State: Colorado

Study No. F243R-11

Title: Water Pollution Studies

Period Covered: July 1, 2004 to June 30, 2005

Project Objective: To develop quantitative chemical and toxicological data on the toxicity of pollutants to aquatic life, investigate water pollution problems in the field, and provide expertise in aquatic chemistry and aquatic toxicology.

STUDY PLAN A: TOXICITY STUDIES

Job A.1. Feminization of Fish by Wastewater Treatment Plant Effluents

Job Objective:

Determine whether feminization of rainbow trout and/or fathead minnows occurs following exposure to wastewater treatment plant effluents and/or receiving waters. If found, tests will be conducted to measure the relative magnitude of feminization. Attempts will be made to identify possible compounds contributing to estrogenic activity and estimates made on the contribution of each compound. Feminized fathead minnows will be raised to sexual maturity and spawned to determine reproductive effects of exposure to estrogenic compounds.

Job A.2. Toxicity of Metals to Fish

Job Objective:

Measure acute (96 hour) and chronic (60 day) effects of zinc, copper and/or cadmium exposure on hatching, survival and growth of different life stages of mottled sculpin, longnose dace and/or other sensitive species. Results from these experiments will compare toxicity thresholds to USEPA metal criteria to ensure that these species are protected.

Job A.3. Effects of Dietary Exposure of Metals to Fish

Job Objective:

Measure the effect of zinc, copper, cadmium and/or selenium from dietary sources on survival and growth of fish in the laboratory. Evaluate the sensitivity of dietary-exposed organisms to waterborne exposure. Relate dietary levels that cause diminished performance in the laboratory with levels found in dietary sources in metal impacted areas such as the upper Arkansas River, Clear Creek and the Eagle River.

Job A.4. Toxicity of Unionized Ammonia to Fish at Cold Water Temperatures

Job Objective:

Determine effects of temperature on toxicity of unionized ammonia to rainbow trout and fathead minnows or other warmwater species at optimal and very cold (less than 5ºC) water temperatures.

Accomplishments

Job A.1.

Exposure of fathead minnows to estrogen and effluent from Ft. Collins wastewater treatment plant was completed during this segment. Surviving organisms have been submitted for histological examination to detect intersex structures in the gonads. Whole-body homogenates are currently maintained at -80ºC for analysis of vitellogenin content.

The project is also providing equipment and support for an onsite bioassay to be conducted by the University of Colorado. The objective of the study is to detect and quantify estrogenic activity in the city of Boulder wastewater treatment plant effluent.

Job A.2.

A manuscript on the toxicity of zinc to mottled sculpin was accepted by the Journal of Environmental Toxicology and Chemistry. Adult mottled sculpin are currently being maintained at the Colorado Division of Wildlife Research Hatchery and the Native Aquatic Restoration Facility where attempts to induce spawning are ongoing.

Job A.3.

No activities during this segment.

Job A.3.

Controllers were designed and installed to control pH of laboratory water on a flow-through basis. A chronic early life stage ammonia toxicity test was initiated using Colorado River strain of rainbow trout. Freshly fertilized eggs were exposed to low levels of ammonia to determine whether recent ammonia criteria developed by the U.S. Environmental Protection agency adequately protect rainbow trout (USEPA 1999). The toxicity test will continue until June 2005. Results of the test will be reported next segment.

STUDY PLAN B: TECHNICAL ASSISTANCE

Job B.1. Development of a Field Test for Rotenone

Job Objective:

To develop a test for rotenone that can measure subpiscidal concentrations in water, can be completed in an hour, and can be used in the field.

Job B.2. Water Quality Assistance to Division of Wildlife Personnel and Other State and Federal Agencies

Job Objective:

To provide expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to Division of Wildlife and other state and federal personnel as requested. Conduct short or long term experiments to produce toxicity data when such data in the literature are lacking or inadequate.

Job B.3. Regulatory and Legal Assistance

Job Objective:

To provide technical assistance to legal and regulatory agencies toward the development, implementation, and enforcement of water quality standards needed to protect or enhance the aquatic resources of Colorado.

Accomplishments

Job B.1.

No activities during this segment

Job B.2.

Acute toxicity tests were conducted on late instar mayflies. The objectives of the tests were to evaluate the protectiveness of USEPA criteria to these taxa and to generate data for use in developing site-specific water quality standards for protection of aquatic organisms. The preliminary results of the tests are reported below.

A study to investigate the effect of incubation temperature on toxicity of zinc to brown trout was initiated. Results from a test conducted in the spring of 2004 indicated that brown trout reared at low temperature exhibited greater tolerance to zinc than trout incubated at 12ºC. To test these observations, brown trout eggs were incubated at 12, 9, 6 3 degrees Celsius. The toxicity of zinc to trout from each of these temperature regimes will be tested using standard acute toxicity testing methodology. A trend of decreasing toxicity with decreasing incubation temperature would support the observations made in 2004. Such a trend, if found, could have cause a reevaluation of toxicity data from fall spawning salmonids such as brown and brook trout. The tests are ongoing and the results will be reported next segment.

A study on the toxicity of zinc and cadmium mixture is being conducted in cooperation with EPA. The objective of the study is to evaluate site-specific water quality characteristics that could influence toxicity of metals to brown trout. An onsite bioassay will be conducted to measure toxicity of California Gulch effluent diluted with upstream Arkansas River water. The results of the onsite study will be compared to a contemporaneous laboratory study using similar test organisms. Differences between the two tests will be attributed to site-specific water quality characteristics in the Arkansas River. The tests will be completed in June 2005 and results reported next segment.

A toxicity tests was initiated to evaluate potential differences in the sensitivity of different strains of rainbow trout to zinc. Rainbow trout eggs from a hatchery strain and eggs from a wild strain (Colorado River) will be raised to 30 days post swimup fry. A zinc toxicity tests will be conducted on both strains and lethal endpoints compared. The test will be conducted near the end of June 2005. The results of the test will be reported next segment.

Job B.3.

Activities under this job include reviewing and commenting on proposals and testimony for aluminum, cadmium, zinc and ammonia standards. Site-specific water quality standards for Mosquito Creek, West Fork of Clear Creek, and South Platte River were also reviewed. A comparison of existing water quality to several possible water quality standards for multiple segments of the Upper Arkansas River was prepared for Vicky Peters of the AGO.

Acute toxicity of dissolved Zn, Cd and Cu to the mayfly *Rhithrogena hageni* (Heptageniidae) in an artificial environment

Abstract

Acute toxicity of dissolved zinc, cadmium and copper to mature nymphs of the mayfly *Rhithrogena hageni* was studied in soft water (hardness < 50 mg/L) with circumneutral pH (7.7). Static-renewal tests were designed to meet ASTM guidelines with some minor modifications. Toxicity endpoints included mortality and moulting. Test duration varied by metal, but all tests ran for at least 96 hours.

Dissolved zinc (up to 16 mg/L) was not acutely toxic to *R. hageni*. Moult frequency showed no relationship to zinc concentration. Acute cadmium toxicity (60% mortality) was observed after 96 hours of exposure to 6.3 mg/L. The estimated median lethal concentration (LC50) did not change after extending the exposure duration to 168 hours. Moult frequency showed no relationship to cadmium concentration. Acute copper toxicity (90%) was observed after 96 hours of exposure to 1.0 mg/L. After 168 hours, acute toxicity was observed in all units where the observed copper concentration was ≥165 µg/L. Total number of exuvia was weakly correlated ($r = -0.64$) to dissolved copper concentration.

R. hageni were expected to be much more sensitive to short-term exposure to elevated concentrations of dissolved zinc and cadmium. Zinc pollution has been implicated as the cause for the decline of this species in contaminated reaches of the Arkansas River in Lake County, Colorado (Clements and Kiffney 1994, Roline 1988, Roline and Boehmke 1981). Though our test results should not be extrapolated to the field, they are clearly inconsistent with field observations (Nelson and Roline 1993) and microcosm experiments (Clements 2004, Clements et al 2002, Courtney and Clements 2002) which demonstrate deleterious effects of metals pollution to populations of this species. The present experimental results demonstrate the feasibility of, and neeed for, further study of the factors that modify dissolved metal toxicity risk to aquatic life in Rocky Mountain streams.

Introduction

Mayflies are a significant component of aquatic ecosystems. The three most important roles of mayflies are: top-down regulation of primary production, daily food resource for trout, and indicators of trace metal contamination. It has been demonstrated that benthic algae proliferate rapidly where grazing literature concerning the use of aquatic insects in biological monitoring studies suggests that mayflies (Ephemeroptera) are generally intolerant of trace metal contamination. Case studies of contaminated rivers in the Rocky Mountain region indicate that most mayfly populations experience reduction in abundance or even local extinction downstream of point-source inputs (Cain et al 1992, Clements and Newman 2002, Winner et al 1980). However, it is commonly observed that total community abundance remains unchanged or even increases at contaminated sites (Clements and Newman 2002). The inferred cause of this phenomenon is temporal, sporadic, acutely toxic levels of trace metals in the stream which selects against grazing ephemeropterans. The reduction of this functional group provides an ecological release for metal-tolerant taxa such as net-spinning trichoptera and orthoclad chironomids (Clements and Rees 1997). Thus, while the total abundance of insect larvae does not change, the taxonomic composition of the benthic community shifts from one dominated by algal grazers to another dominated by filter-feeders and collector-gatherers.

The effect of this benthic community shift on the next highest trophic level (insectivorous fishes) has been studied, but general conclusions are lacking. *In situ* studies of lotic salmonids have focused on diet, bioenergetics and/or foraging behavior. A brief review of this research indicates that, while feeding behaviors vary widely across species, life cycle stages, seasons and locations, drifting aquatic insect larvae are important for satisfying daily energetic needs. It has been documented that during emergence events, salmonid fishes exclusively select emerging adult insects as prey items. These seasonal events are clearly important at the individual level, but population-level consequences (growth, reproduction and recruitment) are unknown. We speculate that severe reduction and/or elimination of metal-sensitive aquatic insects must have negative consequences for fish populations which depend upon seasonal subsidies. Effects at higher levels of biological organization are detectable at broader scales of temporal observation, but are typically confounded by external factors such as weather, climate, and/or anthropogenic disturbance.

Materials and Methods

Test Organism

Mayfly nymphs (*Rhithrogena hageni*) were collected by hand from cobble riffles in mountain streams between February and May of 2005. Nymphs were transported in cooled plastic containers filled with cobble substrate from the collection site. Oxygen and water flow were supplied with an air pump. Holding temperature was kept near ambient conditions with ice. Nymphs were gently transferred with paint brushes to aerated, glass holding tanks and held at 4ºC for a 72-hour acclimatization period. Nymphs were provided with cobble substrate collected from their native stream. After the acclimatization period, the incubator temperature was gradually increased (1ºC per day) to reach test temperature (10ºC). Water was renewed daily (50%) with toxicity test dilution water to slowly acclimate organisms to test water quality. All test subjects were presumed to be *R. hageni* in the field. Nymphs were preserved in 70% ethanol either upon mortality or at termination of the test. The identity of each preserved nymph was verified by two independent taxonomic experts.

General Study Design

Mayflies were exposed to dissolved metals in a 1.25-L, static volume unit constructed from ½-inch polyvinyl chloride (pvc) piping and Rubbermaid (high-density polyethylene) containers. An air-lift system provided flow and dissolved oxygen. Temperature was maintained at the target range (10-12ºC) by a water bath. Exposure water consisted of a mixture of well water and reverse osmosis water to achieve a hardness of around 45 mg $CaCO_3$/L. Exposure water was replaced (90%) every 48 hours. Ten mayfly nymphs were randomly assigned to each experimental unit. Water quality characteristics (dissolved metal, pH, temperature, hardness, alkalinity, conductivity and dissolved oxygen) were measured before and after replacement. Two small, inert, ceramic tiles were placed in each unit for substrate. Nymphs were not fed during the experiments. Mortality and moult events were observed every 24 hours. Corpses and moulted exoskeletons (exuvia) were preserved for identification in 70% isopropyl alcohol. Specific modifications were made in each study; experimental methods follow in chronological order.

Zinc Experiment

Ten mayflies were randomly assigned to each of twelve experimental units. Experimental treatments were assigned in two blocks to control for spatial autocorrelation (small temperature gradient). Nymphs were exposed to one of six nominal levels of dissolved zinc (0, 1, 2, 4, 8 and 16 mg/L). The zinc exposure medium was prepared using zinc sulfate heptahydrate. The exposure medium was replaced (90%) every 48 hours.

Cadmium Experiment

Ten mayflies were randomly assigned to each of nine experimental units. The cadmium exposure medium was prepared from a stock solution made with cadmium chloride and de-ionized water. Nine nominal cadmium levels (0.0, 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5.0 and 10.0 mg/L) were prepared and added to the experimental units. The exposure medium was not replaced during the experiment.

Copper Experiment

Five copper levels (plus a control) were chosen and each level was replicated once. Nominal dissolved copper concentrations were: (0, 125, 250, 500, 1000, 2000 μg/L). Mortalities and exuviae (moulted exoskeletons) were counted, removed and preserved every 24 hours. The exposure medium was replaced (90%) every 48 hours.

Metals Analysis

Zinc, cadmium and copper concentrations in test water samples were determined by flame-atomic absorption spectrometry. Water samples were filtered through a 0.4 µm filter and preserved with 70% nitric acid. The spectrometer was calibrated prior to each use. The calibration was verified with laboratory blanks and NIST traceable QAQC standard from an outside source. The analytical method was validated by analyzing samples spiked with the upper level (2000 µg/L) calibration standard. Some water samples required dilution (1:10) with acidified, de-ionized water.

Results

Zinc Experiment

Physical and chemical conditions varied minimally across the 12 experimental units (Table 1). Measured Zn concentrations were within 6 percent of nominal levels. However, [Zn] measured at the end of the exposure period (168 hrs) were noticeably less (11% on average) than initial [Zn] (Table 2). In the control units, [Zn] increased slightly (18 µg/L) over the duration of the test. *Rhithrogena* nymphs showed no acute toxic response after 96 or 168 hours of exposure to dissolved zinc. Moult frequency (number of exuvia / number of survivors at time t) was varied from 0 to 16 percent across all units. This variation could not be explained by grouping units according to zinc level (Table 3).

Cadmium Experiment

Physical and chemical conditions were similar across the 9 experimental units (Table 4). Cadmium concentrations measured at 96 hours were substantially lower (32% on average) than nominal levels (Table 5). Acute toxicity (60% mortality) was observed in the highest cadmium unit (6.3 mg/L) after 96 hours (Figure 1). The test was extended to 144 hours and mortality in the 6.3 mg/L unit increased to 80% (Figure 2). Additional mortality was observed in the 1.8 and 0.8 mg/L units (30% and 10%, respectively). Number of exuvia did not co-vary with cadmium concentration (Table 5).

Copper Experiment

Conductivity, temperature and pH were similar across all 12 experimental units (Table 6). Hardness measurements are not reported for units other than the control due to interference observed during colorimetric hardness determinations. Copper concentrations measured at 0 hrs were within 3 percent of nominal levels (Table 7). However, dissolved copper was much lower (by as much as 48%) than expected after 96 hours. Acute toxicity (60% mortality) was observed in each copper level greater than (or equal to) 165 µg/L after 96 hours (Figure 3). At 168 hours, only two units had 100% survival (Figure 4). The estimated 168 hr LC50 lies between 50 and 200 µg Cu/L. Total exuvia generally decreased with increasing copper concentration, but the trend was not consistent at intermediate copper levels.

Discussion

Exposure Apparatus

Survival in control exposures exceeded 90% except for one instance. We therefore assert that the apparatus used was able to provide mayfly nymphs with essential flow and dissolved oxygen. However, dissolved metals concentrations fluctuated throughout the three tests. While initial concentrations were very close to nominal levels, dissolved metals measurements decreased over time. This was probably due to adsorption to surfaces and/or uptake by nymphs themselves. Tissue digests performed on nymphs exposed to dissolved copper revealed that *R. hageni* did accumulate copper in their tissues. In order to precisely quantify toxic response in terms of the exposure concentration, we recommend modifying the static-renewal apparatus with a flow-through design to allow continuous volume turnover.

Toxicity

The results of our study demonstrate that middle-instar *R. hageni* were not acutely sensitive to dissolved zinc. Acute toxicity was observed in the cadmium test, but 100% mortality was not achieved in any of the treatment groups. Copper was acutely toxic to *R. hageni* ≥ 185 µg/L after 168 hours. Mayfly survival in the control units was 100% in the copper experiment, thus all mortality events were assumed to have been caused by exposure to copper. These experiments were designed as range-finding tests and thus estimates of acute toxicity risk should be interpreted conservatively until validated with further experimentation.

It was hypothesized that nymphs would moult less frequently if dissolved metals were negatively affecting growth and metabolism. Test results for all three metals were not consistent with this hypothesis, as total number of exuvia did not co-vary with dissolved metals concentration (except in the copper experiment). Thus we conclude that the process that determines when a nymph will moult its exoskeleton is not strongly affected by short-term exposure to dissolved zinc or cadmium.

Aquatic insects have repeatedly shown to be more tolerant of trace metal contamination than salmonid fishes. Trace metal toxicity should be expected to vary widely across members of a diverse biological community. Adaptation to fluctuating conditions of the lotic environment may have afforded aquatic insects the advantage of trace metal tolerance, at least in the short term. However, chronic and sublethal effects have been sparsely investigated. Chronic toxicity of zinc and copper were reported by Hatakeyama in 1989. A 15-year case study of the Arkansas River (Lake Co, CO, USA) suggests that the impacts of mining on benthic communities are severe but not irreversible (Nelson and Roline 1996).

It has been documented that, where strong trophic interactions exist, ecosystem-level changes may be caused by alterations in community composition (Baxter et al 2004, McIntosh and Townsend 1996). One study of brown trout in the Arkansas River demonstrated that shifts in benthic insect species assemblage were reflected in live fish gut contents (Clements and Rees 1997). Suprisingly, brown trout captured at the most contaminated site were, on average, larger than fish sampled at uncontaminated sites. While metal concentrations were elevated in both insect larvae and brown trout tissues, no clear relationship could be established between metals in prey and metals in predators. Effects on individual growth, reproductive success, or population growth could not be elucidated.

Sub-lethal effects of contaminants may have far-reaching consequences for ecosystem structure and function. While it was once thought that simple selection against intolerant organisms would cause shifts in the composition of biological communities, it is becoming clear that anthropogenic effects are often more complex than expected. Additionally, toxicity should be expected to vary across the bioindicator species' life cycle stages. Hatekeyama (1989) demonstrated that nymphs of a grazing mayfly (*Epeorus latifolium*) experienced growth inhibition and failed to reach adulthood when fed copper-contaminated diatoms. Thus it follows that sublethal effects of contaminants (which are often undetected in acute toxicity assays) may lead to decreased organism fitness.

In summary, these studies demonstrate that complex trophic relationships, as well as dynamic environmental conditions, are likely to confound apparent cause-effect relationships that are inferred from field observations. Therefore, in the interest of protecting aquatic life, we recommend that the apparent tolerance to cadmium and zinc exhibited by *R. hageni* in our studies not be extrapolated to their natural habitat. Future investigations of the effects of cadmium, copper and zinc on *R. hageni* should focus on biotic and abiotic modifiers of toxicity, specifically dietary exposure, developmental stage, reproductive success and chemical bioavailability.

Tables

Table 1. Water quality conditions in the zinc exposure medium. Standard deviations are in parentheses.

Nominal Zn (µg/L)	0	1000	2000	4000	8000	16000
Hardness (mg/L)	47.7(0.9)	48.5(2.7)	49.3(1.6)	50.5(0.8)	53.3(2.7)	65.3(1.2)
Alkalinity (mg/L)	41.6(4.5)	40.8(2.8)	40.2(0.3)	39.2(1.1)	39.9(0.7)	38.3(0.4)
pH (standard units)	7.67(.06)	7.65(.02)	7.66(.01)	7.66(.01)	7.66(.02)	7.65(.02)

Table 2. Nominal and observed zinc concentrations (µg/L) and mayfly mortality (%) at 96 and 168 hours. Standard deviations are in parentheses.

Nominal [Zn] (µg/L)	0	1000	2000	4000	8000	16000
Initial [Zn] (µg/L)	4(1)	1061(30)	2103(51)	4183(87)	8277(228)	16413(729)
Final [Zn] (µg/L)	23(6)	872(63)	1763(121)	3893(71)	7427(475)	15467(163)
96 hr mortality (%)	0(0)	5(7)	0(0)	0(0)	10(14)	0(0)
168 hr mortality (%)	10(14)	5(7)	0(0)	0(0)	15(21)	10(0)

Table 3. Moult frequency (% of surviving individuals) of mayflies exposed to zinc.

Nominal Zn (µg/L)	0	1000	2000	4000	8000	16000
48 hrs	0.1	0.26	0.30	0.0	0.10	0.15
72 hrs	0.15	0.11	0.10	0.15	0.05	0.10
96 hrs	0.0	0.11	0.0	0.05	0.00	0.10
120 hrs	0.0	0.0	0.05	0.05	0.05	0.15
168 hrs	0.05	0.05	0.20	0.30	0.05	0.06
216 hrs	0.0	0.16	0.0	0.05	0.17	0.17
Mean	0.05	0.11	0.11	0.10	0.07	0.12
Std Dev	0.06	0.09	0.12	0.11	0.06	0.04

Table 4. Experiment-wide means and standard deviations (s.d.) of physical and chemical parameters of cadmium exposure medium.

hardness (mg/L)		alkalinity (mg/L)		pH (s.u.)		dissolved oxygen (mg/L)		temperature (C)		conductivity (uS/cm)	
mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
43.2	2.1	39.0	2.0	7.6	0.1	9.2	0.1	10.4	0.4	97.2	3.0

Table 5. Nominal and mean measured cadmium concentrations (µg/L) and mayfly mortality (%) at 96 and 144 hours. Standard deviations are in parentheses.

Nominal Cd (µg/L)	Measured [Cd] (µg/L)	96 h mortality (%)	144 h mortality (%)	96 h total exuvia
0	3(3)	0	0	3
80	60 (1)	10	10	4
160	110 (1)	0	0	3
320	200 (1)	0	0	3
640	490 (6)	0	0	3
1250	840 (10)	0	10	2
2500	1800 (10)	0	30	2
5000	3400 (20)	40	40	4
10000	6300 (80)	60	80	4

Table 6. Physical and chemical conditions of copper exposure medium. Mean dissolved copper (µg/L), hardness (mg/L), alkalinity (mg/L), pH (std. units), temperature (Celsius), and conductivity (µS/cm). Standard deviations are in parentheses. NA = not analyzed

Nominal [Cu]	0	125	250	500	1000	2000
Hardness	46.0(0.9)	NA	NA	NA	NA	NA
Alkalinity	46.3(1.5)	NA	NA	NA	NA	NA
pH	7.76(0.16)	7.83(0.08)	7.88(0.06)	7.84(0.07)	7.85(0.07)	7.81(0.07)
Temperature	10.3(0.3)	10.8(0.8)	10.3(0.3)	11.0(.07)	10.3(0.3)	10.6(0.4)
Conductivity	133(11)	124(16)	127(15)	134(21)	126(22)	130(28)

Table 7. Nominal and mean dissolved copper concentrations (µg/L) and mayfly mortality(%) measured at 96 and 168 hours. Standard deviations are in parentheses. Cumulative total of moulted exoskeletons (exuvia) at 168 hours is presented in the bottom row.

Nominal [Cu]	0	125	250	500	1000	2000
Initial [Cu] (µg/L)	<10	149	271	516	1018	1954
96hr [Cu] (µg/L)	<10	103	185	357	679	1020
168hr [Cu] (µg/L)	3	97	165	298	556	963
96 hr mortality (%)	0(0)	35(7)	25(7)	35(7)	35(21)	50(14)
168 hr mortality (%)	0(0)	75(35)	70(14)	100(0)	95(7)	95(7)

Figures

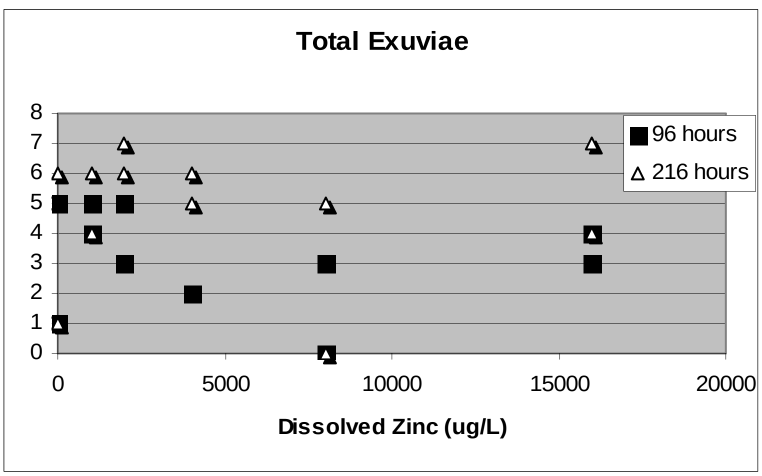

Figure 1. 96hr mortality (%) versus observed [Cd] (mg/L)

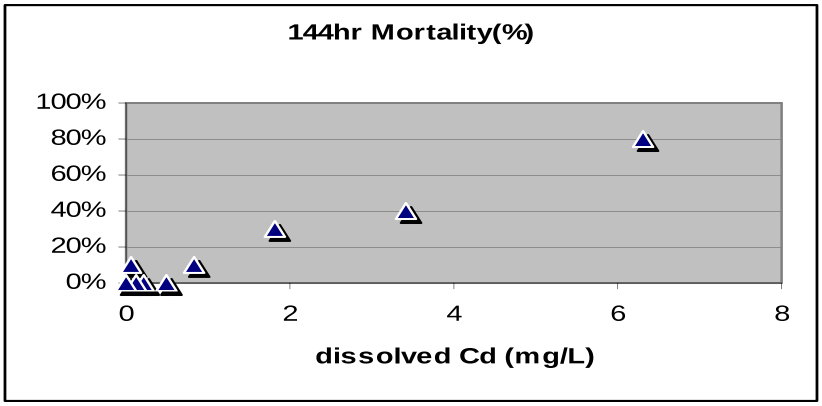

Figure 2. 144hr mortality (%) versus observed [Cd] (mg/L)

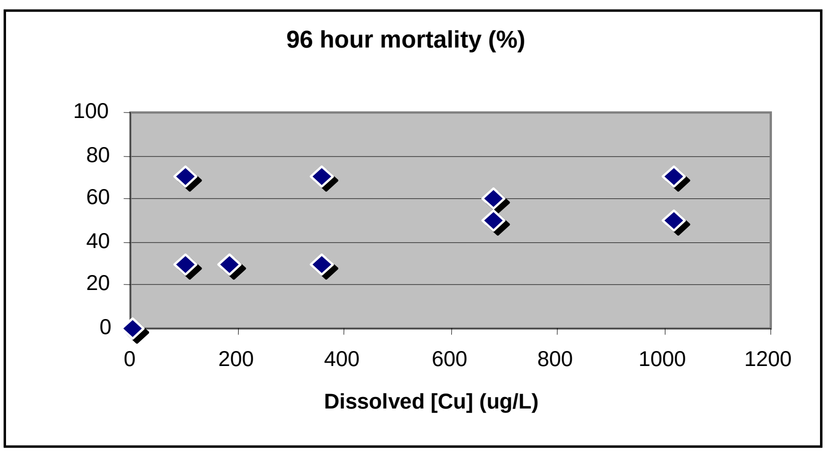

Figure 3. Mortality(%) at 96 hours versus dissolved copper (µg/L)

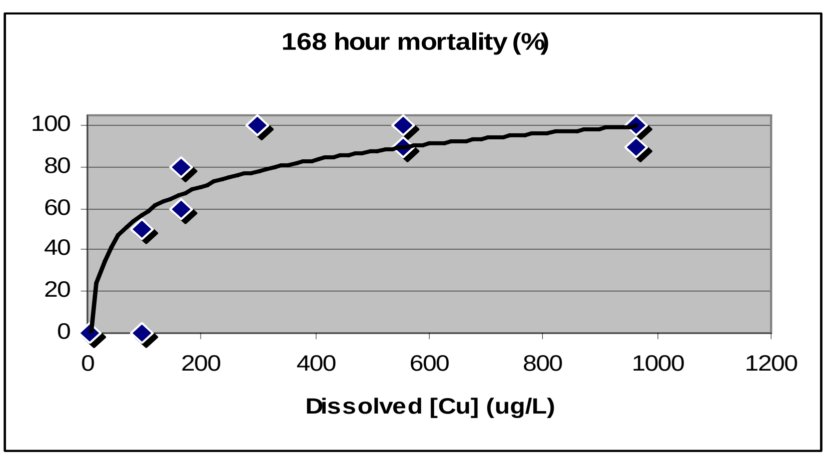

Figure 4. Mortality(%) at 168 hours versus dissolved copper (µg/L)

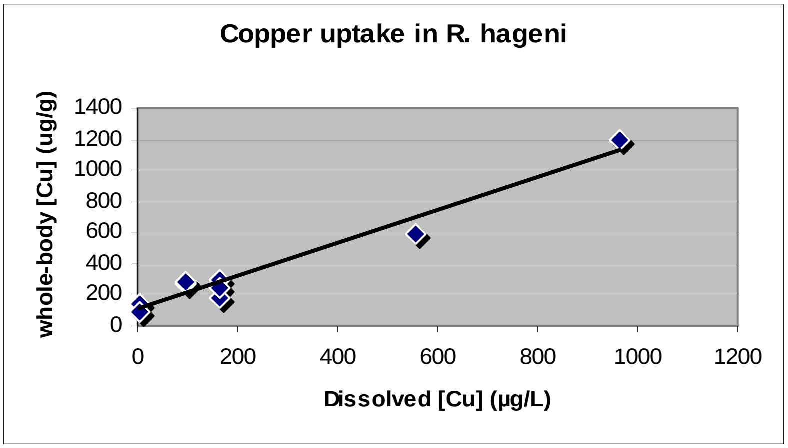

Figure 5. Copper accumulation after 168 hours of exposure to dissolved copper. Whole body concentrations were calculated on a dry mass basis.

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