known to aquatic biologists and entomologists as "clean water species". They generally are the least tolerant of excessive siltation, pH depressions from acid mine drainage (Roback and Richardson, 1969), severe organic pollution from domestic sewage (Avery, 1970), and low pH (Bell, 1971). Warnick and Bell (1969) found Trichoptera larvae to be more tolerant of several heavy metals than mayflies of the genus Ephemerella. Stoneflies (Acroneuria sp.) were about as tolerant as the caddisfly (Hydropsyche) when tested

with the same metals. Surber and Thatcher (1963) found caddisflies (Cheumatopsyche) more tolerant of ABS (alkyl benzine sulfonate) than mayfly naiads of the genera Stenonema and Isonychia.

Will the concentration factor hypothesis still be applicable where chronic exposure to the metal existed prior to the slug discharge of a metal causing a fish-kill? Again, the answer is yes. The mayflies and stoneflies used in these tests were collected from the Rio Grande where they were continuously exposed to zinc levels of 0.0-0.6 mg/liter (Goettl, et al, 1971, 1972). Despite chronic exposure to zinc these naiads did accumulate zinc by a predictable concentration factor over a short period of time and in relative proportion to the zinc levels in Willow Creek.

Practical Application of the Concentration Factor Hypothesis

Aquatic insects as biological monitors of heavy metal pollution offer several advantages over the water sample method of investigating heavy metal fish-kills. Collection of water samples needs to be done as the fish-kill occurs. In many cases the water samples should be collected prior to the time when visual evidence of a fish-kill occurs, because of the latent period (often hours or even days) between first exposure to the toxicant and the occurrence of first mortality. By the time lethal effects on fish are evident the slug of toxicant causing the kill may be many miles downstream from the point of the kill and diluted beyond the point of detection. Water samples will often decrease

in metal concentration the longer they are held prior to analysis. This makes immediate analysis of the samples absolutely necessary.

In contrast, aquatic insect monitors of heavy metal pollution offer many advantages. As was stated in the discussion, aquatic insects accumulate heavy metals over time in relative proportion to the occurrence of that metal in the water. Instead of an instantaneous indication of the level of pollutant present (from the water sample analysis) the aquatic insect gives an average indication of the level of pollutant concentration for the total time period in question. Such a value can be easily, readily, and directly related to the known TL_{50} values for the specific fish and metal contaminant under consideration. The aquatic insect also retains the metal accumulation for a considerable period of time. This gives the investigating biologist "an ace in the hole" because he has at least a 24-48 hour grace period in which to get to the site of the kill and collect the aquatic insect naiads and the data would still be valid.

I believe aquatic insects represent an unexploited resource that can be used as biological monitors of heavy metal pollution.

LITERATURE CITED

Adrian, W. J. 1971. A new wet digestion method for biological materials utilizing pressure. Atomic Absorption Newsletter. 10:96.

American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1971. Standard methods for the examination of water and wastewater. 13th ed. Amer. Public Health Ass. Washington, D. C. 874 p.

Avery, E. L. 1970. Effects of domestic sewage on aquatic insects and salmonids of the East Gallatin River, Montana. Water Res. 4:165-177.

Bell, H. L. 1971. Effects of low pH on the survival and emergence of aquatic insects. Water Res. 5:313-319.

Blaedel, W. J., and V. W. Meloche. 1963. Preoxidants, p. 818-820. In W. J. Blaedel and V. W. Meloche, Elementary quantitative analysis, theory and practice. 2nd ed. Harper and Row, Publ., Inc., New York, N. Y.

Brooks, R. R., and M. G. Rumsby. 1965. The biogeochemistry of trace element uptake by some New Zealand bivalves. Limnol. and Oceanogr. 10:521-527.

Brungs, W. A. 1969. Chronic toxicity of zinc to the fathead minnow, Pimephales promelas Rafinesque. Trans. Amer. Fish. Soc. 98:272-279.

Carlson, C. A. 1966. Effects of three organophosphorus insecticides on immature Hexagenia and Hydropsyche of the upper Mississippi River. Trans. Amer. Fish. Soc. 95:1-5.

Christensen, G. M. 1971. Effects of metal cations and other chemicals upon the in vitro activity of two enzymes in the blood plasma of the white sucker, Catostomus commersoni (Lacepede). Chem.-Biol. Interactions, 4:351-361.

Cooper, C. F., and W. C. Jolly. 1970. Ecological effects of silver iodide and other weather modification agents: a review. Water Res. 6:88-98.

Goettl, J. P. and J. R. Sinley. 1970. Water pollution studies. Colo. Game, Fish and Parks Div. Job Prog. Rep. Fed. Aid Proj. No. F-33-R-5. 59 p.

Goettl, J. P., J. R. Sinley, and P. H. Davies. 1971. Water pollution studies. Colo. Game, Fish and Parks Div. Job. Prog. Rep. Fed. Aid Proj. No. F-33-R-6. 130 p.

_____, _____, and_____. 1972. Water pollution studies. Colo. Division of Wildlife. Job Prog. Rep. Fed. Aid Proj. No. F-33-R-7. 47 p.

Hynes, H. B. N. 1970. The ecology of running waters. Univ. of Toronto Press. 555 p.

Jensen, L. D., and A. R. Gaufin. 1964a. Effects of ten organic insecticides on two species of stonefly naiads. Trans. Amer. Fish. Soc. 93:27-34.

_____, and_____. 1964b. Long-term effects of organic insecticides on two species of stonefly naiads. Trans. Amer. Fish. Soc. 93:357-363.

Johnson, B. T., C. R. Saunders, H. O. Sanders, and R. S. Campbell. 1971. Biological magnification and degradation of DDT and aldrin by freshwater invertebrates. J. Fish. Res. Bd. Canada. 28:705-709.

Kawatski, J. A., and J. C. Schmulbach. 1971. Accumulation of insecticide in freshwater ostracods exposed continuously to sublethal concentrations of aldrin or dieldrin. Trans. Amer. Fish. Soc. 100:565-567.

Kormondy, E. J. 1965. Uptake and loss of zinc-65 in the dragonfly Plathemis lydia. Limnol. and Oceanogr. 10:427-433.

McKim, J. M., and D. A. Benoit. 1971. Effects of long-term exposures to copper on survival, growth and reproduction of the brook trout (Salvelinus fontinalis). J. Fish. Res. Bd. Canada. 28:655-662.

Mount, D. I. 1968. Chronic toxicity of copper to fathead minnows (Pimephales promelas, Rafinesque). Water Res. 2:215-223.

_____, and W. A. Brungs. 1967. A simplified dosing apparatus for fish toxicological studies. Water Res. 1:21-29.

_____, and C. E. Stephan. 1969. Chronic toxicity of copper to the fathead minnow (Pimephales promelas) in soft water. J. Fish. Res. Bd. Canada. 26:2449-2457.

Roback, S. S., and J. W. Richardson. 1969. The effects of acid mine drainage of aquatic insects. Proc. Acad. Nat. Sci. Philadelphia. 121:81-107.

Sanders, H. O., and O. B. Cope. 1968. The relative toxicities of several pesticides to naiads of three species of stoneflies. Limnol. and Oceanogr. 13:112-117.

Sprague, J. B. 1964. Lethal concentrations of copper and zinc for young Atlantic salmon. J. Fish. Res. Bd. Canada. 21:17-26.

_____, 1969. Measurement of pollutant toxicity to fish I. Bioassay methods for acute toxicity. Water Res. 3:793-821.

Surber, E. W., and T. O. Thatcher. 1963. Laboratory studies on the effects of alkyl benzine sulfonate (ABS) on aquatic invertebrates. Trans. Amer. Fish. Soc. 92:152-160.

Tanner, H. A. 1963. Fish-kill in the Rio Grande River below Creede, Colorado, August 2-3, 1963. Report to the Colo. Game, Fish and Parks Comm. 5 p.

Warnick, S. L., and H. L. Bell. 1969. The acute toxicity of some heavy metals to different species of aquatic insects. J. Water Pollution Control Federation. 41(2, part 1):280-284.

Weast, R. C. ed. 1966. Handbook of chemistry and physics. Chemical Rubber Co., Cleveland, Ohio. 1838 p.

Wilkes, F. G., and C. M. Weiss. 1971. The accumulation of DDT by the dragonfly nymph, Tetragoneuria. Trans. Amer. Fish. Soc. 100:222-236.

APPENDICES

APPENDIX A

This appendix lists the average water quality data for all acute bioassays. All parameters were measured according to Standard Methods (1971). Alkalinity is given as mg/liter $CaCO_3$. Hardness is given as mg/liter $CaCO_3$.

Average Water Quality Data for All Acute Bioassays

Test No.	Alkalinity (mg/liter)	Hardness (mg/liter)	pH	Dissolved Oxygen (mg/liter)	Temp. (C)	Conductivity (μmhos/cm)
Copper #1	34	31	6.93	12.2	3.7	197
Copper #2	30	30	6.62	9.5	8.6	198
Copper #3	29	30	6.38	9.6	8.0	207
Copper #4*	63	66	6.83	7.3	7.4	340
Copper #5*	64	69	7.03	7.7	7.4	324
Lead #1	33	32	7.02	12.0	3.5	186
Lead #2*	63	65	7.15	7.5	7.4	329
Silver #1	33	32	7.08	11.6	5.5	132
Silver #2	34	34	7.01	11.7	5.1	141
Silver #3	31	30	7.00	11.8	4.8	141
Silver #4	32	31	7.16	12.0	3.7	163
Silver #5*	63	62	7.07	7.4	7.3	325
Silver #6*	63	65	7.20	7.6	7.3	324
Zinc #1	34	31	7.01	11.9	4.0	199
Zinc #2*	60	61	7.21	7.3	7.3	336

NOTE: Starred (*) tests indicate test run on Ephemerella grandis at Creede. Tests without stars were run on Pteronarcys californica at Fort Collins.

APPENDIX B

Appendix B lists specific information on the mean levels of exposure to the metal(s) and mean levels of accumulation of the metal by the naiads. The heading gives the test description, species used in the test, and length of exposure time in hours. "0.00" values indicate levels beyond the detection limits of normal atomic absorption analysis procedures.

	Metal in Water (mg/liter)			Metal Accumulation in Insect (μg/g)		
Conc. No.	$\bar{X}$	N	Range	$\bar{X}$	N	Range
Copper Acute Bioassay One - Pteronarcys californica - 264 Hours						
1	13.9	12	11.4-16.4	771.6	10	274.4-1498
2	5.64	12	4.60-7.40	1106	10	808.3-1409
3	2.92	12	1.86-4.00	472.5	10	300.7-650.7
4	1.70	12	1.14-1.98	274.9	10	218.0-369.2
5	0.74	12	0.50-0.90	156.8	10	128.5-188.1
C	0.00	12	0.00	92.7	10	62.69-111.0
Copper Acute Bioassay Two - Pteronarcys californica-64 Hours						
1	18.5	3	16.5-21.5	1163	10	793.6-1455
2	10.1	3	9.80-10.5	286.4	10	194.4-412.1
3	8.53	3	7.80-9.00	233.0	10	170.6-343.2
4	7.22	3	6.95-7.35	214.6	10	150.9-355.1
5	5.51	3	5.28-5.97	180.2	10	142.2-227.3
C	0.00	3	0.00	92.7	10	62.79-111.0

Conc. No.	Metal in Water (mg/liter) $\bar{X}$	N	Range	Metal Accumulation in Insect (µg/g) $\bar{X}$	N	Range
Copper Acute Bioassay Three - Pteronarcys californica - 195 Hours						
1	12.2	9	9.78-14.8	2540	9	921.5-3219
2	10.4	9	8.00-14.0	2096	9	1576 -2708
3	8.13	9	5.90-9.30	1767	9	1143 -3218
4	6.47	9	5.52-7.15	1199	9	947.2-1764
C	0.00	9	0.00	122.3	3	81.55-164.9
Copper Acute Bioassay Four - Ephemerella grandis - 178 hours						
1	10.0	7	8.00-10.5	9125	23	2828 -14540
2	4.82	7	3.80-5.38	5787	26	4007 -7625
3	2.51	7	1.80-3.00	3882	27	916.3-5685
4	1.22	7	0.80-2.00	1933	29	1200. -13020
5	0.63	7	0.40-1.00	1240	26	756.6-1830
C	0.00	7	0.00	94.70	16	82.23-120.3
Copper Acute Bioassay Five - Ephemerella grandis - 217 hours						
1	1.06	8	0.93-1.18	1750	28	883.8-2274
2	0.52	8	0.46-0.60	1183	27	909.6-1483
3	0.29	8	0.18-0.35	813.7	24	604.3-1181
4	0.15	8	0.11-0.20	616.3	26	370.1-1643
5	0.08	8	0.06-0.10	429.5	25	203.4-795.3
C	0.00	8	0.00	82.29	25	51.28-117.5

Conc. No.	Metal in Water (mg/liter) $\bar{X}$	N	Range	Metal Accumulation in Insect (µg/g) $\bar{X}$	N	Range
Lead Acute Bioassay One - Pteronarcys californica - 264 Hours						
1	19.2	12	14.0 -23.8	8172	10	6635 -10500
2	7.44	12	6.15-8.35	2249	10	1650 -3634
3	4.43	12	3.83-4.84	1666	10	1147 -1773
4	1.96	12	0.75-4.14	736.6	10	304.5-1805
5	1.08	12	0.65-1.40	716.7	10	462.8-1162
C	0.00	12	0.00	*8.18	10	3.03-18.61

NOTE: (*) starred mean lead due to external contamination of the holding tank from lead base paint chips introduced into holding tank.

Lead Acute Bioassay Two - Ephemerella grandis - 286 Hours						
1	9.24	8	7.73-10.01	104,700	12	56,200 -162,900
2	4.90	8	4.00-5.73	73,200	14	19,890 -111,900
3	2.34	8	2.00-3.15	31,780	10	23,350 -50,110
4	1.32	8	1.00-1.68	14,560	11	11,070 -30840
5	0.69	8	0.50-1.00	5702	9	4217 -6877
C	0.00	8	0.001	*126.6	10	87.91-1077

NOTE: (*) starred mean lead due to external contamination of the holding tank from lead base paint chips introduced into holding tank.

Silver Acute Bioassay One - Pteronarcys californica - 143 Hours						
1	0.738	8	0.615-0.980	53.28	9	29.51-117.7
2	0.399	8	0.365-0.470	30.76	9	16.75-41.67
3	0.217	8	0.185-0.242	22.95	2	18.28-27.62
4	0.105	8	0.100-0.115	13.62	9	5.52-24.12
5	0.050	8	0.045-0.050	9.13	9	4.53-12.41
C	0.00	8	0.00	3.97	2	3.37-4.56

Conc. No.	Metal in Water (mg/liter) $\bar{X}$	N	Range	Metal Accumulation in Insect (µg/g) $\bar{X}$	N	Range
Silver Acute Bioassay Two - Claassenia sabulosa - 73 Hours						
1	0.900	1	--	58.77	13	18.20-162.9
2	0.450	1	--	15.29	14	3.34-26.72
3	0.210	1	--	11.62	13	5.46-19.67
4	0.100	1	--	10.36	12	5.08-18.25
5	0.050	1	--	8.88	14	3.69-19.01
C	0.00	1	--	0.00	11	0.00
Silver Acute Bioassay Three - Pteronarcys californica - 363 Hours						
1	0.104	11	0.074-0.120	11.32	10	7.41-14.43
2	0.043	11	0.030-0.052	11.29	10	7.16-14.49
3	0.022	11	0.016-0.026	9.18	10	3.43-14.52
4	0.012	11	0.008-0.015	7.93	10	1.91-13.21
5	0.006	11	0.004-0.008	7.30	10	4.00-11.62
C	0.00	11	0.00	0.00	10	0.00
Silver Acute Bioassay Four - Pteronarcys californica - 166 Hours						
1	0.067	8	0.052-0.079	20.84	10	8.64-36.96
2	0.028	8	0.024-0.036	16.52	10	9.53-22.93
3	0.015	8	0.013-0.019	14.71	10	6.21-23.26
4	0.008	8	0.007- .012	10.53	10	8.41-16.88
5	0.004	8	0.00 -0.005	7.17	10	4.97-11.97
C	0.00	8	0.00	0.00	10	0.00

Conc. No.	Metal in Water (mg/liter) $\bar{X}$	N	Range	Metal Accumulation in Insect (µg/g) $\bar{X}$	N	Range
Silver Acute Bioassay Five - Ephemerella grandis - 161 Hours						
1	0.75	6	0.63-0.84	65.31	8	33.89-140.0
2	0.40	6	0.31-0.44	36.65	9	3.63-54.49
3	0.23	6	0.19-0.32	47.97	8	8.64-88.75
4	0.12	6	0.10-0.17	28.73	12	0.00-63.95
5	0.06	6	0.05-0.08	25.32	12	0.00-155.1
C	0.00	6	0.00	0.00	5	0.00
Silver Acute Bioassay Six - Ephemerella grandis - 241 Hours						
1	0.15	4	0.14-0.16	66.89	8	16.78-147.0
2	0.07	4	0.06-0.08	48.74	12	0.00-143.2
3	0.04	8	0.03-0.05	60.54	12	32.92-132.9
4	0.02	9	0.01-0.03	22.98	9	0.00-61.88
5	0.01	9	0.00-0.01	53.87	8	0.00-116.1
C	0.00	9	0.00	0.00	8	0.00
Zinc Acute Bioassay One - Pteronarcys californica - 342 Hours						
1	13.6	16	11.4 -15.0	561.2	10	330.3 -777.4
2	5.54	16	4.70-6.20	497.1	10	352.0 -722.3
3	2.83	16	2.16-3.58	415.7	10	278.8 -506.2
4	1.61	16	1.33-2.11	507.7	10	378.4 -630.0
5	0.77	16	0.65-1.12	439.4	10	335.8 -612.0
C	0.00	16	0.00	357.2	10	287.5 -450.7

Conc. No.	Metal in Water (mg/liter) $\bar{X}$	N	Range	Metal Accumulation in Insect (µg/g) $\bar{X}$	N	Range
Zinc Acute Bioassay Two - Ephemerella grandis - 253 Hours						
1	9.20	11	8.88-9.72	2361	5	2164 -2480
2	4.32	11	4.00-4.75	2381	5	2270 -2536
3	2.29	11	1.93-2.57	2187	5	2001 -2375
4	1.04	11	0.85-1.15	2029	5	1951 -2157
5	0.60	11	0.50-0.68	1794	5	1673 -1938
C	0.00	11	0.00	1116	5	1040 -1227
Willow Creek Chronic Bioassay (Zinc) - Pteronarcys californica - 50 Days						
1	1.13	100	0.67-2.20	2009	12	1491 -2699
2	0.58	100	0.37-0.96	1361	11	609.1-2000
3	0.32	100	0.14-0.65	980.9	15	435.1-1483
4	0.20	100	0.05-0.63	1353	15	511.7-3005
C	0.00	100	0.00	415.1	15	258.2-471.2
Willow Creek Chronic Bioassay (Zinc) - Ephemerella grandis - 50 Days						
1	1.13	100	0.67-2.20	--	0	--
2	0.58	100	0.37-0.96	3975	10	1997 -5172
3	0.32	100	0.14-0.65	3739	10	2394 -5420
4	0.20	100	0.05-0.63	3398	10	2690 -4684
C	0.00	100	0.00	1653	10	1474 -2429

APPENDIX C

Appendix C deals with data on metal accumulation with increasing exposure time. However, several tests deal both with metal accumulation vs. increasing exposure time and metal accumulation vs. mean levels of exposure to the metal. The concentration column gives average level of exposure for a 24 hour period. Hours exposure column lists the cumulative exposure time. "0.00" values indicate levels beyond the detection limits of normal atomic absorption analysis procedures.

Conc. (mg/liter)	Hours Exposure	$\bar{X}$ (μg/g)	N	Range (μg/g)
Willow Creek Build-up Test One (Zinc) - Pteronarcys californica				
1.49	12	588.2	10	401.6-931.5
1.58	24	610.3	10	459.5-871.2
1.55	48	776.4	10	604.4-941.7
1.55	96	1159	10	603.1-2232
Willow Creek Build-up Test One (Zinc) - Ephemerella grandis				
1.49	12	1751	55	1262 -1925
1.58	24	1916	51	1851 -1967
1.55	48	2455	49	2356 -2741
1.55	96	2859	64	2351 -3382

Conc. (mg/liter)	Hours Exposure	$\bar{X}$ (µg/g)	N	Range (µg/g)
Willow Creek Build-up Test Two (Zinc) - Ephemerella grandis				
1.07	25	1393	17	1215 -1713
1.08	49	1731	11	1448 -2476
1.14	73	2025	11	1511 -3333
1.26	97	1883	15	1514 -2583
1.28	121	2114	10	1343 -2983
1.40	145	2414	10	1808 -4072
1.37	169	2292	10	1554 -2896
1.43	193	2461	13	1871 -3957
1.24	217	2432	15	1930 -4763
1.34	241	2245	16	1175 -3024
1.73	308	3160	12	2234 -6579
1.72	404	3130	20	2263 -5296
Willow Creek Build-up Test Three (Zinc) - Pteronarcys californica				
1.07	13	598.6	10	385.4-853.3
--	25	412.2	7	303.7-527.1
1.41	49	819.0	10	398.4-1447
1.14	73	940.0	10	421.3-3013
1.26	96	1424	5	480.7-2221
Willow Creek Build-up Test Four (Zinc) - Pteronarcys californica				
1.34	24	632.1	10	254.8-1293
1.07	48	699.1	11	430.0-1123
1.47	72	--	--	--
1.16	96	1309	11	407.4-2277
1.24	120	--	--	--
1.77	144	--	--	--

Willow Creek Build-up Test Four (Zinc) - Pteronarcys californica cont.

Conc. (mg/liter)	Hours Exposure	$\bar{X}$ (µg/g)	N	Range (µg/g)
--	168	--	--	--
--	192	1035	8	539.2-1630
1.57	216	--	--	--
1.80	240	--	--	--
--	264	--	--	--
--	288	1406	10	625.0-2305
1.21	213	--	--	--
--	400	2038	14	118.4-3761
	Copper Build-up Over Time - Pteronarcys californica			
5.5	0	50,80	10	36.92-64.52
5.5	64	180.2	10	142.2-227.3
5.5	264	1106	10	808.3-1409
	Silver Build-up Over Time - Pteronarcys californica			
0.738	58	33.15	2	29.51-36.79
0.738	70	37.41	2	33.83-40.99
0.738	93	44.88	1	44.88
0.738	109	76.77	3	44.20-117.7
0.738	143	107.4	1	107.4
	Silver Build-up Over Time - Claassenia sabulosa			
0.90	30	22.11	2	18.20-26.01
0.90	47	48.84	2	44.55-53.13
0.90	56	40.32	3	23.75-67.88
0.90	72	91.86	3	84.26-105.9

APPENDIX D

Appendix D lists the linear regression equation by the least squares method, the standard error of y and the correlation coefficient (r) for each acute bioassay, all Willow Creek build-up bioassays and the regression of metal accumulation over exposure time. The regression equation is in the standard form $y_i = a + bx_i \pm e_i$. In the regression equation "a" is the point where the regression line crosses the y axis, "b" is the slope of the regression line, and "e_i" is the standard error of y_i.

Test Description	Regression Equation $y_i = a + bx_i$	$\pm e_i$	Correlation Coefficient (r)
Copper Acute Bioassay One	$y = 19.025 + 182.51x_i$	± 366.11	0.986
Copper Acute Bioassay Two	$y = -127.9 + 58.91x_i$	± 363.24	0.901
Copper Acute Bioassay Three	$y = 75.40 + 196.90x_i$	± 834.99	0.994
Copper Acute Bioassay Four	$y = 872.7 + 877.26x_i$	± 3054.6	0.982
Copper Acute Bioassay Five	$y = 300.8 + 1461.0x_i$	± 537.63	0.974
Lead Acute Bioassay One	$y = -111.25 + 416.5x_i$	± 2740.8	0.991
Lead Acute Bioassay Two	$y = 1696.0 + 11893x_i$	± 38200	0.985
Silver Acute Bioassay One	$y = 6.055 + 64.535x_i$	± 16.415	0.996
Silver Acute Bioassay Two	$y = 1.087 + 57.834x_i$	± 18.958	0.950
Silver Acute Bioassay Three	$y = 7.954 + 38.783x_i$	± 1.668	0.830
Silver Acute Bioassay Four	$y = 9.383 + 187.34x_i$	± 4.737	0.909
Silver Acute Bioassay Five	$y = 24.48 + 52.253x_i$	± 14.523	0.893
Silver Acute Bioassay Six	$y = 38.563 + 203.18x_i$	± 15.107	0.666

Appendix D cont.

Test Description	Regression Equation $y_i = a + bx_i$	$\pm e_i$	Correlation Coefficient (r)
Zinc Acute Bioassay One	$y = 417.26 + 11.289x_i$	±66.82	0.779
Zinc Acute Bioassay Two ...	$y = 1699.0 + 95.96x_i$	±434.5	0.694
Willow Creek Chronic Bioassay			
Pteronarcys californica	$y = 686.09 + 1202.2x_i$	±522.53	0.897
Ephemerella grandis	$y = 2142.4 + 3793.0x_i$	±911.22	0.878
Willow Creek Build-up Test One			
Pteronarcys californica (96hr)	$y = 465.97 + 7.055x_i$	±228.67	*0.993
Ephemerella grandis (96 hrs). .	$y = 1645.9 + 13.321x_i$	±439.63	*0.975
Willow Creek Build-up Test Two			
Ephemerella grandis (97 hrs). .	$y = 1238.8 + 8.282x_i$	±281.3	*0.905
Ephemerella grandis (404 hrs). .	$y = 1472.1 + 4.590x_i$	±540.73	*0.936
Ephemerella grandis	$y = 828.83 + 2311.5x_i$	±508.23	0.937
Willow Creek Build-up Test Three			
Pteronarcys californica (96hr).	$y = 301.10 + 10.461x_i$	±344.34	*0.934
Pteronarcys californica	$y = 73.847 + 714.4x_i$	±302.27	0.305
Willow Creek Build-up Test Four			
Pteronarcys californica (96hrs).	$y = 327.13 + 9.873x_i$	±304.51	*0.970
Pteronarcys californica (404 hr).	$y = 626.02 + 3.210x_i$	±475.7	*0.910
Pteronarcys californica	$y = 123.35 + 569.47x_i$	±311.93	0.321
Copper Build-up Over Time			
Pteronarcys californica	$y = -7.784 + 4.146x_i$	±469.67	*0.993
Silver Build-up Over Time			
Pteronarcys californica	$y = -26.278 + 0.9112x_i$	±28.241	*0.968
Claassenia sabulosa	$y = -28.096 + 1.5391x_i$	±25.606	*0.913

NOTE (*) indicates regression of metal accumulation over exposure time.