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DISSERTATION

ECOTOXICOLOGICAL AND GENETIC EFFECTS OF A MIXTURE OF HEAVY METALS ON SELECTED AQUATIC MACROINVERTEBRATES

Submitted by

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Department of Fishery and Wildlife Biology

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2000

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COLORADO STATE UNIVERSITY

March 24, 2000

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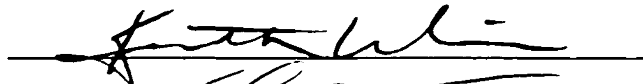

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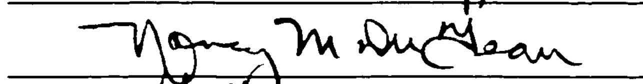

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ABSTRACT OF DISSERTATION

ECOTOXICOLOGICAL AND GENETIC EFFECTS OF A MIXTURE OF HEAVY METALS ON SELECTED AQUATIC MACROINVERTEBRATES

This research investigated the ecotoxicological and genetic effects of exposure to a mixture of heavy metals on selected aquatic macroinvertebrates. Studies centered on macroinvertebrates collected from, or concentrations found in, the upper Arkansas River. The upper Arkansas River receives wastes from mines and tailings piles in Leadville, Colorado and has been recognized as a site of poor water quality for many years.

To investigate the potential of sediments in the Arkansas River to act as a source of metals to benthic organisms, *Chironomus tentans* (Diptera: Chironomidae) were exposed to synthetic sediment spiked with a mixture of metals at concentrations similar to those found in the Arkansas River in a series of toxicity and bioaccumulation experiments. Results of these studies showed that a mixture of cadmium, copper, lead, and zinc readily accumulated in *C. tentans*, and were toxic at realistic concentrations measured in the field.

Because mining began in California Gulch 1859, it is likely that populations have been exposed to heavy metals for over 130 years. This site provided a unique opportunity to investigate both ecotoxicological and genetic effects of long-term exposure to heavy metals. Populations of the mayfly *Baetis tricaudatus* (Ephemeroptera: Baetidae) previously exposed to a mixture of metals in the field exhibited higher survival and growth, and bioaccumulated more cadmium than naive populations. In addition, previously exposed populations had significantly higher metallothionein concentrations.

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These findings suggest that long-term exposure to metals has resulted in tolerance, and that metallothionein may act as an underlying mechanism for this tolerance.

To determine if there were underlying genetic differences among these populations, a population genetic study was conducted. Mayflies were collected from seven sites on the Arkansas River at four different time points. A region of the ND1 mitochondrial DNA gene was amplified by polymerase chain reaction and analyzed by single strand conformation polymorphism analysis to determine haplotype. Haplotype diversity was generally lower immediately following spring runoff than in late summer, and generally lowest at the most contaminated site. Haplotype diversity was significantly correlated with concentrations of zinc in water, and copper and zinc in periphyton for one of the time points.

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GENERAL INTRODUCTION

Mining activities have had a major impact on watersheds in the Rocky Mountain region since the discovery of gold and other minerals in the mid-1800s. A recent analysis of randomly selected 3rd and 4th-order streams in Colorado, indicated that approximately one-fourth of them were at least moderately polluted with heavy metals (Clements et al., 2000). It is estimated that at least 2,600 km of streams have been affected by heavy metals from over 5,000 abandoned mines or tailings piles in this state alone (Colorado Department of Health 1992). A large number of these mines are located in or near the town of Leadville (Figure 1; U.S. Bureau of Mines 1992).

Mining began in this area in 1859, after A.G. Kelly discovered gold in the Arkansas River at Granite (Griswold and Griswold 1996). Several prospectors moved upstream until they got to California Gulch (so named because it had all the promise of the California gold rush), located near present-day Leadville. Within two months, 4,000 men had staked 400 separate mineral claims, each 100 feet long, for placer mining in the gulch. Within six months, miners had retrieved over one-million dollars worth of gold (Voynick 1984). Just a few years later however, the gravels were exhausted, and by 1865 the California Gulch boom was over (Blair 1980). Miners moved up into the hills and began hardrock mining. Focus shifted from gold to the massive lead-silver deposits they discovered there. In 1878, "Slabtown" was given the more suitable name of "Leadville" (Blair and Churchill 1997). Within a few years, miners had driven 150 miles of tunnels through solid rock in search of silver ore. The silver boom lasted until 1893, when the price of silver fell, following repeal of the Sherman Silver Purchase Act (Griswold and

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Griswold 1996). Because of the diverse mineralization underlying the region however, miners were able to continue working, extracting copper and zinc instead. The last great years for the Leadville Mining District were the early 1950s, although as late as 1980, its mines produced thirty-nine million dollars worth of precious and base metals (Voynick 1984).

This wealth did not come without great costs however. Stephen Voynick (1984: p.67) wrote:

> "...as the smelters worked overtime casting 100-pound bars of silver and lead, industrialization was visible in another way— in clouds of noxious, poisonous sulfur and metal fumes and smoke that drifted over the city while tons of metal-based chemical wastes were dumped into the Arkansas River."

Much of this waste enters the Arkansas River through two tunnel systems: the Leadville Mine Drainage Tunnel and the Yak Tunnel. The Leadville Mine Drainage Tunnel was begun in 1943 and completed in 1952, and drains several of the downtown mines (Voynick 1984). It flows directly into the Arkansas River just north of Leadville. The Yak Tunnel was constructed in 1906 to provide drainage and haulage service, as well as electricity, to several other mines (Blair 1980). The Yak Tunnel drains water containing high concentrations of heavy metals from these mines into California Gulch, which flows into the Arkansas River just southwest of Leadville.

California Gulch was placed on the U.S. Environmental Protection Agency's (EPA's) National Priorities (Superfund) List in 1983. Acute toxicity (96-hour toxicity test with *Ceriodaphnia dubia*) has been measured >50 km downstream from California Gulch in the Arkansas River (Clements and Kiffney 1994), and metals levels have

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exceeded federal criteria for over 400 km downstream (Lewis 1987). In 1990, a water treatment plant was constructed to treat the water draining the Yak Tunnel (U.S. EPA Region VIII 1999). However, during high flow events, such as occur during spring runoff, water bypasses the treatment plant and flows directly into California Gulch and then the Arkansas River, carrying with it high concentrations of cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn).

During low flow periods, this site is not unlike many others, where regulations have forced a decrease in effluent concentrations of contaminants. When concentrations in the overlying water decrease however, sediments may become the predominant source of contaminants to the system, especially in areas where contaminants had been released for many years (Brannon et al. 1980, Jennett et al 1980, Larsson 1985). Sediments often contain complex mixtures of contaminants at concentrations that are orders of magnitude greater than in the overlying water (Chapman 1989, Luoma 1989). For example, in the Arkansas River, water collected from a site just downstream from California Gulch contained 0.3 parts-per million (ppm) Zn, while sediment collected from the same site contained 750 ppm Zn (Chapter 3).

Sediments in the Arkansas River may act as a source of metals, particularly for benthic organisms (those living on or in the substrate). To test the hypotheses that benthic invertebrates bioaccumulate metals from sediment, and that bioaccumulation results in mortality, I exposed *Chironomus tentans* (Diptera: Chironomidae) to synthetic sediment spiked with a mixture of Cd, Cu, Pb, and Zn at concentrations similar to what we find in the Arkansas River, in a series of toxicity and bioaccumulation experiments (Chapter 1). Uptake and depuration (or elimination) kinetics were used to model

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bioaccumulation of each of the four metals and to test the hypothesis that bioaccumulation depends on duration of exposure.

Results of these studies showed that a mixture of heavy metals readily accumulated in a benthic invertebrate, and were toxic at realistic concentrations measured in the field (Harrahy and Clements 1997). Assuming similar bioavailability, these results suggest that sediments in the Arkansas River may be toxic to benthic invertebrates. Indeed, field studies indicate that benthic communities in the Arkansas River are severely degraded (Roline 1988, Clements 1994, Clements and Kiffney 1994). We do however, find some species surviving in even the most contaminated reaches. Based on these results and observations, I hypothesized that a) metals are more bioavailable in synthetic sediment than in Arkansas River sediment, and/or b) benthic invertebrates surviving in the most contaminated reaches of the Arkansas River have become tolerant to metals. Frugis (1995) investigated the bioavailability question and found that metals are indeed more bioavailable in synthetic sediment than in spiked natural sediment. I investigated the tolerance question.

Some populations chronically exposed to heavy metals have exhibited enhanced tolerance relative to naive populations (Antonovics et al. 1971; Bryan and Hummerstone 1971; Fraser et al. 1978; Wentsel et al. 1978; Weis et al. 1981; Diamond 1989; Maltby 1991). To test the hypothesis that previously exposed populations in the Arkansas River are more tolerant to subsequent exposure than previously unexposed populations, I conducted a series of laboratory toxicity tests. *Baetis tricaudatus* (Ephemeroptera: Baetidae) mayflies were collected from sites located upstream and downstream of the California Gulch Superfund site on the Arkansas River, and from the Cache la Poudre

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River (reference site), and exposed to a mixture of Cd, Cu, and Zn in flow-through microcosms.

B. tricaudatus was chosen as the study organism for these, as well as the genetic studies, for several reasons. First, while mayflies are generally sensitive to heavy metals, this particular species can survive in areas with relatively high concentrations of metals (Roline 1988, Clements 1994). Because *B. tricaudatus* consumes detritus and diatoms in sediment and periphyton (Merritt and Cummins 1996), it is exposed to metals through both aqueous (gills) and dietary routes. Its survival under such circumstances raises some interesting questions with regard to tolerance, mechanisms of tolerance, and costs of tolerance. Second, it is considered an r-selected species (Robinson et al. 1992, Williams and Feltmate 1992), and makes up a large proportion of the drift in streams (Kohler 1985, Peckarsky and Cowan 1995). In a test of the colonization cycle hypothesis, a stable isotope tracer experiment revealed that approximately one-third to one-half of a *Baetis* nymph population drifted at least 2.1 km downstream over summer, and that approximately the same proportion of the population flew 1.6 to 1.9 km upstream from where they emerged (Hershey et al. 1993). These are important characteristics to consider in assessing whether a population has acclimated or adapted to metals, or is continually replenished through colonization. Third, *B. tricaudatus* is widely distributed in streams and rivers throughout the United States (Morihara and McCafferty 1979), making results of these studies potentially applicable to other metal contaminated areas. And finally, it can be collected in the large numbers required for conducting toxicity tests.

Results of the toxicity tests are presented in Chapter 2. Acute effects (on survival) were mixed with respect to tolerance. Because recent studies have shown that

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tolerance may be more evident at the sublethal level (Posthuma et al. 1993, Vuori 1994, and Diamond et al. 1995). I conducted a sublethal experiment to test the hypotheses that previously exposed populations have lower growth rates and higher bioaccumulation rates than previously unexposed populations. In addition, to determine a possible underlying mechanism for tolerance and increased bioaccumulation of metals, I compared concentrations of the metal-binding protein metallothionein. I hypothesized that previously exposed populations had higher concentrations of metallothionein than previously unexposed populations.

Metallothionein is a low molecular weight, cysteine-rich protein that functions in both metal homeostasis (primarily through storage and transfer of Cu and Zn) and detoxification (by outcompeting more critical macromolecules in binding) (Roesijadi 1992). Its synthesis may be induced by exposure to metals, particularly Cd, Cu, and Zn. Such induction may occur through acclimation (a physiological response) or adaptation (a genetic response) (Mulvey and Diamond 1991). For example, prior induction may result in the accumulation of a pool of metallothionein, available for binding in subsequent exposures (McCarter and Roch 1984 and Berger et al. 1995). Alternatively, the metallothionein gene may be amplified, resulting in increased capacity for this metal-binding protein's synthesis (Maroni et al. 1987). In either case, metallothionein may be a useful biomarker of metal exposure.

The field of biomarker research is developing rapidly and recent advances in molecular biology have greatly enhanced our ability to study the genetics of natural populations. In particular, polymerase chain reaction (PCR)-based assays have increased our ability to detect differences in genetic variability within and among populations

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(Simon et al. 1994). Use of neutral genetic markers, such as mitochondrial DNA (mtDNA), allows us to take a phylogenetic approach to population genetics, and to examine the effects of historic factors, such as population bottlenecks (Bickham and Smolen 1994). Analysis of mtDNA is a powerful tool because the mitochondrial genome is primarily maternally inherited and does not recombine. In addition, it evolves at a faster rate than the nuclear genome (Norris et al. 1996); thus, intraspecific variation is frequently detectable.

Placer mining of gold deposits began in California Gulch in 1859 and it is likely that populations in the Arkansas River have been exposed to heavy metals for over 130 years. Some populations may have been entirely eliminated for some time following periods of heavy mining when water quality was very low. Therefore, in addition to ecotoxicological effects, this study site provided an opportunity to investigate the molecular genetic effects of long-term exposure to heavy metals. To test the hypothesis that populations previously exposed to heavy metals have less genetic diversity than populations previously unexposed, I conducted a population genetic study of *B. tricaudatus*, using primers I designed for a protein-coding mitochondrial DNA gene (NADH dehydrogense subunit 1, or ND1) (Chapter 3).

While some ecotoxicologial studies have provided indirect evidence for a genetic component to heavy metal tolerance (Wilson 1988, Maltby 1991, Klerks and Levinton 1993, Postma et al. 1995), few studies have combined ecotoxicological and molecular genetic approaches to provide direct evidence. Additionally, little research has been conducted to investigate the mechanisms responsible for increased tolerance and the costs associated with that tolerance. Anderson et al. (1994) called for a multidisciplinary

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approach to understanding ecological consequences of exposure to contaminants, and proposed a research framework that integrates ecology with population genetics to form a broader model for assessing and predicting effects of pollution. Use of the same species in my toxicity and genetic studies allowed unique insight into both differential susceptibility to a mixture of heavy metals, and the underlying genetic differences between previously exposed and naive populations.

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LITERATURE CITED

Anderson, S. et al. 1994. Genetic and molecular ecotoxicology: a research framework. *Environmental Health Perspectives* **102**:3-8.

Antonovics, J., A.D. Bradshaw, and **R.G. Turner.** 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* **7**:1-85.

Berger, B., R. Dallinger, and **A. Thomaser.** 1995. Quantification of metallothionein as a biomarker for cadmium exposure in terrestrial gastropods. *Environmental Toxicology and Chemistry* **14**:781-791.

Bickham, J.W. and **M.J. Smolen.** 1994. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. *Environmental Health Perspectives* **102**:25-28.

Blair, E. 1980. Leadville: Colorado's Magic City. Fred Pruett Books, Boulder, CO. 247 p.

Blair, E. and **E.R. Churchill.** 1997. Everybody Came to Leadville. First Light Publishing, Fort Collins, CO. 40 p.

Brannon, M.J., R.H. Plumb, Jr., and **I. Smith, Jr.** 1980. Long-term release of heavy metals from sediments. In R.A. Baker, ed., *Contaminants and Sediments*, **Vol. 1.** Ann Arbor Science, Ann Arbor, MI, pp. 221-266.

Bryan, G.W. and **L.G. Hummerstone.** 1971. Adaptation of the polychaete *Nereis diversicolor* to estuarine sediments containing high concentrations of zinc and cadmium. *Journal of the Biological Association of the U.K.* **53**:839-857.

Chapman, P.M. 1989. Current approaches to developing sediment quality criteria. *Environmental Toxicology and Chemistry* **8**:589-599.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Clements, W.H. 1994. Benthic invertebrate community responses to heavy metals in the Upper Arkansas River Basin, Colorado. *Journal of the North American Benthological Society* **13**:30-44.

Clements, W.H., D.M. Carlisle, J.M. Lazorchak, and **P.C. Johnson.** 2000. Heavy metals structure benthic communities in Colorado mountain streams. *Ecological Applications*. In press.

Clements, W.H. and **P.M. Kiffney.** 1994. Integrated laboratory and field approach for assessing impacts of heavy metals at the Arkansas River, Colorado. *Environmental Toxicology and Chemistry* **13**:397-404.

Colorado Department of Health. 1992. Water quality in Colorado. Water Quality Control Division.

Diamond, S.A., M.C. Newman, M. Mulvey, P.M. Dixon, and **D. Martinson.** 1989. Allozyme genotype and time-to-death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute exposure to inorganic mercury. *Environmental Toxicology and Chemistry* **8**:613-622.

Diamond, S.A., J.T. Oris, and **S.I. Guttman.** 1995. Adaptation to fluoranthene exposure in a laboratory population of fathead minnows. *Environmental Toxicology and Chemistry* **14**:1393-1400.

Fraser, J., D.T. Parkin, and **E. Vespoor.** 1978. Tolerance to lead in the freshwater isopod *Asellus aquaticus*. *Water Research* **12**:637-641.

Frugis, M. 1995. Bioavailability of metal mixtures in natural and synthetic sediments. M.S. thesis. Colorado State University, Fort Collins, CO.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Griswold, D.L. and **J.H. Griswold.** 1996. *History of Leadville and Lake County, Colorado: From Mountain Solitude to Metropolis.* Volume 1. Colorado Historical Society in cooperation with the University Press of Colorado, Boulder, CO. 1303 p.

Harrahy, E.A. and **W.H. Clements.** 1997. Toxicity and bioaccumulation of a mixture of heavy metals in *Chironomus tentans* (Diptera: Chironomidae) in synthetic sediment. *Environmental Toxicology and Chemistry* **16**:317-327.

Hershey, A.E., J. Pastor, B.J. Peterson, and **G.W. Kling.** 1993. Stable isotopes resolve the drift paradox for *Baetis* mayflies in an Arctic River. *Ecology* **74**:2315-2325.

Jennett, J.C., S.W. Effler, and **B.G. Wixson.** 1980. Mobilization and toxicological aspects of sedimentary contaminants. In R.A. Baker, ed., *Contaminants and Sediments*, Vol. 1. Ann Arbor Science, Ann Arbor, MI, pp. 429-444.

Klerks, P.L. and **J.S. Levinton.** 1993. Evolution of resistance and changes in community composition in metal-polluted environments: A case study on Foundry Cove. In *Ecotoxicology of Metals in Invertebrates*. R. Dallinger and P.S. Rainbow (eds.). SETAC Special Publications Series, Ann Arbor, MI. P.223-241.

Kohler, S.L. 1985. Identification of stream drift mechanisms: an experimental and observational approach. *Ecology* **66**:1749-1761.

Larsson, P. 1985. Contaminated sediments of lakes and oceans act as sources of chlorinated hydrocarbons for release to water and atmosphere. *Nature* **317**: 347-349.

Lewis, W.S. 1987. An evaluation of mining related metals pollution in the upper Arkansas River basin. M.S. thesis. Colorado School of Mines, Golden, CO.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Luoma, S.N. 1989. Can we determine the biological availability of sediment-bound trace elements? *Hydrobiologia* **176/177**:379-396.

Maltby, L. 1991. Pollution as a probe of life-history adaptation in *Asellus aquaticus* (Isopoda). *OIKOS* **61**:11-18.

Maroni, G., J. Wise, J.E. Young and **E. Otto.** 1987. Metallothionein gene duplications and metal tolerance in natural populations of *Drosophila melanogaster. Genetics* **117**:739-744.

McCarter, J.A. and **M. Roch.** 1984. Chronic exposure of Coho salmon to sublethal concentrations of copper— III. Kinetics of metabolism of metallothionein. *Comparative Biochemistry and Physiology* **77C**:83-87.

Merritt, R.W. and **K.W. Cummins.** 1996. *An Introduction to the Aquatic Insects of North America*. Third Edition. Kendall/Hunt Publishing Company, Debuque, Iowa.

Morihara, D.K. and **W.P. McCafferty.** 1979. The *Baetis* larvae of North America (Ephemeroptera: Baetidae). *Transactions of the American Entomological Society* **105**:139-221.

Mulvey, M. and **S.A. Diamond.** 1991. Genetic factors and tolerance acquisition in populations exposed to metals and metalloids. In: *Ecotoxicology of Metals: Current Concepts and Applications*. M.C. Newman and A.W. McIntosh (eds.). Lewis Publishers, Chelsea, MI.

Norris, D.E., J.S.H. Klompen, J.E. Keirans, and **W.C. Black IV.** 1996. Population genetics of *Ixodes scapularis* (Acari: Ixodidae) based on mitochondrial 16S and 12S genes. *Journal of Medical Entomology* **33**:78-88.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Peckarsky, B.L. and **C.A. Cowan.** 1995. Microhabitat and activity periodicity of predatory stoneflies and their mayfly prey in a western Colorado stream. *OIKOS* 74:513-521.

Posthuma, L., R.A. Verweij, B. Widianarko, and **C. Zonneveld.** 1993. Life history patterns in metal-adapted Collembola. *OIKOS* **67**:235-249.

Postma, J.F., S. Mol, H. Larsen, and **W. Admiraal.** 1995. Life-cycle changes and zinc shortage in cadmium-tolerant midges, *Chironomus riparius* (Diptera), reared in the absence of cadmium. *Environmental Toxicology and Chemistry* **14**:117-122.

Robinson, C.T., L.M. Reed, and **G.W. Minshall.** 1992. Influence of flow regime on life history, production, and genetic structure of *Baetis tricaudatus* (Ephemeroptera) and *Hesperoperla pacifica* (Plecoptera). *Journal of the North American Benthological Society* **11**:278-289.

Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology* **22**:81-114.

Roline, R.A. 1988. The effects of heavy metals pollution of the upper Arkansas River on the distribution of aquatic macroinvertebrates. *Hydrobiologia* **160**:3-8.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and **P. Flook.** 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**:651-701.

U.S. Bureau of Mines. 1992. Minerals availability system non-proprietary (MASNP) database. U.S. Bureau of Mines, Division of Statistics and Information Services, Washington, D.C.

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U.S. Environmental Protection Agency. 1999. California Gulch fact sheet. http://www.epa.gov/unix0008/sf/fact/r80829.html.

Vuori, K. 1994. Rapid behavioural and morphological responses of hydropsychid larvae (Trichoptera, Hydropsychidae) to sublethal cadmium exposure. *Environmental Pollution* **84**:291-299.

Voynick, S.M. 1984. *Leadville: A Miner's Epic.* Mountain Press Publishing Company. Missoula, MT. 165 p.

Williams, D.D. and **B.W. Feltmate.** 1992. *Aquatic Insects.* CAB International, Wallingford, UK. 358 p.

Weis, J.S., P. Weiss, M. Heber, and **S. Vaidya.** 1981. Methylmercury tolerance of killifish (*Fundulus heteroclitus*) embryos from a polluted vs. a nonpolluted environment. *Marine Biology* **65**:283-287.

Wentsel, R., A. McIntosh, and **G. Atchinson.** 1978. Evidence of resistance to metals in larvae of the midge *Chironomus tentans* in a metal contaminated lake. *Bulletin of Environmental Contamination and Toxicology* **20**:451-455.

Wilson, J.B. 1988. The cost of heavy-metal tolerance: an example. *Evolution* **42**:408-413.

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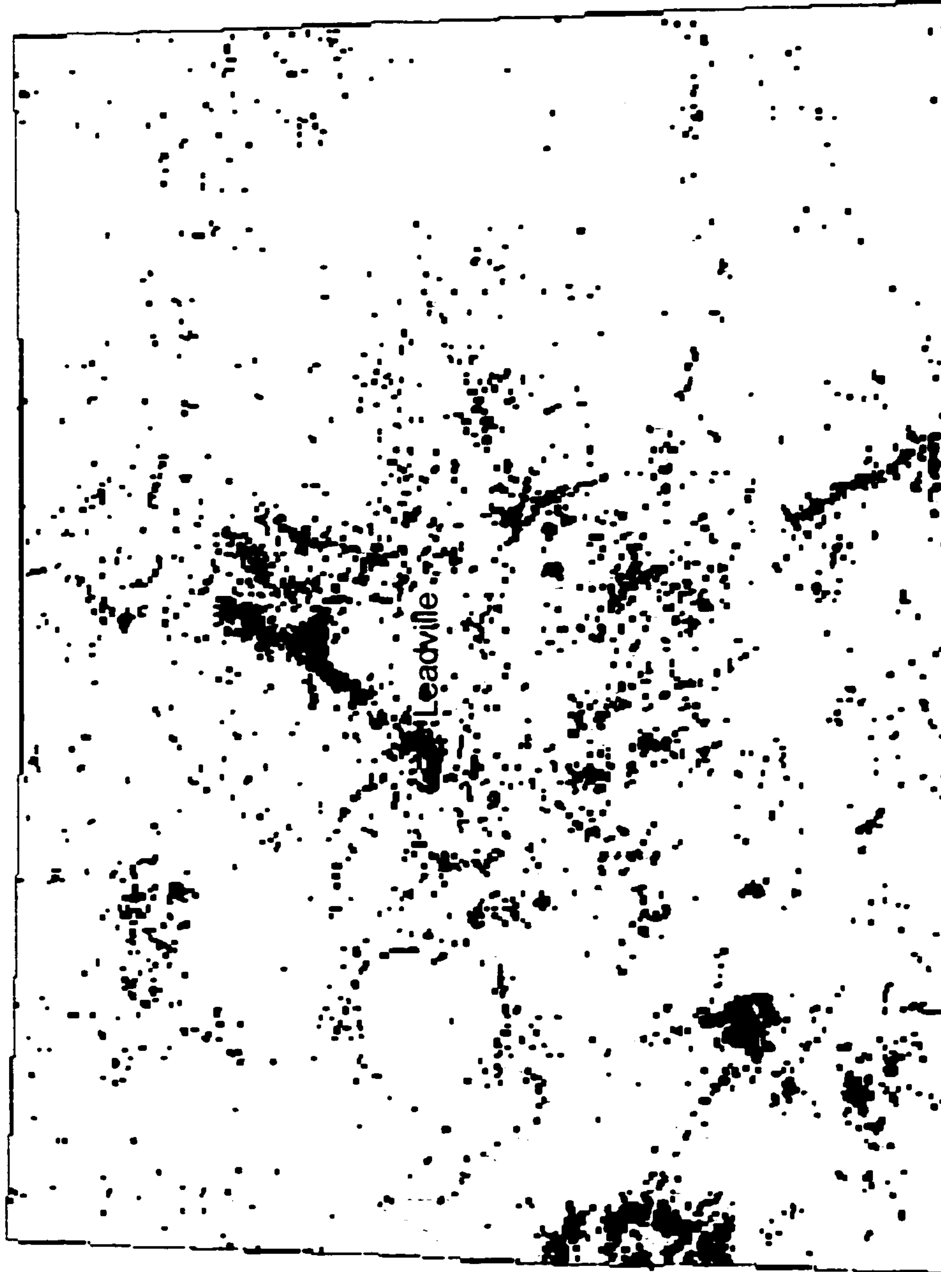

Figure 1. Map of Colorado showing watersheds and the locations of historic and current mines (U.S. Bureau of Mines 1992).

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CHAPTER 1

TOXICITY AND BIOACCUMULATION OF A MIXTURE OF HEAVY METALS IN *CHIRONOMUS TENTANS* (DIPTERA: CHIRONOMIDAE) IN SYNTHETIC SEDIMENT

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ABSTRACT

This research investigated toxicity and bioaccumulation of a mixture of Cd, Cu, Pb, and Zn in *Chironomus tentans* in synthetic sediment, and compared predicted to measured steady state bioaccumulation factors (BAFs). In a toxicity test, *C. tentans* were exposed to various dilutions of a base concentration (1.0 X) of a mixture of the four metals (5 µg/g Cd, 10 µg/g Cu, 70 µg/g Pb, and 300 µg/g Zn) in synthetic sediment. Mortality ranged from 17 to 100%. To measure bioaccumulation of the metals, *C. tentans* were exposed to 0.35 X the base concentration for a period of up to 14 d in two uptake tests. Bioaccumulation of all four metals increased over the 14 d uptake phases. Concentrations of metals in chironomids were significantly correlated with exposure time in the uptake phases. Only concentrations of copper approached background levels after 7 d depuration. Uptake rate coefficients and elimination rate constants were determined for each metal. Bioaccumulation factors were highest for Cd and lowest for Pb. With the exception of Pb, steady-state BAFs were within a factor of about two of those calculated using the first-order kinetic model. The high BAFs calculated may indicate greater bioavailability in synthetic sediment. Studies comparing toxicity and bioaccumulation of natural and synthetic sediments are necessary before the use of synthetic sediments is widely adopted.

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INTRODUCTION

Sediments often contain complex mixtures of contaminants at concentrations orders of magnitude greater than in the overlying water (Chapman 1989 and Luoma 1989). However, much of the information regarding effects of contaminants in aquatic systems is based on aqueous exposure alone (Cairns et al. 1984, Burton 1991, and McIntosh 1991). Sediments may act as a source of contaminants (Brannon et al. 1980, Jennett et al. 1980, and Larsson 1985), particularly for benthic organisms (Wentsel et al. 1977, Cairns et al. 1984, and Chapman 1989). Contaminants may be transferred from benthic invertebrates to higher trophic levels through the food chain (Landrum and Robbins 1990, Thomann et al. 1992, and Timmermans et al. 1992).

Bioaccumulation of sediment contaminants can be modeled as the net result of uptake and depuration kinetics (Lee 1992) using the first-order equation

$$dCt/dt = k_s * Cs - k2 * Ct \quad (1)$$

where:

Ct = tissue residue (μg/g tissue)

k_s = sediment uptake rate coefficient (g sediment/(g tissue*time))

Cs = contaminant concentration in sediment (μg/g sediment);

$k2$ = depuration rate constant ($time^{-1}$)

t = time

Sediment bioaccumulation factors (BAFs) may be estimated using the first-order kinetic model as

$$BAF = k_s/k2 \quad (2)$$

Alternatively, BAFs may be directly calculated [13,14] as $BAF = Ct_{ss}/Cs$ (3)

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where:

C_{tss} = tissue concentration at steady state (μg/g tissue)

C_s = sediment concentration (μg/g sediment)

The first-order kinetic model assumes no significant growth of test organisms, which may be reasonable for some short-term laboratory tests, and constant uptake and depuration rates. It can be used to predict nonsteady-state tissue residues and time course of uptake, under the exposure conditions at which measurements were made (Lee 1992). In contrast, direct calculation of BAFs assumes that contaminant concentration in a benthic organism is at steady-state with the contaminant concentration in sediment. This assumption may be violated in short-term laboratory tests, resulting in underestimation of predicted tissue residues (Boese and Lee 1992).

The first-order kinetic model is theoretically applicable to metals, but has typically been used with organics (Landrum and Scavia 1983, Landrum 1989, and Landrum et al. 1994). Its application to metals may be limited because metal bioavailability may influence uptake kinetics, and induction of metal-binding proteins may influence depuration kinetics (Lee 1992 and U.S. EPA 1994). However, kinetic models are needed to predict non-steady-state, nonequilibrium accumulation, particularly when there are multiple routes of uptake (e.g., sediment and interstitial water) (Landrum et al. 1992).

Prompted by the need for uncontaminated and non-toxic reference and dilution sediment, standard synthetic sediments have been developed (Walsh et al. 1992 and Suedel and Rodgers 1994a). Although synthetic sediments have been shown to support survival and growth of a variety of plants and animals (Walsh et al. 1992, Suedel and

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Rodgers 1994a, and Suedel and Rodgers 1994b), and have been used in toxicity tests (Walsh et al. 1991, Walsh et al. 1991, and Watzin et al. 1994), few bioaccumulation studies have been conducted in synthetic sediments (Titus and Pfister 1982 and Ciborowski et al. 1991). Although it may be difficult to reproduce all the properties of a field-collected sediment (Walsh et al. 1992 and Suedel and Rodgers 1994a), particularly those which affect bioavailability, synthetic sediments offer several advantages over natural sediments. They are contaminant free and are reproducible (Walsh et al. 1991, Walsh et al. 1991, and Walsh et al. 1992) which allows comparison of results among tests. In contrast, the physical, chemical, and biological characteristics of natural sediments vary both spatially and temporally.

Most sediment studies have focused on responses to single chemicals, although few sites in nature are polluted with only one chemical (Sorensen 1991). Experiments conducted with chemical mixtures more realistically reflect polluted environments and may be important in the development of ecologically relevant criteria (Kraak et al. 1994).

The purpose of this research was to investigate toxicity and bioaccumulation of a mixture of cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) in the benthic invertebrate *Chironomus tentans* (Diptera: Chironomidae) in synthetic sediment, and to compare predicted to measured steady state BAFs. These four metals are the predominant contaminants in several metal-polluted streams in Colorado. At the time of this study, the synthetic sediment was being considered for use as a standard reference and dilution sediment. Modified versions of its formulation (with respect to source of organic matter) have since been used successfully in toxicity tests (Watzin et al. 1994 and N.E. Kemble, personal communication). Tests were conducted later in our laboratory to

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compare toxicity between synthetic and natural (Arkansas River) sediment (Frugis 1995).

METHODS

Test organism culture

Chironomus tentans used in toxicity and uptake tests were obtained from stock cultures that originated from the National Fisheries Contaminant Research Center, National Biological Survey (Columbia, MO). Chironomids were cultured in 20 L glass aquaria containing a paper towel substrate and approximately 10 L of dechlorinated tap water, and were maintained at approximately 20°C. *C. tentans* were fed a slurry of TetraMin fish flakes. Each aquarium received approximately 6 ml of the food slurry twice weekly. Culture water was replaced weekly. This procedure is similar to that recommended by the U.S. EPA (1994).

Sediment dosing

Synthetic sediment was prepared following Walsh et al. (1992). Briefly, for each batch, 850 g washed medium quartz sand, 150 g of a clay and silt mixture, 22 g *Sphagnum* moss, 0.1 g soluble humic acids, and 0.5 g dolomitic limestone were added to a 3.8-L glass jar and rolled for 1 h on a rolling mill. Batches of sediment were composited and stored dry in a plastic bucket. Maximum storage time was one month. The sand had been washed with tap water until the water ran clear, rinsed with Milli-Q water, and then spread on a tarp to dry. The *Sphagnum* moss had been soaked in deionized water, with daily replacement, for five days to remove acids, and spread on a tarp to dry. Dried moss was ground in a blender and sifted through a series of sieves to

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give a mean particle size of 840 μm. This synthetic sediment contains 3% organic matter. Total organic carbon and sulfides were not measured. Particle size distribution and other characteristics have been described by Walsh et al. (1992).

Synthetic sediment was spiked with a mixture of heavy metals. The base concentration (1.0 X) was equal to 5 μg/g Cd, 10 μg/g Cu, 70 μg/g Pb, and 300 μg/g Zn, and was based on sediment data collected from the Arkansas River, downstream from the California Gulch Superfund site, located near Leadville, CO. Zinc acetate, lead acetate, and copper and cadmium atomic absorption spectrophotometry (AA) standard solutions (1000 ppm; Sigma Chemical Company) were added to Milli-Q water to make up solutions used to spike sediment. Copper and cadmium AA standards were used to avoid precipitation problems caused by addition of chlorides or sulfates to the lead (personal observation). Solutions were prepared separately for each treatment. Spiked sediments were rolled for 1 h on a rolling mill and then left uncovered in a fume hood until excess water had evaporated (48 h). Moist, spiked sediments were mixed by hand prior to distribution to beakers.

Toxicity

C. tentans were exposed to a mixture of heavy metals in synthetic sediment for 10 d in a toxicity test (U.S. EPA 1994). A preliminary range-finding test showed 100% mortality in the 10.0 X treatment. The toxicity test was conducted with treatments equal to 0 X (control), 0.0875 X, 0.175 X, 0.35 X, 0.70 X, 1.0 X, 1.4 X, 2.8 X, and 5.6 X the base concentration of the four metals.

Sediment (200 mL) was added to each of 45 1-L plastic beakers. There were 3

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replicates of 9 treatments for mortality and bioaccumulation determination, plus 2 extra beakers of 9 treatments for initial and final sediment and interstitial water metals analysis. Moderately hard (alkalinity 65±5; hardness 90±10) reconstituted water (800 mL) was slowly added to each beaker using a buret. The beakers were placed in an incubator (Precision, model 818) at 20°C (+/-1°C) for 48 h to allow any resuspended sediment to settle. Light was provided on a 16-h on/8-h off schedule. Beakers were aerated using pipette tips.

After 48 h, basic water chemistry parameters (pH, dissolved oxygen, and conductivity) were measured in each beaker. One of the extra beakers from each of the nine treatments was removed to determine initial metals concentrations in the overlying water, interstitial water, and sediment. Forty second-instar *C. tentans* were randomly assigned to each of the 36 remaining beakers. Larvae were not fed during the exposure period because we thought the organic matter in the sediment would provide sufficient food.

Basic water chemistry parameters were measured every two days in each beaker throughout the test period. At the end of the test, the remaining extra beaker from each of the nine treatments was sampled to determine final metals concentrations in the interstitial water and sediment. Overlying water was sampled from all beakers to determine metals concentrations.

Mortality and bioaccumulation were determined after 10 d in each of the nine treatments (n=3 beakers). Larvae were considered dead if they did not respond to gentle prodding, or were missing. Larvae visible on the surface of the sediment were removed first, using plastic forceps, and then contents of the beaker were washed through a No. 30

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(0.6 mm) plastic sieve to recover remaining larvae.

Uptake

We conducted two uptake tests to measure temporal changes in metals concentrations and to estimate uptake rate coefficients. *C. tentans* were exposed to 0.35 X the base concentration of a mixture of Cd, Cu, Pb, and Zn in synthetic sediment for 14 d. The 0.35 X treatment was equal to 1.75 µg/g Cd, 3.5 µg/g Cu, 24.5 µg/g Pb, and 105 µg/g Zn, and was chosen based on results of the toxicity test.

Synthetic sediment was spiked with 0.0 X and 0.35 X (first test) or 0.35 X (second test) the base concentration of the four metals, and added to 1-L plastic beakers, as in the toxicity test. After 48 h, basic water chemistry parameters (pH, dissolved oxygen, and conductivity) were measured in each beaker. Two extra beakers containing spiked sediment were destructively sampled to determine mean initial metals concentrations in the overlying water, interstitial water, and sediment. Thirty second-instar *C. tentans* were randomly assigned to each of the remaining beakers for the first uptake test. Forty second-instar *C. tentans* were randomly assigned to each of the remaining beakers for the second uptake test. Additional organisms were included in the second test to allow for losses during transfer to beakers containing clean sediment for the depuration test.

C. tentans exposed to spiked sediment were sampled (n=3 beakers) on days 1, 2, 4, 7, 10, and 14. *C. tentans* exposed to control sediment (n=3 beakers) were sampled on days 1 and 14. Dissolved oxygen, pH, and conductivity were measured, and overlying water samples were taken from all beakers sampled at each time point. Interstitial water

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and sediment samples were taken from two extra beakers at each time point in the first test, and from two extra beakers on days 1, 7, and 14 in the second test. These extra beakers contained chironomids to account for the effects of the test organisms upon interstitial and overlying water quality.

Depuration

We conducted a depuration test to measure temporal changes in metals concentrations and to estimate depuration rate constants. After 14 d exposure, chironomids were transferred to beakers containing clean sediment and sampled (n=3 beakers) after 0, 0.5, 1, 2, 5, and 7 d. Dissolved oxygen, pH, and conductivity were measured, and overlying water samples were taken from all beakers at each time point. Interstitial water and sediment samples were taken from two extra beakers on days 1 and 7.

Bioaccumulation factor determination

Bioaccumulation factors (BAFs, g dry sediment/g dry tissue) were calculated for each of the four metals using the first-order kinetic model (Eqn. 2). Uptake rate coefficients (k_s, g dry sediment/(g dry tissue*day)) were determined for each metal as the slope of the linear regression of the tissue residue (μg/g dry tissue) versus time in days (for the linear portion of the uptake phase), divided by the mean measured sediment concentration (μg/g dry sediment) over the 14 d uptake phase. Depuration rate constants ($k2$, day^{-1}) were determined as the absolute value of the slope of the regression of the natural log of the tissue residue versus time.

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Steady-state BAFs (g dry sediment/g dry tissue) were also calculated (Eqn. 3) for each of the four metals using mean tissue residue (μg/g dry tissue) attained by *C. tentans* at 14 d and mean measured sediment concentration (μg/g dry sediment) over the 14 d uptake phase.

Metals analysis

Concentrations of Cd, Cu, Pb, and Zn were measured in overlying water, interstitial water, and sediment in all tests. Both dissolved and total metals were analyzed in the overlying water. To determine dissolved metals concentrations, overlying water was collected with a syringe and passed through an Acrodisc 0.45 μm filter. I did not attempt to account for potential binding of dissolved metals to the filter. Interstitial water was collected by centrifuging sediment at 6,000 rpm for approximately 10 min. Water and interstitial water samples were acidified to pH 2, and sediment samples were dried and digested in nitric acid and hydrogen peroxide prior to analysis by flame atomic absorption spectrophotometry.

C. tentans sampled in the tests were immediately placed in 7 mL plastic test tubes, and dried in an oven at 55°C, until they reached a constant weight. Dried chironomids were digested in nitric acid and then analyzed on an atomic absorption spectrophotometer to determine concentrations of each of the four metals. Samples (n=3) of 40 second-instar *C. tentans* were also taken directly from the cultures to determine concentrations of metals at the start of each test.

Quality-control and quality assurance procedures included analysis of National Institute of Standards & Technology (NIST) bovine liver (standard reference material

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1577b) and Buffalo River sediment (standard reference material 2704), HNO_3 blanks, water blanks, and Cd, Cu, Pb, and Zn standards. Average recovery of Cd, Cu, Pb, and Zn from NIST bovine liver tissue (n=6) was 77, 100, 83, and 105%, respectively (Figure 1.1). Average recovery of Cd, Cu, Pb, and Zn from NIST Buffalo River sediment (n=6) was 86, 79, 93, and 76%, respectively (Figure 1.1). Metals in HNO_3 and water blanks were consistently below detection. Metal standards were within 95% of nominal concentrations. To minimize contamination, all labware was acid washed and plastic materials were used wherever possible.

Statistical analysis

All statistical analyses were performed using a PC version of Statistical Analysis System (SAS Institute 1985). Results from the toxicity test were analyzed using a one-way analysis of variance (ANOVA) technique to compare treatments. Tukey's Honest Significant Difference (HSD) multiple comparison test was performed to analyze all pairwise comparisons, with alpha equal to 0.05. HSD tests were also performed to compare bioaccumulation of each metal among treatments in the toxicity test. An LC50 value was calculated for the toxicity test using a trimmed Spearman-Karber method.

Linear and polynomial regression analyses were used to examine the relationship between concentration of metal in *C. tentans* and exposure time in the uptake and depuration tests. Due to heterogeneity of variances, analyses were performed on square-root transformed data. A square-root transformation was chosen over the more common log transformation based on comparisons of scatter plots and r^2 values. Linear, quadratic, and cubic terms were tested for significance, for each metal.

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RESULTS

Physicochemical characteristics

In general, conductivity increased (from 258 to 488 µmho) with increasing metals concentrations in the toxicity test. pH averaged 7.25 (±0.22) for all treatments except 5.6 X. pH for this treatment averaged 6.07 (±0.33). There was little variability in dissolved oxygen (7.4 ± 0.3 mg/L) across treatments. Concentrations of metals measured in the sediment were generally within 70% of nominal concentrations, and concentrations of metals in the interstitial water were typically an order of magnitude greater than in the overlying water (Table 1.1).

There was little variability in conductivity (415 ± 18 µmho; 396 ± 18 µmho), pH (7.4 ± 0.5; 7.0 ± 0.6), and dissolved oxygen (5.9 ± 0.3 mg/L; 6.7 ± 0.3 mg/L) in the first and second uptake tests. Concentrations of metals measured in the sediment were generally within 80% of nominal concentrations (Tables 1.2 and 1.3). There was also little variability in conductivity (400 ± 16 µmho), pH (6.8 ± 0.4) and dissolved oxygen (6.4 ± 0.2 mg/L) in the depuration test.

Toxicity

Control mortality averaged 17.5%, indicating that the synthetic sediment was capable of supporting *C. tentans* over the course of the test (Figure 1.2). *C. tentans* exposed to metals in synthetic sediment at concentrations of 0.7 X and higher exhibited significantly greater mortality than controls. All chironomids exposed to the 5.6 X treatment died within six days. The calculated LC50 value was 0.53 X, with a 95% confidence interval of 0.46 X to 0.62 X.

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Metals concentrations in *C. tentans* increased with sediment levels (Figure 1.3). Chironomids exposed to the 1.0 X treatment accumulated significantly greater concentrations of Cd, Pb, and Zn than controls. Bioaccumulation data are not presented for the 2.8 X and 5.6 X treatments because of high mortality in these treatments.

Differences in behavior among treatments were observed in the toxicity test. *C. tentans* exposed to low concentrations of metals (0 X through 0.35 X) burrowed 2 to 4 cm into the sediment within a few hours and were typically not visible on the surface throughout the test period. Chironomids exposed to higher concentrations of metals (0.7 to 2.8 X) generally remained on the surface of the sediment or did not burrow as deep. Chironomids exposed to the highest concentration of metals (5.6 X) were generally inactive within one day of being placed into the beakers.

Uptake

C. tentans were exposed to 0.35 X in two uptake tests. This concentration was the highest treatment tested where survival did not differ significantly from the control. Survival averaged 77.6% (±12.3, n=24) and 76.4% (±18.1, n=18) in the first and second uptake tests, respectively.

Concentrations of Cd, Cu, and Zn in *C. tentans* increased over the 14 d uptake tests (Figures 1.4 and 1.5). Concentrations of Pb initially increased sharply, decreased by 4 d, and then increased more gradually. Linear and polynomial regression analyses were performed to examine the relationship between concentration of each metal in the chironomids and exposure time. Linear regression models were selected for Cd, Cu, Pb, and Zn based on the significance of r^2 values (Table 1.4). Concentrations of metals in *C.*

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tentans were highly correlated with exposure time, and r^2 values ranged from 0.67 to 0.96. The r^2 values were highest for Cd in both uptake tests.

Patterns of uptake for each metal were similar between the two experiments except that uptake of the metals appeared to reach a maximum at 14 d in the first test and at 10 d (with the exception of Pb), in the second test. Concentrations of Pb increased more gradually over time than the other metals.

Accumulation of metals was fit to the first-order kinetic model. Uptake rate coefficients (k_s) were highest for Cd and lowest for Pb in both uptake tests (Table 1.5). There was good agreement between the two experiments for all k_s values.

Depuration

Concentrations of Cu in *C. tentans* approached levels measured in the cultures (background levels) within 4 d (Figure 1.5). However, tissue concentrations of Cd and Zn were still significantly greater than those measured in the cultures after 7 d depuration. Tissue concentrations of Cd leveled off by day four. Depuration of Pb was initially rapid then slowed, which is consistent with a first-order depuration model (Lee 1992). The chironomids depurated Zn, but concentrations were not significantly correlated with time (Table 1.4).

Depuration of the metals was fit to the first-order kinetic model. Pb had the highest depuration rate constant ($k2$) (Table 1.5) due to the steep initial portion of its curve. Zn had the lowest $k2$; however, this parameter was not significant (p=0.201).

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Bioaccumulation factors

Measured steady-state BAFs (Eqn. 3) were similar for both uptake tests, with BAFs highest for Cd and lowest for Pb (Table 1.6). These steady-state values compared favorably to values predicted by the kinetic model (Table 1.6). With the exception of Pb, steady-state BAFs were within a factor of about two of those calculated using the first-order kinetic model. BAFs for Pb were within about one order of magnitude.

DISCUSSION

Toxicity and Bioavailability

C. tentans exposed to Cd, Cu, Pb, and Zn in synthetic sediment at concentrations measured at the Arkansas River (1.0 X treatment) had significantly greater mortality than controls. If we could assume similar bioavailability, these results would suggest that sediments at the Arkansas River Superfund site are toxic to some benthic invertebrates. Field studies do indicate that benthic communities at the Arkansas River are severely degraded (Roline 1988 and Clements 1994). However, assumptions of similar bioavailability are problematic for many reasons.

Metals may be more bioavailable in the synthetic sediment used in our tests than in Arkansas River sediment (Frugis 1995), limiting comparisons between laboratory and field observations. This is a potential disadvantage associated with the use of synthetic sediment, particularly in metals toxicity testing. Redox potential of sediments may strongly influence metal bioavailability (Gambrell and Patrick 1988 and Suedel and Rodgers 1994a). For example, in reduced sediments, acid volatile sulfide (AVS) has been shown to play a significant role in regulating the toxicity of Cd (DiToro et al 1990).

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AVS was recently shown to be important in controlling metal solubility in the Clark Fork River, Montana (Brumbaugh et al. 1994). However, because the synthetic sediment is generally in an oxidized state (Eh value of 462 mV) (Walsh et al. 1992), AVS probably did not modify toxicity and bioaccumulation of metals in our studies. Because synthetic sediments are generally oxidized, metals may be more bioavailable than in natural sediment.

Total organic carbon (TOC) is another factor that has been shown to influence the bioavailability of metals in sediment (Breteler and Saksa 1985 and Luoma 1989). While the nominal amount of organic matter (3%) for the synthetic sediment is known, TOC was not measured in the experiments. Since measured values for metals concentrations did not always closely agree with nominal concentrations, it may be reasonable to suspect that measured values for TOC deviated from the nominal value as well. This further limits comparisons to the field.

Metals may be considered bioavailable if they are transferred from sediment to a benthic invertebrate (Lee 1991). We observed significant increases in concentrations in *C. tentans* with increases in concentrations in sediment for each metal except Cu. It may be that the chironomids were able to regulate this essential metal. Other studies have shown that animals regulate concentrations of essential metals better than non-essential metals (Amiard et al. 1987 and Krantzberg and Stokes 1989). Although concentrations of the essential metals Cu and Zn in *C. tentans* exposed to the 1.0 X treatment were similar to those measured in Orthocladiinae chironomids from the field, concentrations of the non-essential metal Cd were generally an order of magnitude greater in the laboratory (W.H. Clements, unpublished data). These results may indicate that Cd is more

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bioavailable in this synthetic sediment than in natural sediment.

The effects of mixtures cannot always be predicted from the effects of single metals (Ahsanullah et al. 1988 and Kraak et al. 1994). The relative proportions of the four metals in the mixture may have influenced toxicity and bioaccumulation of each individual metal. In an experiment in which Zn was removed from the mixture, survival of *C. tentans* exposed to the 1.0 X treatment of the three-metal mixture was significantly greater than in the four-metal mixture (73% vs. 27.5%) (E.A. Harrahy, unpublished data). While an additive model of toxicity may, in general, adequately describe the combined effects of metals, the type of action may deviate from additivity when low concentrations of one metal are combined with high concentrations of another. Parrott and Sprague (1993) showed an antagonistic relationship between low concentrations of copper and high concentrations of zinc. On the other hand, although Cd was present in low concentration relative to the other metals, it has been shown to be quite toxic to second-instar *Chironomus* (Williams et al. 1986), and its relative contribution in causing the toxicity may have been high. Khangarot and Ray (1989) showed the decreasing order of toxicity to *C. tentans* to be Cu > Cd > Zn > Pb in 48 h EC50 water-only tests.

While mixture experiments more realistically reflect polluted environments, interactions among metals have been shown to depend also on concentration (Sprague and Ramsay 1965), water hardness (Lloyd 1961), and production of metal binding proteins (Wicklund et al. 1988). To determine the relative contribution of Cd, Cu, Pb, and Zn in causing the observed toxicity, additional experiments would need to be conducted with single metals and mixtures of metals in the synthetic sediment.

A lower pH was measured in the highest (5.6 X) treatment. Khangarot and Ray

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(1989) also noted decreased pH values with higher metals test concentrations. While it is possible that the lower pH adversely affected *C. tentans*, *Chironomus* has been shown to survive in water with a pH as low as 4.4 (Krantzberg and Stokes 1988). If the lower pH contributed to the observed toxicity, it is more likely to have occurred through increased bioavailability of the metals.

Toxicokinetics

Concentrations of metals in *C. tentans* were significantly correlated with exposure time. Kinetic studies suggest that concentrations of metals in aquatic insects increase linearly over time until an apparent steady state is reached between influx and efflux (Hare et al. 1991, Timmermans et al. 1992, and Timmermans et al. 1992). With the exception of Pb, an apparent steady state was reached within about ten days. A longer exposure period may be necessary to estimate the time to steady state for Pb. However, given the relatively short life span of *C. tentans* (24-28 d at 20°C) (ASTM 1994) it is possible that a steady state may not be attained.

After 7 d depuration, concentrations of Cd, Pb, and Zn in *C. tentans* were still significantly greater than background concentrations measured in the cultures. Levels of Cd and Pb were approximately 12 and 25 times these initial concentrations. The steep initial portions of the depuration curves, particularly for Pb, may have been due to evacuation of the gut contents. Metals in gut contents of aquatic insects have been reported to represent up to 65% of the total quantity of metal in the body (Elwood et al. 1976 and Chapman 1985). Gut contents were not eliminated prior to metal analysis. The flatter portion of the depuration curves may have been due to induction of metal binding

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proteins, which have low exchange rates (Lee 1992). However, regression analyses indicated that the curves were significantly linear for Cu and Pb, and not significantly biphasic for any of the metals.

Uptake rate coefficients and depuration rate constants were calculated using the first-order kinetic model. In general, the assumption of constant uptake and depuration rates was not significantly violated. Uptake and depuration curves were generally linear. Although changes in bioavailability and induction of metal binding proteins have been cited as possible interferences in application of this model to metals (Lee 1992 and U.S. EPA 1994), they did not appear to be a significant factor in the present studies. Uptake rate coefficients and depuration rate constants were used to determine BAFs. Single compartment models have been successfully used to estimate transfer between whole aquatic insects and their environments (Hare 1991, Timmermans et al. 1992, and Timmermans et al. 1992). However, BAFs for metals are usually measured directly (Eqn. 3), rather than calculated using the first-order kinetic model, probably because it is a simpler alternative.

Direct calculation of BAFs assumes that metal concentrations in organisms are at steady state with metal concentrations in sediment. I calculated BAFs using the concentration of metal in *C. tentans* after 14 d exposure. The assumption of steady state may have been violated for Pb since concentrations of this metal may still have been increasing at 14 d, giving an underestimated BAF when the lower tissue concentration is divided by the sediment concentration. BAFs calculated for the first and second uptake tests were similar.

BAFs calculated using the first-order kinetic model were similar to measured

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steady-state BAFs. BAFs calculated both ways were highest for Cd and lowest for Pb. The high BAFs for Cd may have been due to the inability of the chironomids to regulate this non-essential metal. As noted above, the high BAFs for Cd may have also been due to its greater relative bioavailability in synthetic sediment. The low BAFs for Pb indicate that this metal was relatively less bioavailable. Studies have shown that Pb has a low bioaccumulation potential relative to other metals (Johnson 1987 and Hare et al. 1991).

Conclusions

Results of this study show that a mixture of heavy metals in a synthetic sediment readily accumulated in a benthic invertebrate, allowing comparisons of predicted to steady-state BAFs. To establish a strong causal relationship between heavy metal contamination and ecological effects, and to develop sediment quality criteria, a triad of information that combines data from chemistry, ecological surveys, and toxicity tests, has been recommended (Chapman 1989 and Canfield et al. 1994). Synthetic sediments may serve as reference and dilution sediments in toxicity tests (Suedel and Rodgers 1994a), and may be useful for studies designed to examine abiotic factors that control bioavailability. However, studies comparing toxicity and bioaccumulation of natural and synthetic sediments are necessary before the use of synthetic sediments is widely adopted. Frugis (1995) demonstrated greater bioaccumulation of metals in *C. tentans* exposed to spiked synthetic sediment (83% sand) than spiked natural sediment. The high BAFs calculated in the present studies may also be indicative of greater bioavailability, and limit inferences to the Arkansas River. Thus, although the ability to prepare synthetic sediments to match natural sediments with respect to particle size and organic

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matter content is an advantage (Walsh et al. 1992, Suedel and Rodgers 1994a, and Suedel and Rodgers 1994b), it may be difficult to match synthetic sediments with respect to all of the properties that may affect bioavailability of metals. Thus, inferences concerning toxicity and bioaccumulation in field sediments may be limited.

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LITERATURE CITED

Ahsanullah, M. M.C. Mobley and **P. Rankin.** 1988. Individual and combined effects of zinc, cadmium and copper on the marine amphipod *Allorchestes compressa*. *Aust. J. Mar. Freshwater Res.* **39**:33-37.

American Society for Testing and Materials. 1994. Standard guide for conducting sediment toxicity tests with freshwater invertebrates. ASTM E 1383-94. In *Annual Book of ASTM Standards*, Vol. 11.04 Philadelphia, PA, USA, pp. 1-19.

Amiard, J.C., C. Amiard-Triquet, B. Berthet and **C. Metayer.** 1987. Comparative study of the patterns of bioaccumulation of essential (Cu, Zn) and non-essential trace metals in various estuarine and coastal organisms. *J. Exp. Mar. Biol. Ecol.* **106**:73-89.

Boese, B.L. and **H. Lee II.** 1992. Synthesis of methods to predict bioaccumulation of sediment pollutants. ERL-N Contribution No. N232. Bioaccumulation/Stratozone Team, Pacific Ecosystems Branch, Environmental Research Laboratory-Narragansett, U.S. Environmental Protection Agency, Newport, OR.

Brannon, M.J., R.H. Plumb, Jr. and **I. Smith, Jr.** 1980. Long term release of heavy metals from sediments. In R.A. Baker, ed., *Contaminants and Sediments*, Vol.1. Ann Arbor Science, Ann Arbor, MI, pp. 221-266.

Breteler, R.J. and **F.I. Saksa.** 1985. The role of sediment organic matter on sorption-desorption reactions and bioavailability of mercury and cadmium in an intertidal ecosystem. In R.D. Cardwell, R. Purdy and R.C. Bahner, eds., *Aquatic Toxicology and Hazard Assessment: Seventh Symposium*. ASTM STP 854. American Society for Testing and Materials, Philadelphia, PA, USA, pp. 454-468.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Brumbaugh, W.G., C.G. Ingersoll, N.E. Kemble, T.W. May and **J.L. Jajicek.** 1994. Chemical characterization of sediments and pore water from the upper Clark Fork River, Montana. *Environ. Toxicol. Chem.* **13**:1971-1983.

Burton, G.A., Jr. 1991. Assessing the toxicity of freshwater sediments. *Environ. Toxicol. Chem.* **10**:1585-1627.

Cairns, M.A., A.V. Nebeker, J.H. Gakstatter and **W.L. Griffis.** 1984. Toxicity of copper-spiked sediments to freshwater invertebrates. *Environ. Toxicol. Chem.* **3**:435-445.

Canfield, T.L., N.E. Kemble, W.G. Brumbaugh, F.J. Dwyer, C.G. Ingersoll, and J.F. Fairchild. 1994. Use of benthic invertebrate community structure and the sediment quality triad to evaluate metal-contaminated sediment in the upper Clark Fork River, Montana. *Environ. Toxicol. Chem.* **13:**1999-2012.

Chapman, P.M. 1985. Effects of gut sediment contents on measurements of metal levels in benthic invertebrates- a cautionary note. *Bull. Environ. Contam. Toxicol.* **35**:345-347.

Chapman, P.M. 1989. Current approaches to developing sediment quality criteria. *Environ. Toxicol. Chem.* **8**:589-599.

Ciborowski, J.J.H., E.C. Hanes and **L.D. Corkum.** 1991. Standardized materials and procedures for *Hexagenia*, a benthic bioassay organism: application to 21-day sediment bioassays. *Proceedings*, Ontario Ministry of the Environment Technology Transfer Conference on Environmental Research, Toronto, ON, Canada, Nov. 25-26, 1991, **II**:676-684.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Clements, W.H. 1994. Benthic invertebrate community responses to heavy metals in the Upper Arkansas River Basin, Colorado. *J. North Am. Benthol. Soc.* **13**:30-44.

DiToro, D.M., J.D. Mahony, D.J. Hansen, K.J. Scott, M.B. Hicks, S.M. Mayr and **M.S. Redmond.** 1990. Toxicity of cadmium in sediments: the role of acid volatile sulfide. *Environ. Toxicol. Chem.* **9**:1487-1502.

Elwood, J.W., S.G. Hildebrand and **J.J. Beauchamp.** 1976. Contribution of gut contents to the concentration and body burden of elements in *Tipula* spp. from a spring-fed stream. *J. Fish. Res. Board Can.* **33**:1930-1938.

Frugis, M. 1995. Bioavailability of metal mixtures in natural and synthetic sediments. M.S. thesis. Colorado State University, Fort Collins, CO.

Gambrell, R.P. and **W.H. Patrick, Jr.** 1988. The influence of redox potential on the environmental chemistry of contaminants in soils and sediments. In D.D. Hook et al. eds., *The Ecology and Management of Wetlands*, Vol.1-Ecology of Wetlands. Timber Press, Portland, OR, pp. 319-333.

Hare, L., P.G.C. Campbell, A. Tessier and **N. Belzile.** 1989. Gut sediments in a burrowing mayfly (Ephemeroptera, *Hexagenia limbata*): their contribution to animal trace element burdens, their removal, and the efficacy of a correction for their presence. *Can. J. Fish. Aquat. Sci.* **46**:451-456.

Hare, L., E. Saouter, P.G.C. Campbell, A. Tessier, F. Ribeyre and **A. Boudou.** 1991. Dynamics of cadmium, lead, and zinc exchange between nymphs of the burrowing mayfly *Hexagenia rigida* (Ephemeroptera) and the environment. *Can. J. Fish. Aquat. Sci.* **48**:39-47.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Hare, L., A. Tessier and **P.G.C. Campbell.** 1991. Trace element distributions in aquatic insects: variations among genera, elements, and lakes. *Can. J. Fish. Aquat. Sci.* **48**:1481-1491.

Jennett, J.C., S.W. Effler and **B.G. Wixson.** 1980. Mobilization and toxicological aspects of sedimentary contaminants. In R.A. Baker, ed., *Contaminants and Sediments*, Vol. 1. Ann Arbor Science Publishers, Inc., Ann Arbor, MI. pp. 429-444.

Johnson, M.G. 1987. Trace element loadings to sediments of fourteen Ontario lakes and correlations with concentrations in fish. *Can. J. Fish. Aquat. Sci.* **44**:3-13.

Khangarot, B.S. and **P.K. Ray.** 1989. Sensitivity of midge larvae of *Chironomus tentans* Fabricius (Diptera: Chironomidae) to heavy metals. *Bull. Environ. Contam. Toxicol.* **42**:325-330.

Kraak, M.H.S., D. Lavy, H. Schoon and **M. Toussaint.** 1994. Ecotoxicity of mixtures of metals to the zebra mussel, *Dreissena polymorpha*. *Environ. Toxicol. Chem.* **13**:109-114.

Krantzberg, G. and **P.M. Stokes.** 1988. The importance of surface adsorption and pH in metal accumulation by chironomids. *Environ. Toxicol. Chem.* **7**:653-670.

Krantzberg, G. and **P.M. Stokes.** 1989. Metal regulation, tolerance, and body burdens in the larvae of the genus *Chironomus*. *Can. J. Fish. Aquat. Sci.* **46**:389-398.

Landrum, P.F. 1989. Bioavailability and toxicokinetics of polycyclic aromatic hydrocarbons sorbed to sediments for the amphipod *Pontoporeia hoyi*. *Environ. Sci. Technol.* **23**:588-595.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Landrum, P.F., W.S. Dupuis and **J. Kukkonen.** 1994. Toxicokinetics and toxicity of sediment-associated pyrene and phenanthrene in *Diporeia* spp.: examination of equilibrium-partitioning theory and residue-based effects for assessing hazard. *Environ. Toxicol. Chem.* **13**:1769-1780.

Landrum, P.F., H. Lee II and **M.J. Lydy.** 1992. Toxicokinetics in aquatic systems: model comparisons and use in hazard assessment. *Environ. Toxicol. Chem.* **11**:1709-1725.

Landrum, P.F. and **J.A. Robbins.** 1990. Bioavailability of sediment-associated contaminants to benthic invertebrates. In R. Baudo, J.P. Geisy and H. Muntau, eds., *Sediments: Chemistry and Toxicity of In-Place Pollutants*. Lewis Publishers, Inc., Ann Arbor, MI, pp. 237-257.

Landrum, P.F. and **D. Scavia.** 1983. Influence of sediment on anthracene uptake, depuration, and biotransformation by the amphipod *Hyalella azteca*. *Can. J. Fish. Aquat. Sci.* **40**:298-305.

Larsson, P. 1985. Contaminated sediments of lakes and oceans act as sources of chlorinated hydrocarbons for release to water and atmosphere. *Nature* **317**:347-349.

Lee, H., II. 1991. A clam's eye view of the bioavailability of sediment-associated pollutants. In R. Baker, ed., *Organic Substances in Water*, Volume 3- Biological. Lewis Publishers, Inc., Chelsea, MI, pp. 73-93.

Lee, H., II. 1992. Models, muddles, and mud: predicting bioaccumulation of sediment-associated pollutants. In G.A. Burton, Jr., ed., *Sediment Toxicity Assessment*. Lewis Publishers, Inc., Ann Arbor, MI, pp. 267-293.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Lloyd, R. 1961. The toxicity of mixtures of zinc and copper sulfates to rainbow trout (*Salmo gairdneri* Richardson). *Ann. Appl. Biol.* **49**:535-538.

Luoma, S.N. 1989. Can we determine the biological availability of sediment-bound trace elements? *Hydrobiologia* **176/177**:379-396.

McIntosh, A. 1991. Trace metals in freshwater sediments: a review of the literature and an assessment of research needs. In M.C. Newman and A.W. McIntosh, eds., *Metal Ecotoxicology- Concepts and Applications*. Lewis Publishers, Chelsea, MI. pp. 243-260.

Parrott, J.L. and **J.B. Sprague.** 1993. Patterns in toxicity of sublethal mixtures of metals and organic chemicals determined by Microtox and by DNA, RNA, and protein content of fathead minnows (*Pimephales promelas*). *Can. J. Fish. Aquat. Sci.* **50**:2245-2253.

Roline, R.A. 1988. The effects of heavy metals pollution of the upper Arkansas River on the distribution of aquatic macroinvertebrates. *Hydrobiologia* **160**:3-8.

SAS Institute. 1985. *SAS Procedures Guide for Personal Computers, Version 6 Edition.* Cary, NC.

Sorensen, E.M. 1991. *Metal Poisoning in Fish.* CRC Press, Ann Arbor, MI.

Sprague, J.B. and **B.A. Ramsay.** 1965. Lethal levels of mixed copper-zinc solutions for juvenile salmon. *J. Fish. Res. Board Can.* **22**:425-432.

Suedel, B.C. and **J.H. Rodgers, Jr.** 1994a. Development of formulated reference sediments for freshwater and estuarine sediment testing. *Environ. Toxicol. Chem.* **13**:1163-1175.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Suedel, B.C. and **J.H. Rodgers, Jr.** 1994b. Responses of *Hyalella azteca* and *Chironomus tentans* to particle-size distribution and organic matter content of formulated and natural freshwater sediments. *Environ. Toxicol. Chem.* **13**:1639-1648.

Thomann, R.V., J.P. Connolly and **T.F. Parkerton.** 1992. An equilibrium model of organic chemical accumulation in aquatic food webs with sediment interaction. *Environ. Toxicol. Chem.* **11**:615-629.

Timmermans, K.R., W. Peeters and **M.Tonkes.** 1992. Cadmium, zinc, lead, and copper in larvae of *Chironomus riparius* (Meigen) (Diptera, Chironomidae): uptake and effects. *Hydrobiologia* **241**:119-134

Timmermans, K.R., E. Spijkerman, M. Tonkes and **H. Govers.** 1992. Cadmium and zinc uptake by two species of aquatic invertebrate predators from dietary and aqueous sources. *Can. J. Fish. Aquat. Sci.* **49**:655-662.

Titus, J.A. and **R.M. Pfister**. 1982. Effects of pH, temperature, and Eh on the uptake of cadmium by bacteria and an artificial sediment. *Bull. Environm. Contam. Toxicol.* **28**:697-704.

U.S. Environmental Protection Agency. 1994. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA 600/R-94/024. Office of Research and Development. Duluth, MN.

Walsh, G.E., D.E. Weber, L.K. Esry, M.T. Nguyen, J. Noles and **B. Albrecht.** 1992. Synthetic substrata for propogation and testing of soil and sediment organisms. *Pedobiologia* **36**:1-10.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Walsh, G.E., D.E. Weber, M.T. Nguyen and **L.K. Esry**. 1991. Responses of wetland plants to effluents in water and sediment. *Environ. Exp. Bot.* **31**:351-358.

Walsh, G.E., D.E. Weber, T.L. Simon and **L.K. Brashers.** 1991. Toxicity tests of effluents with marsh plants in water and sediment. *Environ. Toxicol. Chem.* **10**:517-525.

Watzin, M.C., P.F. Roscigno and **W.D. Burke.** 1994. Community-level field method for testing the toxicity of contaminated sediments in estuaries. *Environ. Toxicol. Chem.* **13**:1187-1193.

Wentsel, R., A. McIntosh and **G. Atchison.** 1977. Sublethal effects of heavy metal contaminated sediment on midge larvae (*Chironomus tentans*). *Hydrobiologia* **56**:153-156.

Wicklund, A., P. Runn and **L. Norrgren.** 1988. Cadmium and zinc interactions in fish: effects of zinc on uptake, organ distribution, and elimination of ^{109}Cd in the zebrafish, *Brachydanio rerio*. *Arch. Environ. Contam. Toxicol.* **17**:345-354.

Williams, K.A., D.W.J. Green, D. Pascoe and **D.E. Gower.** 1986. The acute toxicity of cadmium to different larval stages of *Chironomus riparius* (Diptera: Chironomidae) and its ecological significance for pollution regulation. *Oecologia* **70**:362-366.

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Table 1.1. Concentrations (in ug/g for sediment and mg/L for interstitial and overlying water) of metals measured in the *C. tentans* toxicity test. Concentrations of metals in each phase were measured on day 0 (48 hours after addition of overlying water, before addition of chironomids) and day 10.

		Day 0				Day 10			
Phase	Trt. (X)	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
Sediment	0.0	0.15	1.00	BD	2.04	BD	1.02	0.42	2.29
	0.0875	0.98	1.17	5.14	20.51	0.44	1.58	5.33	20.69
	0.175	1.18	1.57	9.10	39.52	0.68	2.19	8.09	37.05
	0.35	2.50	3.27	18.57	75.49	1.48	3.67	18.12	68.12
	0.70	3.45	4.93	28.65	106.35	2.92	6.69	34.63	148.85
	1.0	8.02	12.93	67.75	254.50	4.12	9.32	51.83	208.98
	1.4	1.57	23.00	29.76	136.49	3.93	9.64	51.12	213.48
	2.8	16.16	26.93	141.86	463.28	7.51	20.58	182.75	356.48
	5.6*	22.03	30.04	214.30	857.23	11.5	34.16	224.99	596.33
Interstitial	0	0.00	0.02	0.19	1.33	0.00	0.04	0.06	0.13
	0.0875	0.07	0.05	0.27	1.00	0.02	0.05	0.17	0.64
	0.175	0.07	0.03	0.30	1.86	0.03	0.06	0.26	0.33
	0.35	0.08	0.08	0.41	4.11	0.10	0.15	0.83	3.91
	0.70	0.29	0.25	1.65	18.57	0.15	0.18	0.88	7.38
	1.0	0.71	0.64	3.84	10.64	0.27	0.19	0.11	15.50
	1.4	1.58	1.36	7.39	18.44	0.32	0.15	0.67	18.20
	2.8	7.16	2.24	17.36	812.00	1.00	2.00	11.00	124.00

	5.6*	17.50	8.82	60.90	720.00	9.00	2.00	19.00	391.00
Overlying	0	0.00	BD	BD	BD	BD	BD	BD	0.01
	0.0875	0.00	BD	BD	0.03	0.00	BD	0.02	0.03
	0.175	0.00	0.00	BD	0.10	0.00	BD	0.02	0.14
	0.35	0.01	0.00	BD	0.46	0.01	BD	0.01	0.58
	0.70	0.03	0.00	BD	1.77	0.03	0.00	0.03	1.96
	1.0	0.05	BD	BD	3.15	0.08	0.00	0.03	3.51
	1.4	0.07	0.00	BD	5.14	0.15	0.00	0.02	5.26
	2.8	0.36	0.12	0.37	4.88	0.95	0.04	0.12	36.28
	5.6*	0.72	0.24	3.17	6.48	3.67	0.47	6.90	160.23

*The 5.6 X beakers were broken down after 6 d due to mortality.

BD = Below detection

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Table 1.2. Concentrations (in ug/g for sediment and mg/L for interstitial water) of metals and (standard deviation) measured over time in the first *C. tentans* uptake test.

	Phase	Time (d)	Cd	Cu	Pb	Zn
Control	Sediment	1	0.08	0.70	BD	9.23
		14	0.08	0.85	BD	3.10
	Interstitial	1	BD	0.06	BD	0.18
		14	BD	0.02	BD	0.07
Spiked	Sediment	0	1.35	3.42	15.56	71.37
			± 0.06	± 0.22	± 0.02	± 4.15
		1	1.13	3.07	13.94	63.26
			± 0.04	± 0.20	± 1.02	± 6.33
		2	1.05	2.85	13.15	58.26
			± 0.13	± 0.30	± 0.98	± 9.72
		4	1.50	3.72	18.96	76.64
			± 0.11	± 0.30	± 1.80	± 3.91
		7	0.98	2.69	11.48	53.50
			± 0.10	± 0.09	± 1.25	± 5.28
		10	1.33	3.40	15.35	72.86
			± 0.15	± 0.25	± 1.45	± 13.63

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	14	1.95	4.94	25.65	123.83
		± 0.11	± 0.21	± 0.16	± 24.11
Interstitial	0	0.08	0.23	1.69	8.00
		± 0.11	± 0.25	± 0.88	± 4.40
	1	0.02	0.09	0.60	4.78
		± 0.03	± 0.09	± 0.75	± 0.27
	2	0.01	0.05	0.35	3.60
		± 0.00	± 0.02	± 0.00	± 0.35
	4	0.04	0.11	0.53	5.30
		± 0.02	± 0.07	± 0.21	± 0.58
	7	BD	0.07	0.23	2.31
			± 0.03	± 0.02	± 0.06
	10	0.12	0.24	1.43	5.52
		± 0.02	± 0.06	± 0.68	± 1.10
	14	0.04	0.10	1.09	3.70
		± 0.01	± 0.06	± 0.14	± 0.83

Note: Dissolved metals in the overlying water were generally 70-90% of total. BD = Below detection; n= 1 for control sediment and interstitial samples; n= 2 for spiked sediment and interstitial samples.

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Table 1.3. Concentrations (in ug/g for sediment and mg/L for interstitial water) of metals and (standard deviation) measured over time in the second *C. tentans* uptake test and depuration test.

	Phase	Time (d)	Cd	Cu	Pb	Zn
Uptake	Sediment	0	1.40	3.15	17.98	70.52
			± 0.25	± 0.42	± 2.92	± 17.02
		1	1.69	3.99	21.75	82.19
			± 0.05	± 0.09	± 0.76	± 0.87
		7	1.72	4.32	22.74	82.08
			± 0.14	± 0.60	± 2.22	± 6.27
		14	1.54	3.53	20.19	77.05
			± 0.03	± 0.01	± 0.19	± 2.32
	Interstitial	0	0.10	0.19	0.84	6.85
			± 0.05	± 0.15	± 0.58	± 1.97
		1	0.11	0.20	0.90	6.38
			± 0.01	± 0.02	± 0.03	± 0.62
		7	0.23	0.40	2.15	6.48
			± 0.04	±0.08	± 0.24	± 0.16

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		14	0.10	0.17	1.12	4.17
			± 0.02	± 0.02	± 0.22	± 0.86
Depuration	Sediment	1	0.18	0.84	0.75	1.71
			± 0.03	± 0.09	± 0.18	± 0.65
		7	0.20	0.76	0.90	1.49
			± 0.00	± 0.23	± 0.25	± 0.44
	Interstitial	1	0.00	0.02	0.00	0.05
			± 0.00	± 0.00	± 0.00	± 0.01
		7	0.00	0.03	0.04	0.10
			± 0.00	± 0.00	± 0.00	± 0.00

Note: In addition to total metals reported above, dissolved metals were measured in the overlying water. Dissolved metals were generally 70-90% of total.
BD= Below detection; *n*= 2 for sediment and interstitial water samples

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Table 1.4. Results of linear regression analyses showing the relationship between concentration of metal in *C. tentans* (square-root transformed) and exposure time. Pearson r^2-values and p-values are presented.

Test	Metal	r^2	p-value
Uptake 1	Cd	0.912	0.0001
	Cu	0.668	0.0193
	Pb	0.902	0.0001
	Zn	0.787	0.0018
Uptake 2	Cd	0.964	0.0001
	Cu	0.882	0.0001
	Pb	0.802	0.0011
	Zn	0.852	0.0001
Depuration	Cd	0.560	0.0523
	Cu	0.652	0.0152
	Pb	0.617	0.0373
	Zn	0.183	0.7439

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Table 1.5. Estimated kinetic parameters (±SE) for heavy metal accumulation by *C. tentans* exposed to 0.35 X the base concentration.

Test	Parameter	Cd	Cu	Pb	Zn
Uptake 1	k_s[1]	5.092 ±0.430	1.136 ±0.276	0.263 ±0.061	0.526 ±0.211
Uptake 2	k_s	3.083 ±0.264	1.971 ±0.360	0.313 ±0.101	0.574 ±0.085
Depuration	$k2$[2]	0.079 ±0.037	0.103 ±0.027	0.715 ±0.320	0.023 ±0.017

[1] g dry sediment/(g dry tissue*day)
[2] day^{-1}

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Table 1.6. Comparison of measured steady-state to predicted bioaccumulation factors.

Test	Metal	BAF[1] ($k_s/k2$)	BAF[1] (Ct_{ss}/Cs)
Uptake 1	Cd	NA[2]	41.55
	Cu	NA	16.63
	Pb	NA	3.51
	Zn	NA	10.52
Uptake 2	Cd	39.02	23.73
	Cu	19.14	12.99
	Pb	0.44	7.08
	Zn	24.96	10.42

[1]g dry sediment/g dry tissue
[2]Not applicable because a depuration test was not conducted in conjunction with this uptake test.

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Figure 1.1. Average recovery of of Cd, Cu, Pb, and Zn from National Institute of Standards and Technology (NIST) bovine liver (standard reference material 1577b) and Buffalo River sediment (standard reference material 2704). Samples (*n*=6) of each standard were digested in nitric acid and analyzed by flame atomic absorption spectrophotometry.

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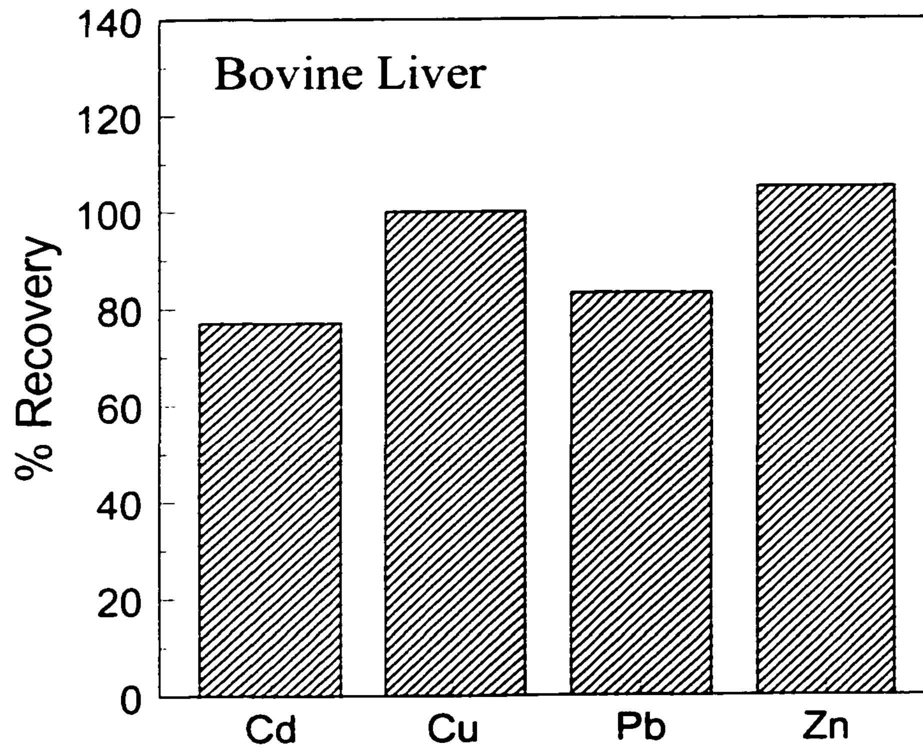

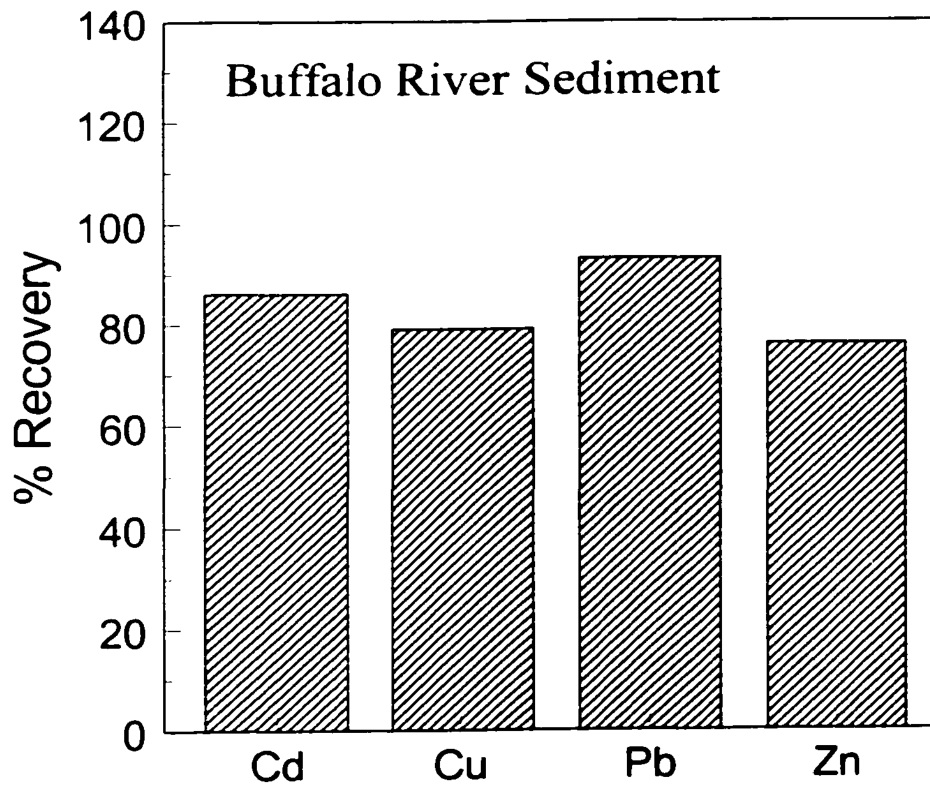

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Figure 1.2. Mean (±SD) percent mortality (n=3) of *C. tentans* larvae exposed to 0 through 5.6 X the base concentration of a mixture of Cd, Cu, Pb and Zn in synthetic sediment for 10 d. Means with the same letter were not significantly different (Tukey's HSD: $p<0.05$). The LC50 value (and 95% confidence interval) was calculated using the trimmed Spearman Karber method. All chironomids exposed to the 5.6 X treatment were dead in 6 d.

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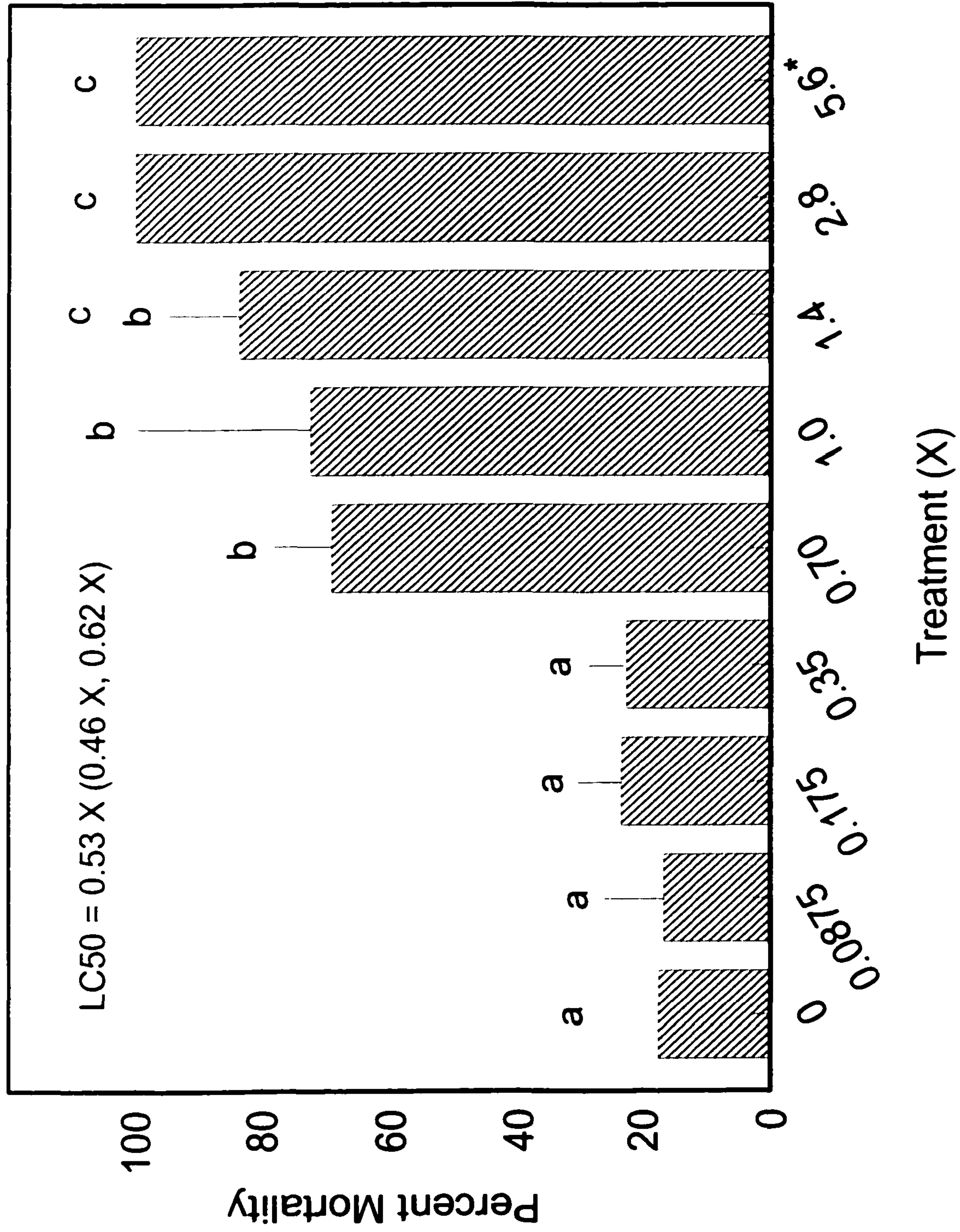

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Figure 1.3. Bioaccumulation of Cd, Cu, Pb, and Zn by *C. tentans* larvae after 10 d exposure to a mixture of the metals in synthetic sediment. F and p values are presented for ANOVA. Means (n=3) with the same letter were not significantly different (Tukey's HSD: $p<0.05$).

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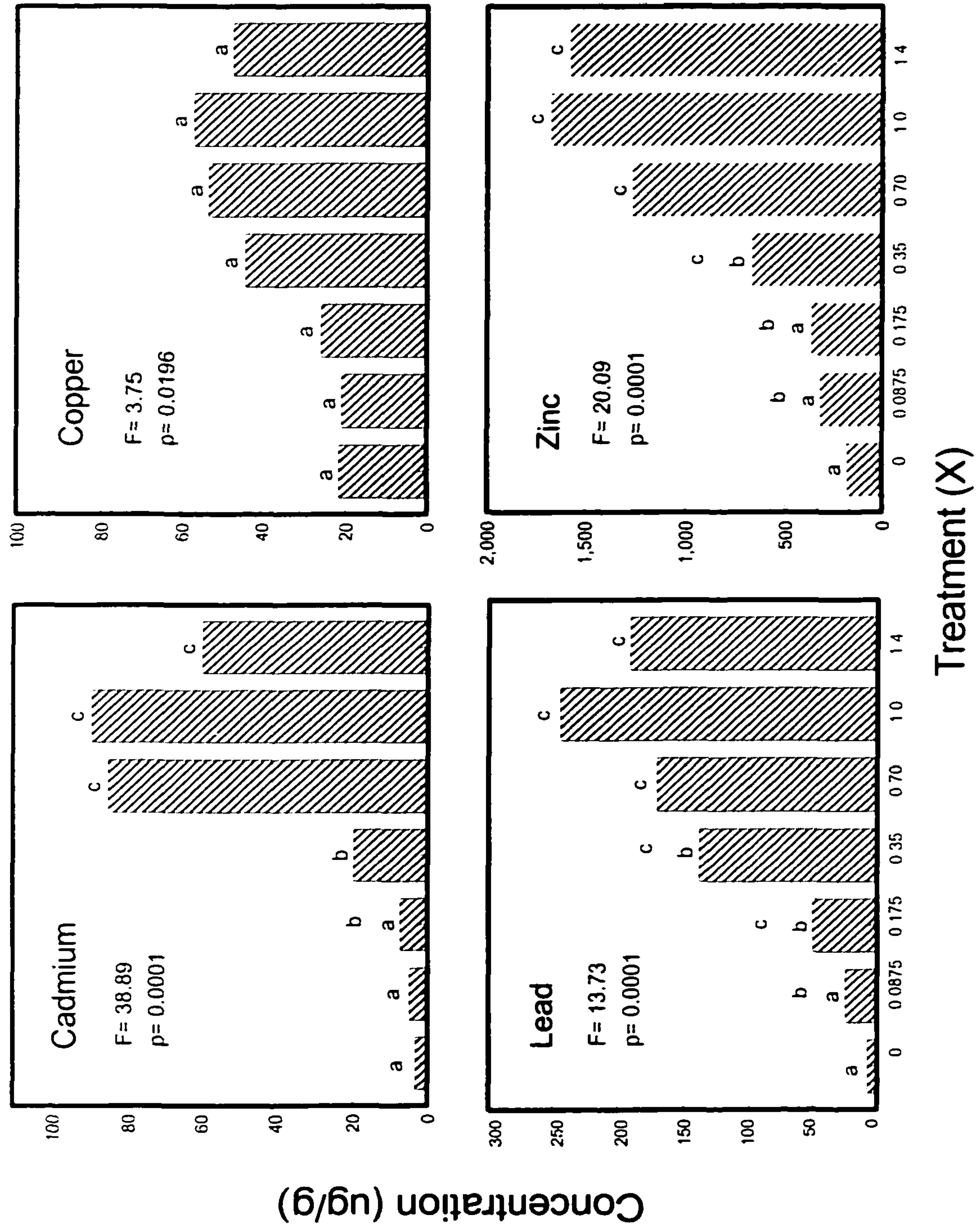

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Figure 1.4. Mean (±SD) concentrations of Cd, Cu, Pb, and Zn in *C. tentans* larvae over time, in the first uptake experiment. Chironomids were exposed to 0.35 X a base concentration of a mixture of the four metals in synthetic sediment.

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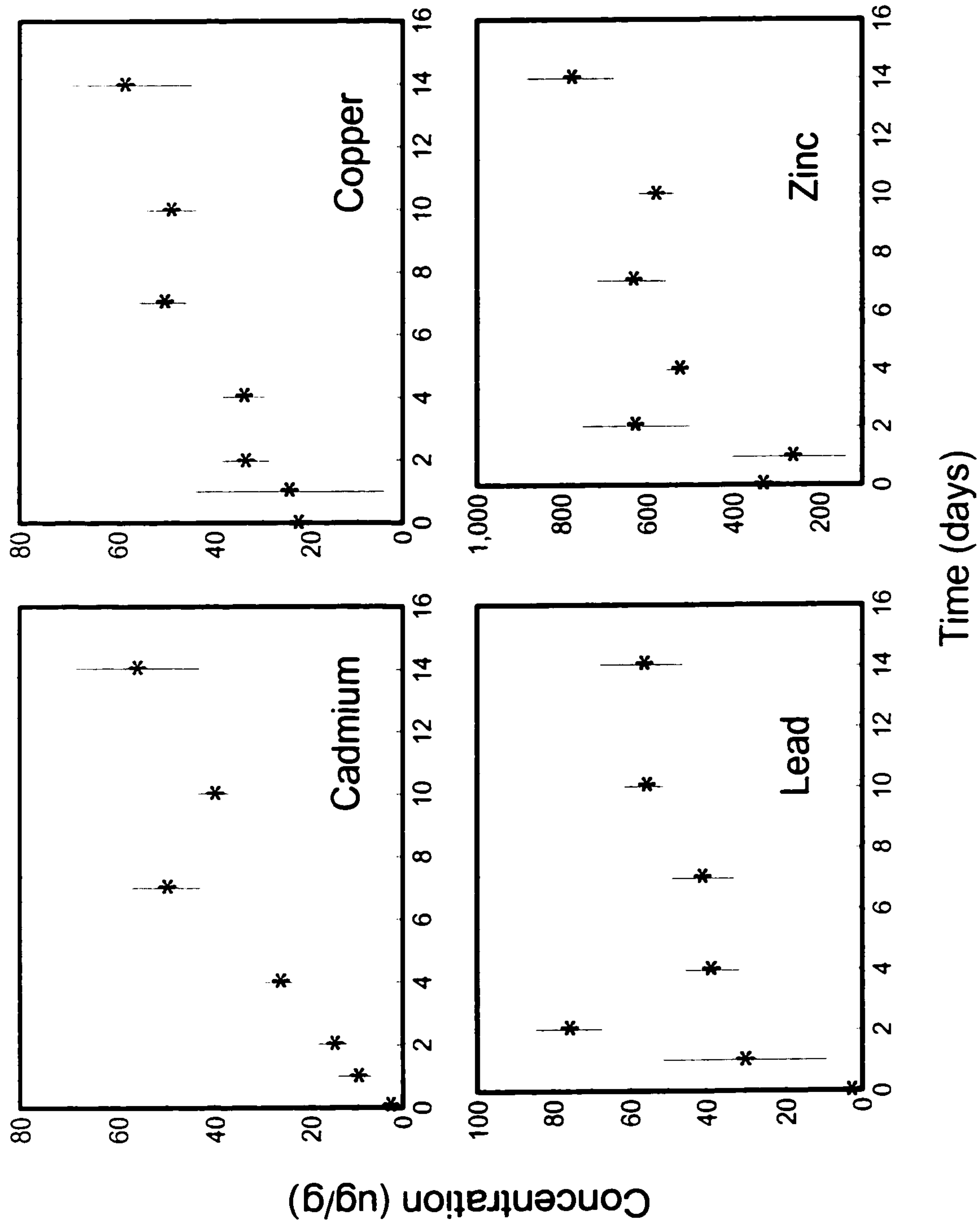

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Figure 1.5. Mean (±SD) concentrations of Cd, Cu, Pb, and Zn in *C. tentans* larvae over time, in the second uptake and the depuration experiments. Chironomids were exposed to 0.35 X a base concentration of a mixture of the four metals in synthetic sediment for 14 d and then transferred to beakers containing clean sediment.

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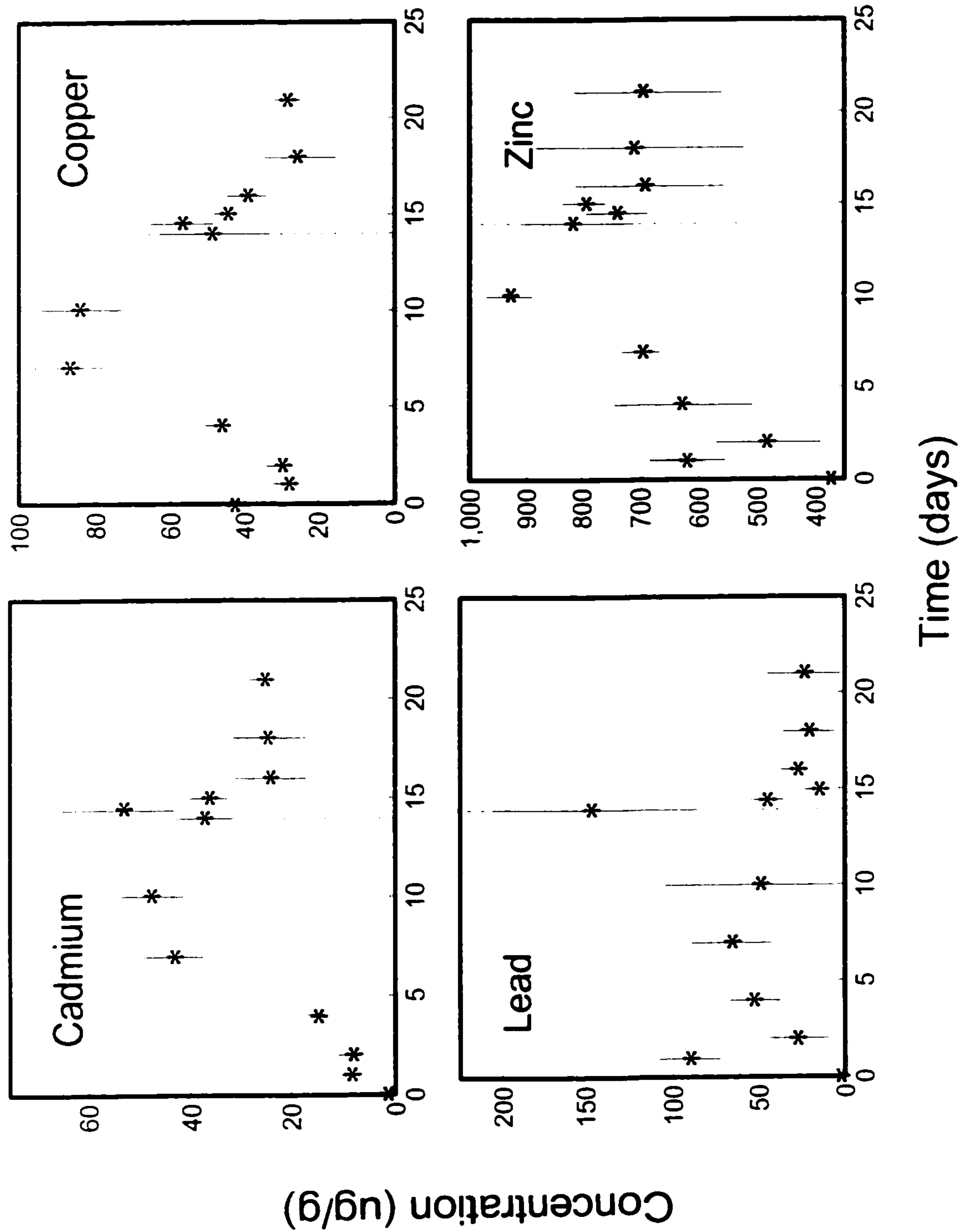

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CHAPTER 2

INFLUENCE OF PREVIOUS EXPOSURE TO METALS

ON SURVIVAL, GROWTH AND METALLOTHIONEIN CONCENTRATIONS

IN *BAETIS TRICAUDATUS* (EPHEMEROPTERA: BAETIDAE):

A COMPARISON OF POPULATIONS

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ABSTRACT

The purpose of this research was to compare survival and growth among populations of the mayfly *Baetis tricaudatus* (Ephemeroptera: Baetidae) previously exposed or naive to metals upon subsequent exposure to metals in the laboratory, and to compare concentrations of metallothionein among populations in the field. Mayflies were collected from sites located upstream (moderate concentrations of metals) and downstream (high concentrations of metals) of a U.S. Environmental Protection Agency (EPA) Superfund site on the Arkansas River and exposed to five treatments of a mixture of Cd, Cu, and Zn in a static acute toxicity test. Results indicated mayflies previously exposed to higher concentrations of metals in the field were significantly more tolerant than mayflies previously exposed to lower concentrations. In a second acute toxicity test mayflies were collected from the same upstream site, a downstream site located closer to the U.S. EPA Superfund site, and a true reference site and exposed to three treatments of a mixture of Cd, Cu, and Zn in flow-through microcosms. This experiment did not demonstrate significant tolerance in the previously exposed populations. Tolerance and costs of tolerance may be better detected at sublethal concentrations. A sublethal toxicity test conducted with these same populations of mayflies indicated that previously exposed populations bioaccumulated significantly higher concentrations of Cd. In addition, only the population previously exposed to moderate concentrations of metals was able to grow under subsequent exposure to metals. In addition, this population had significantly higher concentrations of the metal-binding protein metallothionein than either the reference population or the more highly contaminated population. While this protein may act as an underlying mechanism of tolerance, the majority of total metals present in these

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mayflies was not bound to metallothionein. Given the magnitude of total metals present in mayflies in the Arkansas River, the potential for adverse effects therefore, remains high.

INTRODUCTION

Populations chronically exposed to heavy metals often exhibit enhanced tolerance relative to naive populations (Antonovics et al., 1971; Bryan and Hummerstone, 1971; Stokes at al., 1973; Fraser et al., 1978; Wentsel et al., 1978; Weis et al., 1981; Nevo et al., 1984; Wilson, 1988; Diamond, 1989; Maltby, 1991; Miller and Hendricks, 1996; Groenendijk, 1999). Mechanisms associated with increased tolerance in natural populations include changes in metal uptake rates, the ability to bind, compartmentalize, or sequester metals, and differences in enzyme sensitivity to metals (Mulvey and Diamond, 1991). Two general explanations have been provided to account for observed tolerance in exposed populations: physiological acclimation and genetic adaptation (Klerks and Levinton, 1989; Niederlehner and Cairns, 1992; and Klerks and Levinton, 1993).

Physiological acclimation to heavy metals has been reported in invertebrates (Miller and Hendricks, 1996) and fish (Duncan and Klaverkamp, 1983) and has been associated with induction of metal-binding proteins such as metallothionein (Roch and McCarter, 1984; Benson and Birge, 1985; Klaverkamp and Duncan, 1987; Aoki et al., 1989). Metallothionein is a low molecular weight, cysteine-rich protein that functions in both metal homeostasis (primarily through transport and storage of the essential metals copper (Cu) and zinc (Zn)) and detoxification (by outcompeting more critical

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macromolecules in binding) (Roesijadi, 1992). Its synthesis may be induced by exposure to metals, particularly cadmium (Cd), Cu, and Zn.

In contrast to physiological acclimation, which demonstrates the phenotypic plasticity of aquatic organisms, adaptation to heavy metals implies changes in gene frequencies resulting from increased survival of tolerant genotypes. Some studies suggest tolerance to heavy metals has a genetic basis (Wilson, 1988; Diamond et al., 1989; Maltby, 1991; Klerks and Levinton, 1993); however, the distinction between acclimation and adaptation is not always clear because physiological responses to contaminants may have a genetic basis (Mulvey and Diamond, 1991). For example, prior induction may result in the accumulation of a pool of metal-binding protein, available for binding in subsequent exposures. Alternatively, the metal-binding protein gene may be amplified, increasing the capacity for metal-binding protein synthesis.

Tolerance to heavy metals, whether derived through physiological or genetic mechanisms, may incur specific metabolic costs. Tolerant populations that spend more energy on detoxification by sequestering or eliminating metals, or producing metal binding proteins, may have less energy to devote to other processes. For example, springtails exposed to Cu and lead (Pb) exhibited reduced growth rates due to increased molting to eliminate the metals (Bengtsson et al., 1983). Further, production of storage organelles or metal binding proteins may expend energy even under uncontaminated conditions. For example, decreased productivity was observed in metal-tolerant plants grown on more favorable soils (Wilson, 1988), and Cd-tolerant populations of chironomids reared in the absence of Cd continued to show low growth rates and high mortality rates (Postma et al., 1995).

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The purpose of this research was to compare survival and growth among populations previously exposed or naive to metals upon subsequent exposure to metals in the laboratory, and to compare concentrations of metallothionein among populations in the field. Studies were conducted using the benthic macroinvertebrate *Baetis tricaudatus* Dodds (Ephemeroptera: Baetidae). While mayflies are generally sensitive to metals, this particular species can survive in areas known to have relatively high concentrations of Cd, Cu, Pb, and Zn (Roline, 1988; Clements, 1994). Because *Baetis* consumes detritus and diatoms in sediment and periphyton (Merritt and Cummins, 1984), and because of its relatively large gill surface area, it is exposed to metals through both aqueous and dietary routes. Its survival under such circumstances makes it an ideal organism with which to study mechanisms and costs of tolerance. For the studies presented here, *B. tricaudatus* were collected from the Cache la Poudre River (representing a naive population) and from the Arkansas River (representing previously-exposed populations).

The upper Arkansas River has been recognized as a site of poor water quality for many years. It receives waste from mines and tailings piles in Leadville, Colorado, predominantly through two tunnel systems: the Leadville Mine Drainage Tunnel and the Yak Tunnel. The Leadville Mine Drainage Tunnel discharges contaminated water into the Arkansas River just north of Leadville. The Yak Tunnel, a U.S. Environmental Protection Agency (EPA) Superfund site, discharges more highly contaminated water into California Gulch, which flows directly into the Arkansas River just southwest of Leadville (Figure 2.1). Concentrations of metals are orders of magnitude higher in California Gulch than in the Leadville Mine Drainage Tunnel. Acute toxicity (96-hour toxicity test with *Ceriodaphnia dubia*) has been measured >50 Km downstream from California Gulch in

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the Arkansas River (Clements and Kiffney, 1994), and metals levels have exceeded federal criteria for over 400 Km downstream (Lewis, 1987). In 1992, two water treatment plants began treating the water draining these tunnels. Metals concentrations in the Arkansas River have decreased below the Leadville Mine Drainage Tunnel (Nelson and Roline 1996), but there has been little change in water quality below California Gulch. During high flow events, such as occur during spring runoff, water bypasses the treatment plant and flows directly into California Gulch and then the Arkansas River, carrying with it high concentrations of Cd, Cu, Pb, and Zn.

Because placer mining of gold deposits began in California Gulch in 1859 (Griswold and Griswold, 1996), it is likely that populations in the Arkansas River have been exposed to heavy metals for over 130 years. Some populations may have been entirely eliminated for some time following periods of heavy mining. This study site provided a unique opportunity to investigate both ecotoxicological and molecular genetic effects of long-term exposure to heavy metals. Results of the ecotoxicological studies are presented here; results of the population genetic study are presented elsewhere (Harrahy et al., in prep).

METHODS

Experimental animal

Baetis tricaudatus is a mayfly with a widespread distribution (Morihara and McCafferty, 1979). It has a bivoltine life cycle and emerges in the spring and fall. Considered to be a rapid colonizer (Robinson et al., 1992), it exhibits a relatively high degree of plasticity in its life history to allow for changing environmental conditions

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(Clifford, 1982; Minshall, 1988). *Baetis* larvae are classified trophically as collector-gatherers and scrapers (Merritt and Cummins, 1996), and generally feed by grazing periphyton communities for diatoms and algae.

Collection sites

The Arkansas River is located between the Sawatch and Mosquito mountain ranges in central Colorado. Its primary source of metals is California Gulch, in Leadville; however, other sources include the Leadville Mine Drainage Tunnel and several tributary streams. Metals of primary concern in the Arkansas River are Cd, Cu, Pb, and Zn. Concentrations of metals in water are typically greater than the chronic criterion value for Zn at all sites below California Gulch, and less than the chronic criterion value at all sites above (Harrahy, unpublished data). *Baetis tricaudatus* were collected from three sites (AR1, AR5, and AR8; Figure 2.1) for use in these studies. Site AR1 is located approximately 4.5 km upstream of California Gulch, but 5 km below the Leadville Mine Drainage Tunnel. It was chosen because metal concentrations here are above background, but always lower than concentrations at sites located downstream of California Gulch. Thus, mayflies collected from AR1 represent a population with a moderately contaminated exposure history. Site AR5 is located approximately 12 km downstream of California Gulch. Metal concentrations here are often quite high, particularly following spring runoff. Site AR8 is located approximately 50 km downstream of California Gulch. Despite this distance, metal concentrations frequently remain high. Mayflies collected from sites AR5 and AR8 represent populations with a more heavily contaminated exposure history.

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The Cache la Poudre River is located in north central Colorado where its basin is bounded on the west by the Laramie and Medicine Bow Mountain ranges. It flows east to join the South Platte River east of Fort Collins. Concentrations of metals in the Cache la Poudre River are generally below detection. Site PR3 is located approximately 14 km northwest of Fort Collins. Mayflies collected from PR3 represent a naive (unexposed) population.

Mayfly larvae were collected using kick nets and by hand, and transported in aerated coolers to the laboratory, where they were acclimated prior to toxicity testing, or homogenized in buffer prior to metallothionein analysis. Physicochemical characteristics (temperature, dissolved oxygen, conductivity, pH, and hardness) were measured and water samples were collected for metals analysis from each field site whenever mayflies were collected.

Toxicity tests

To test the hypotheses that previously exposed populations are more tolerant to a mixture of metals than naive populations, and that there are costs associated with tolerance, a series of laboratory toxicity tests were conducted using early-instar *B. tricaudatus*.

Static acute toxicity test

Baetis tricaudatus were collected from sites located upstream (AR1) and downstream (AR8) of California Gulch on the Arkansas River, and exposed to a mixture of Cd, Cu, and Zn in water for 96 h. For each population, 10 mayflies were randomly

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assigned to each of five treatments (*n*=3) in 1-L plastic beakers. Treatments were 0 X, 6.25 X, 12.5 X, 25 X, and 50 X, where 1 X was equal to the average (three year) concentration of each metal measured at site AR5 on the Arkansas River, or 1 μg/L Cd, 3 μg/L Cu, and 250 μg/L Zn. Substrate and food were provided by placing a periphyton-coated rock (from the Cache la Poudre River) in the bottom of each beaker. The beakers were aerated and placed in water baths in the Stream Research Laboratory (SRL), a greenhouse located at the Colorado State University Foothills Campus. Water chemistry parameters (temperature, dissolved oxygen, conductivity, pH, hardness) were measured daily. Larvae were checked every twelve hours during the 96-hour exposure period for mortality. Total and dissolved metals concentrations were measured in water samples, collected at 0 and 96 hours.

Flow-through acute toxicity test

Baetis tricaudatus were collected from sites located upstream (AR1) and downstream (AR5) of California Gulch on the Arkansas River, and from a site on the Cache la Poudre River (PR3), and exposed to a mixture of Cd, Cu, and Zn in water for seven days. For each population, 20 mayflies were randomly assigned to each of three treatments (*n*=4) in flow-through microcosms in the SRL. Each circular (12" diameter, 10" high), clear-plastic microcosm received untreated water from nearby Horsetooth Reservoir at a rate of 1.0 L/min through small holes drilled in plastic t-valves to create a current of velocity of 10 cm/s. Substrate and food were provided by placing four periphyton-coated rocks (from the Cache la Poudre River) in the bottom of each microcosm. After 24 h acclimation, experimental microcosms were dosed with a mixture

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of Cd, Cu, and Zn. Treatments were 0 X, 12.5 X, and 25 X, where 1 X was equal to the chronic criterion value (adjusted for water hardness) for each metal, or 0.44 µg/L Cd, 4.22 µg/L Cu, and 38.22 µg/L Zn. Stock solutions of a mixture of $CdCl_2$, $CuSO_4$, and $ZnSO_4$ were delivered to treated microcosms from separate 20-L carboys using peristaltic pumps. Water chemistry parameters (temperature, dissolved oxygen, conductivity, pH, and hardness) and total and dissolved metals concentrations were measured on days 1, 3, 5, and 7 in each microcosm. At 7 d, the number of mayflies surviving in each microcosm was recorded. Larvae were considered dead if they did not respond to gentle prodding, or were missing.

Flow-through sublethal toxicity test

Baetis tricaudatus were collected from sites located upstream (AR1) and downstream (AR5) of California Gulch on the Arkansas River, and from a site on the Cache la Poudre River (PR3), and exposed to a mixture of Cd, Cu, and Zn in water for eleven days. For each population, 20 mayflies were randomly assigned to each of two treatments (*n*=6) in flow-through microcosms in the SRL. Each microcosm was set up as described above, except that treatments were 0 X and 5 X. Water chemistry parameters (temperature, dissolved oxygen, conductivity, pH, and hardness) and total and dissolved metals concentrations were measured on days 1, 4, 7, 10, and 11 in each microcosm. At 11 d, the number of mayflies surviving in each microcosm was recorded. Surviving mayflies were pooled for each microcosm to determine growth and metal bioaccumulation. Growth was measured as dry weight. Dry weights were divided by the number of surviving mayflies to give a final average individual weight for each

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microcosm. Relative growth rates were then calculated for each microcosm using the equation

$$\text{Final} - \text{Initial} / \text{Initial}$$

where the initial average individual weight was calculated as the mean of six replicates of twenty mayflies each, collected from each population at the start of the test.

Metallothionein analysis

Total metallothionein, natively-bound metallothionein, and total metals concentrations were measured in mayflies with different metal exposure histories. Total metallothionein refers to all metallothionein capable of binding to metals (some of which may already be bound), whereas natively-bound metallothionein refers to that portion already bound to metals (at the time of collection). Total metals refers to the total amount of metals in whole bodies (not just that associated with metallothionein). *Baetis tricaudatus* were collected (four replicates of 200 mayflies each) from three sites (AR1, AR5, and PR3), brought back to the laboratory alive, and immediately homogenized on ice in 4 mL of 50 mM HEPES buffer (Tissue Tearer model 985-370, BioSpec Products, Inc., Racine WA, USA; HEPES Ultra Pure, Sigma-Aldrich Co., St. Louis, MO, USA). Each homogenized sample was divided into four 1-mL subsamples for separate total metallothionein, natively-bound metallothionein, total metals, and protein analyses.

Metallothionein was measured using a cadmium saturation technique (Eaton and Toal 1982) as modified by Gasser (1998). Each homogenized subsample for total metallothionein and natively-bound metallothionein analysis was centrifuged at 15,000 *g* (4°C), for 10 min to remove chitin and isolate the cytosol and microsomes. The

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supernatant of each subsample was transferred to a clean tube, heated at 100°C in a dry bath for 10 min, cooled on ice, and then centrifuged at 15,000 *g* (4°C), for 10 min to separate heat labile (precipitate) from heat stable (supernatant) proteins. The supernatant was transferred to a clean tube and mixed with 10 mM $CdCl_2$ (cadmium saturation), or 10 mM NaCl (control saturation) for total metallothionein or natively-bound metallothionein analysis, respectively. Each tube was mixed on a mechanical rotator for 10 min. Chelex 100 resin (Sigma-Aldrich Co., St. Louis, MO, USA) was added to each tube to remove excess cadmium and other unbound metals. (Total metallothionein is quantified as the amount of Cd remaining.) Each tube was rotated for 15 min, then centrifuged for 2 min at 14,000 *g*. The supernatant was transferred to a clean tube and diluted with 1% nitric acid (HNO_3) to 4 mL. Buffer blanks were treated simultaneously as negative controls for this protocol. Tubes were allowed to stand for at least 12 h prior to analysis of metals by flame or graphite furnace atomic absorption spectrophotometry (AAS). Total metallothionein was calculated for the Cd-saturated samples by adjusting the Cd concentration given by AAS for volume, converting to moles, and normalizing to protein content. Natively-bound metallothionein was calculated for the NaCl saturated samples by adjusting the Cd, Cu, or Zn concentration given by AAS for volume, converting to moles, and normalizing to protein content. Final units for each of these determinations were µmoles metal/g protein.

Homogenized subsamples for total metals analysis were digested in 1.5 mL of Instra-analyzed HNO_3 (70%; J.T. Baker, Phillipsburg, NJ, USA) in a dry bath at 100°C. After cooling, 1 mL 30% trace metals grade hydrogen peroxide (H_2O_2; J.T. Baker, Phillipsburg, NJ, USA) was added to each tube. Buffer blanks were digested

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simultaneously as negative controls. Tubes were diluted to 6 mL with Nanopure water and allowed to stand at room temperature for at least 12 h prior to analysis of metals by flame or furnace AAS. Total metal was calculated by adjusting the Cd, Cu, or Zn concentration given by AAS for volume, converting to moles, and normalizing to protein content, to give final units of μmoles metal/g protein.

Homogenized subsamples for protein analysis were centrifuged at 15,000 *g* for 10 min. The supernatant was transferred to a clean tube and diluted with Nanopure water prior to analysis by the Bradford Method (Coomassie Plus protein kit; Bio-Rad Laboratories, Hercules, CA, USA). Assay dye reagent was mixed with diluted supernatant in a microtiter plate. The plate was read at 600 nm on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). Ultra pure bovine serum albumin was used as a standard. Protein concentrations were calculated from a curve prepared from standards in each plate.

Metals analysis

Total and dissolved concentrations of Cd, Cu, and Zn were measured in water samples collected in the field and during toxicity tests. To determine dissolved metal concentrations, water was collected with a syringe and passed through an Acrodisc (Gelman Sciences, Ann Arbor, MI, USA) 0.45-μm filter. All water samples were acidified to pH 2 prior to analysis by flame AAS.

Baetis tricaudatus sampled from the field (for initial weight) or at the end of the sublethal toxicity test were immediately placed in 7-mL plastic test tubes and dried in an oven at 55°C until they reached a constant weight (used in growth determinations). Dried

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mayflies were digested in HNO_3 and H_2O_2 prior to analysis by flame AAS to determine concentrations of Cd, Cu, and Zn.

Preparation for metals analysis associated with the metallothionein study is described above. All samples were analyzed by flame AAS, with the exception of total metals and natively-bound metallothionein for mayflies collected from the Cache la Poudre River, which required the lower detection limits of graphite furnace AAS.

Quality-control and quality-assurance procedures included analysis of National Institute of Standards & Technology (NIST, Gaithersburg, MD, USA) bovine liver (standard reference material 1577b), HNO_3 blanks, water blanks, and Cd, Cu, and Zn standards. Average recovery of Cd, Cu, and Zn from NIST bovine liver tissue (n=3) was 90, 100, and 100%, respectively. Metals in HNO_3 and water blanks were consistently below detection. Metal standards were within 95% of nominal concentrations. To minimize contamination, all labware was acid-washed and plastic materials were used wherever possible.

Statistical analysis

All statistical analyses were performed using a PC version of Statistical Analysis System (SAS, 1996). Results of the static acute toxicity test were analyzed using logistic regression with analysis of covariance to examine equivalence of slopes and intercepts between populations. LC10, LC50, and LC90 values were estimated for each population. Use of a single endpoint (e.g., LC50) is meaningful only when concentration-response relationships among populations are parallel (Oris and Bailer, 1997). Results of the two flow-through toxicity tests were analyzed using a completely randomized factorial

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design, with location (PR3 vs. AR1 vs. AR5) and treatment (control vs. metal dosed) as the main effects. Two-way analysis of variance (ANOVA) was used to examine the effects of location and treatment on survival (flow-through acute) or the effects of location and treatment on survival, relative growth rate, and bioaccumulation of each metal (flow-through sublethal). Mayflies that emerged were not included in these calculations since the duration of their exposure varied. All survival data were arcsine square-root transformed. Relative growth rate and bioaccumulation data were log transformed where necessary due to heterogeneity of variances. A significant interaction term was interpreted as indicating that effects of treatment depended on location (or exposure history). Results of the metallothionein study (total metals, natively-bound metallothionein, and total metallothionein) were analyzed using one-way ANOVA to compare results by location. Where the F-test was significant, the Ryan-Einot-Gabriel-Welsch-Q (REGWQ) multiple comparison test was used to test for differences among treatments. REGWQ controls for maximum experiment-wise error rate.

RESULTS

Field measurements

Water samples were collected in the field for metals analysis each time mayflies were collected. Concentrations of total Cd and Cu were generally below detection (<5 ppb), but concentrations of total Zn ranged from below detection at site PR3 to 258 μg/L at site AR5 (Table 2.1). Dissolved metals were generally 70-90% of total. Concentrations of Zn increased by site in the following order: PR3 < AR1 < AR8 < AR5. Site AR5 had concentrations of Zn that were always greater than the chronic criterion

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value of 59 µg/L (adjusted for water hardness).

Physicochemical data were also collected each time mayflies were collected and are presented in Table 2.2. Temperature and dissolved oxygen were similar across sites. Conductivity, pH, and water hardness (as $CaCO_3$) were all higher in the Arkansas River than in the Cache la Poudre River. In general, conductivity was higher at sites with greater metals concentrations.

Toxicity tests

Physicochemical characteristics measured in the toxicity tests were similar to those measured in the field, with the exception of conductivity and water hardness (Table 2.2). Conductivity increased with increasing concentrations of metals, especially in the static acute toxicity test, in which it ranged from about 40 to 80 µmho. Hardness in the laboratory was similar to hardness measured in the Cache la Poudre River, but was lower than in the Arkansas River. There was little variability in temperature, dissolved oxygen, or pH over the course of each toxicity test.

Static acute toxicity test

Average concentrations of metals measured in water in the static acute toxicity test were generally lower than nominal (Table 2.3). Initial concentrations were very close to nominal, but final concentrations were lower, probably due to adsorption and possibly bioaccumulation (water was not renewed). *Baetis tricaudatus* were exposed to approximately 0, 6.25, 12.5, 25, or 50 X the average (three year) concentration of Cd, Cu, and Zn measured in water at site AR5. Survival after 96 h exposure ranged from 8.5

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to 96.7% (Figure 2.2). Slopes were significantly different between populations (p=0.001), and indicated that the population at AR8 (downstream of California Gulch) was more tolerant to metals than the population at AR1 (upstream). Fitted parameters from logistic regressions on treatment-mortality data and predicted LC10, LC50, and LC90 values for the two populations are presented in Table 2.4. LC10 values were not significantly different between the populations, but LC50 and LC90 values were nearly significantly different.

Flow-through acute toxicity test

Average concentrations of metals measured in water in the flow-through acute toxicity test were very close to nominal for Cd, though lower than nominal for Cu and Zn (Table 2.3). *B. tricaudatus* were exposed to approximately 0, 12.5, or 25 X the chronic criterion value (adjusted for hardness) of a mixture of Cd, Cu, and Zn. Survival after 7 d exposure ranged from 7.1 to 95.0% (Figure 2.3). Two-way ANOVA indicated a significant treatment effect (p=0.0001) and a nearly significant location effect (p=0.0701), but no significant interaction between the two (p=0.9086). In other words, the previously exposed populations were not shown to be more tolerant to metals than the naive population.

Flow-through sublethal toxicity test

Because some studies suggest tolerance may be more evident at the sublethal level, a flow-through sublethal toxicity test was conducted. Average concentrations of metals measured in this test were close to nominal (Table 2.3). *Baetis tricaudatus* were

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exposed to 0 or 5 X the chronic criterion value (adjusted for hardness) of a mixture of Cd, Cu, and Zn. Survival after 11 d exposure was significantly different among locations (p=0.0111) (probably due to lower control survival of mayflies collected from the Cache la Poudre River), but not between treatments (p=0.1245) (indicating the 5 X treatment was, in fact, sublethal) (Figure 2.4).

Surviving mayflies were analyzed for bioaccumulation of Cd, Cu, and Zn. Two-way ANOVA indicated significant treatment effects (p<0.05) for all three metals. However, there was a significant location effect (p=0.0001) and a significant interaction between treatment and location (p=0.0012) only for the non-essential metal Cd (Figure 2.5). Mayflies collected from the more contaminated site (AR5) bioaccumulated more Cd than mayflies from either of the other sites.

Growth data collected on surviving mayflies indicated that in the controls, each population significantly grew over the course of the test (p<0.05). However, a one-way ANOVA indicated there were significant differences in initial weights among locations at the start of the test (p<0.0001). Mayflies collected from site AR5 were significantly larger than those from AR1 or PR3. To take initial weight into account, growth rates were calculated. Two-way ANOVA on growth rate data indicated significant location (p=0.0004) and treatment (p=0.0004) effects, and a nearly significant interaction (p=0.0719) (Figure 2.6). Only mayflies from AR1 (moderately contaminated) maintained a positive growth rate under subsequent exposure to metals.

Metallothionein analysis

As part of the metallothionein analysis, concentrations of Cd, Cu, and Zn were

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measured in *B. tricaudatus* collected from sites with different exposure histories. Concentrations of Cd and Zn were significantly different among populations PR3, AR1, and AR5 ($p<0.05$). Concentrations of Cu were not significantly different between populations AR1 and AR5, though they were significantly greater in each of these populations than concentrations in population PR3 ($p<0.05$). Concentrations of Cd and Zn increased with exposure history (Figure 2.7).

Concentrations of Cu and Zn natively-bound to metallothionein were not significantly different between populations AR1 and AR5, though they were significantly greater in each of these populations than in population PR3 ($p<0.05$). Concentrations of Cd natively-bound to metallothionein were significantly different among populations ($p<0.05$), and increased with exposure history (Figure 2.8). Percentage of total metals (in whole bodies) natively-bound to metallothionein generally increased with exposure history (Table 5), but ranged from only 0.1 to 19.0%. Thus, only a fraction of the metals found in whole bodies are actually associated with metallothionein.

Concentrations of total metallothionein (bound plus unbound) differed significantly among populations PR3, AR1, and AR5 ($p<0.05$). Interestingly, concentrations were greater in mayflies collected from AR1 (the moderately contaminated site) than in those collected from AR5 (the more heavily contaminated site) (Figure 2.9). However, percentage of total metallothionein natively-bound to metals increased with exposure history (Table 2.5).

The order of equilibrium affinity of metallothionein for metals is generally reported to be Cu > Cd > Zn, meaning Cd will replace Zn, but not Cu. In this study, the percentage of Cu natively-bound to metallothionein that was displaced by Cd using the

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Cd-saturation technique was 34.8, 4.0, and 0% for populations PR3, AR1, and AR5 respectively. Percentage of Zn natively-bound to metallothionein that was displaced by Cd using the Cd-saturation technique was 53.2, 51.0, and 30.6% for populations PR3, AR1, and AR5 respectively.

DISCUSSION

This research provided some evidence that populations previously exposed to metals are more tolerant to subsequent exposure to metals. Results of the first acute toxicity test indicated greater tolerance to metals in a population of mayflies located downstream of California Gulch than in a population located upstream. However, results of the second acute toxicity test, conducted with a reference population from the Cache la Poudre River, did not indicate greater tolerance in previously exposed populations than in a naive population. Failure to detect tolerance in the second acute toxicity test may have been due to the relatively low (75%) control survival of mayflies collected from the Cache la Poudre River (which added to the variability of control survival among populations) or to the low power (to detect a significant interaction) associated with this experimental design.

Alternatively, failure to detect tolerance in the second test may have been due to larger body size. The first acute toxicity test was conducted in May, and the second in July, when mayflies in the Arkansas River were larger. In addition, when collecting mayflies for the population studies, larger instars were collected to insure accurate species identification. This is in contrast to collection of whole communities which allows inclusion of smaller instars which can be identified under a microscope at the end of a

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test. A laboratory study conducted by Clements (1999) that compared tolerance between whole communities collected from the Arkansas River and the unpolluted Cache la Poudre River indicated greater tolerance in the Arkansas River community, primarily through greater survival of *B. tricaudatus* and *Rhithrogena hageni* mayflies. Smaller instars of *B. tricaudatus* have been shown to be more sensitive to metals than larger instars (Kiffney and Clements, 1996).

Another variable which may have affected our ability to detect tolerance in the second acute toxicity test is concentration. High mortality rates associated with acute exposures may preclude the operation of genetically or physiologically-based tolerance mechanisms. For example, even if there are underlying differences in ability to produce metallothionein among populations, it may take some time before induction of metallothionein imparts a differential response (Diamond et al., 1995). While Lloyd (1960) found that rainbow trout (*Salmo gairdneri*) were more resistant to lethal concentrations of Zn following exposure to sublethal concentrations, other studies indicate tolerance and costs of tolerance are better detected at sublethal concentrations (Posthuma et al., 1993; Diamond et al., 1995).

In the sublethal toxicity test, *B. tricaudatus* collected from the moderately contaminated site (AR1) maintained a positive growth rate during subsequent exposure to metals. In contrast, mayflies collected from both the reference site (PR3) and the more heavily contaminated site (AR5) did not grow. Bengtsson et al. (1983) found a similar pattern of growth for springtails (*Onychiurus armatus*) feeding on metal-polluted fungi, with a higher growth rate at moderate metals concentrations than at either control or high metals concentrations. According to Holloway et al. (1990), if there is a trade-off

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between growth and survival in a contaminated environment but no such trade-off in an uncontaminated environment, the optimal strategies are no resistance in the uncontaminated and some degree of resistance in the contaminated. Mayflies collected from PR3 may not have evolved mechanisms of resistance that would allow them to maintain energy for growth in the presence of metals (especially the non-essential metal Cd) at concentrations above background levels. Mayflies collected from AR5, while capable of surviving in the presence of higher concentrations of metals, may have had to devote more energy to resistance, leaving less for growth than those collected from AR1. Thus, the difference in growth rates between the two Arkansas River populations may be due to the degree of previous exposure.

According to the trade-off hypothesis (Sibly and Calow, 1989), the ability to tolerate increased concentrations of metals may come at the expense of energy for other processes such as growth and reproduction, thereby reducing fitness even in an uncontaminated environment. Growth rates in the sublethal toxicity test supported this hypothesis. Mayflies collected from the more heavily contaminated site had significantly lower growth rates than mayflies from either the uncontaminated or moderately contaminated sites, under control conditions. However, caution should be taken in citing these results as evidence of the trade-off hypothesis since previously exposed populations were not independent (taken from the same river), and not replicated at the level of previous exposure. When Harper et al. (1997) compared a metal-tolerant plant (*Mimulus guttatus*) from three different mine sites, they found no clear evidence for the trade-off hypothesis.

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Sequestering of metals and/or production of metallothionein may also involve metabolic costs. *B. tricaudatus* collected from the more heavily contaminated site (AR5) bioaccumulated more Cd than mayflies from either of the other sites when exposed to metals. These results suggest that mayflies from AR5 may be expending more energy to produce greater amounts of metallothionein. While this would allow them to survive in a more contaminated environment, the more divergent that environment becomes from an uncontaminated environment, the greater the cost of acclimation/adaptation (energy expended for metallothionein production) (Mulvey and Diamond, 1991). However, when concentrations of total metallothionein were compared in mayflies collected from these field sites, those from the moderately contaminated site (AR1) had significantly greater concentrations than mayflies from either the unpolluted or the more heavily contaminated sites. It may be that the population at site AR5 was too stressed to produce as much metallothionein as the population at site AR1. Further, the greater concentrations of total metallothionein in mayflies at AR1 may have been partially responsible for continued growth under subsequent exposure to metals. Again, the differences in metallothionein concentration between the two Arkansas River populations may be due to differences in degree of previous exposure. Clements and Rees (1997) found higher concentrations of metals in liver and kidney tissues in brown trout collected from AR1 than from AR5. Livers and kidneys are the primary organs of metal storage and regulation (Heath 1995) and the higher concentrations of metals in these tissues in brown trout at AR1 may have been associated with greater concentrations of metallothionein.

The inducibility of metallothionein may also differ among these populations and may be a more interesting question than amount of total metallothionein present at time

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of collection. Determination of relative inducibility would require exposing mayflies from the three sites to metals and comparing concentrations of metallothionein over time. Researchers who studied a species of *Baetis* (*B. thermicus*) from metal contaminated rivers in Japan showed metal tolerance to be associated with a metallothionein-like protein (Suzuki et al., 1988; Aoki et al., 1989). This protein could be induced in the laboratory under Cd exposure by the metal-tolerant species but not by two non-tolerant species (*B. yoshinensis* and *B. sahoensis*). In addition, these studies showed that Cu readily displaced the more toxic metal Cd bound to metallothionein (Suzuki et al., 1989).

Because metallothionein has a higher affinity for Cu than for Cd (Petering et al., 1990), Cu natively-bound to metallothionein is not displaced by Cd in the Cd-saturation technique. Thus, total metallothionein is underestimated by that amount bound to Cu. The term "cadmium binding potential" may be a better descriptor of what is actually measured using this approach. In the present studies, the percentage of Cu natively-bound to metallothionein that was displaced by Cd was low (0 to 4%) for the Arkansas River mayflies, but relatively high (35%) for mayflies from the Cache la Poudre River. Because concentrations of total Cu in mayflies from the Cache la Poudre River were initially very low, Cd-saturation may have resulted in relatively greater displacement for this population. Zn natively-bound to metallothionein was more readily displaced by Cd (30-53%) than was Cu, but not to the extent expected (closer to 100%) based on known binding affinities. Because Arkansas River mayflies are exposed to much higher concentrations of Zn than Cu or Cd, it is possible that Zn natively-bound to metallothionein was better able to compete with Cd in the saturation step. Hamilton et al. (1987) found that both Cu and Zn could compete for binding sites with Cd in brook trout

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(*Salvelinus fontinalis*), with the outcome of competition depending on both binding affinities and relative abundance of competing metals.

Results of the metallothionein analysis indicate that a greater proportion of the total metallothionein was natively-bound to metals in the AR5 population than in either of the other populations. Thus, while the AR1 population had significantly more total metallothionein available, the AR5 population seemed to make more efficient use of its total metallothionein. Regardless, only a fraction of total metals found in whole bodies was actually associated with metallothionein (natively-bound). Very little of the total Zn or Cu was natively-bound to metallothionein. The greatest proportion of Cd natively-bound to metallothionein was 19%, and this was in the AR5 population.

This research provided an opportunity to test the spillover hypothesis (Winge et al., 1974). According to this hypothesis, toxic effects should not occur until all metallothionein is saturated, and there is spillover. However, Hamilton et al. (1987) showed that while there was large Cd-binding capacity present in liver of brook trout exposed to Cd, approximately 85% of the total Cd measured in the liver was unbound, resulting in sublethal effects. More recently, Deeds and Klerks (1999) showed that in freshwater oligochaetes (*Limnodrilus udekemianus*) exposed to Cd, only about 10% of the metallothionein available was bound to Cd, leaving enough free Cd to cause toxicity. The majority of total metals present in the Arkansas River populations was not bound to metallothionein. Some portion of these total metals may have been associated with gut contents and the exoskeleton. However, given the magnitude of total metals present in whole bodies, the bioavailable fraction was likely still much greater than the bound (to metallothionein) fraction. Therefore, the potential for adverse effects may be quite high.

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Summary and conclusions

Populations of *B. tricaudatus* previously exposed to a mixture of metals in the field exhibited higher survival and growth, and bioaccumulated more Cd after exposure to metals in the laboratory than naive populations. In addition, previously exposed populations had significantly higher metallothionein concentrations. These findings suggest that long-term exposure to metals has resulted in the development of tolerance, and that metallothionein may be an underlying mechanism of this tolerance. However, the degree of tolerance and associated costs, depended on the degree of previous exposure. For example, mayflies previously exposed to moderate concentrations of metals at site AR1 were able to grow under subsequent exposure to metals. In contrast, mayflies previously exposed to high concentrations of metals at site AR5 were not able to grow.

While metal-adapted populations may be better equipped to survive in metal contaminated areas, they may exhibit increased susceptibility to other stressors. For example, Courtney and Clements (2000) found that Arkansas River communities were more sensitive to acidification than Cache la Poudre River communities, primarily due to greater sensitivity of *Baetis* spp. In addition, *B. tricaudatus* in the Arkansas River were more susceptible to predation by stoneflies than those in the Cache la Poudre River (Clements, 1999).

Studies indicate that treatment of mine drainage at the Arkansas River has resulted in decreased metal concentrations in the overlying water and recovery of some biotic measures (taxa richness, number of EPT taxa, number of shredders) (Nelson and Roline, 1996). However, because water treatment plants are bypassed under high flow conditions, and because concentrations of metals remain high in sediment, periphyton

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(Harrahy, unpublished data), and in the hyporheic zone (Nelson and Roline, 1999), the potential for continued long-term exposure and costs associated with long-term exposure of *B. tricaudatus* and other benthic invertebrates to metals remains high.

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LITERATURE CITED

Antonovics, J., A.D. Bradshaw, and **R.G. Turner**. 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* **7**:1-85.

Aoki, Y.S. Hatakeyama, N. Kobayashi, Y. Sumi, T. Suzuki, and **K.T. Suzuki**. 1989. Comparison of cadmium-binding protein induction among mayfly larvae of heavy metal resistant (*Baetis thermicus*) and susceptible species (*B. yoshinensis* and *B. sahoensis*). *Comparative Biochemistry and Physiology* **93C**:345-347.

Bengtsson G., T. Gunnarsson, and **S. Rundgren**. 1983. Growth changes caused by metal uptake in a population of *Onychiurus armatus* (Collembola) feeding on metal polluted fungi. *OIKOS* **40**:216-225.

Benson, W.H. and **W.J. Birge**. 1985. Heavy metal tolerance and metallothionein induction in fathead minnows: Results from field and laboratory investigations. *Environmental Toxicology and Chemistry* **4**:209-217.

Bryan, G.W. and **L.G. Hummerstone**. 1971. Adaptation of the polychaete *Nereis diversicolor* to estuarine sediments containing high concentrations of zinc and cadmium. *Journal of the Biological Association U.K.* **53**:839-857.

Clements, W.H. 1994. Benthic invertebrate community responses to heavy metals in the Upper Arkansas River Basin, Colorado. *Journal of the North American Benthological Society* **13**:30-44.

Clements, W.H. 1999. Metal tolerance and predator-prey interactions in benthic macroinvertebrate stream communities. *Ecological Applications* **9**:1073-1084.

Clements, W.H. and **P.M. Kiffney**. 1994. Integrated laboratory and field approach for assessing impacts of heavy metals at the Arkansas River, Colorado.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Environmental Toxicology and Chemistry **13**:397-404.

Clements, W.H. and **D.E. Rees**. 1997. Effects of heavy metals on prey abundance, feeding habits, and metal uptake of brown trout in the Arkansas River, Colorado. *Transactions of the American Fisheries Society* **126**:774-785.

Clifford, H.F. 1982. Life cycles of mayflies (Ephemeroptera), with special reference to voltinism. *Quaestiones Entomologicae* **18**:15-90.

Courtney, L.A. and **W.H. Clements**. 2000. Sensitivity to acidic pH in benthic invertebrate assemblages with different histories of exposure to metals. *Journal of the North American Benthological Society* **19**:112-127.

Deeds, J.R. and **P.L. Klerks**. 1999. Metallothionein-like proteins in the freshwater oligochaete *Limnodrilus udekemianus* and their role as a homeostatic mechanism against cadmium toxicity. *Environmental Pollution* **106**:381-389.

Diamond, S.A., M.C. Newman, M. Mulvey, P.M. Dixon, and **D. Martinson**. 1989. Allozyme genotype and time-to-death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute exposure to inorganic mercury. *Environmental Toxicology and Chemistry* **8**:613-622.

Diamond, S.A., J.T. Oris, and **S.I. Guttman**. 1995. Adaptation to fluoranthene exposure in a laboratory population of fathead minnows. *Environmental Toxicology and Chemistry* **14**:1393-1400.

Duncan, D.A. and **J.F. Klaverkamp**. 1983. Tolerance and resistance to cadmium in white suckers (*Catostomus commersoni*) previously exposed to cadmium, mercury, zinc, or selenium. *Canadian Journal of Fisheries and Aquatic Sciences* **40**:128-138.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Eaton, D.L. and **B.F. Toal**. 1982. Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. *Toxicology and Applied Pharmacology* **66**:134-142.

Fraser, J., D.T. Parkin, and **E. Vespoor**. 1978. Tolerance to lead in the freshwater isopod *Asellus aquaticus*. *Water Research* **12**:637-641.

Gasser, L. 1998. Acclimation, metallothionein levels, and adenosine triphosphatase activity in brown trout, *Salmo trutta*, chronically exposed to both cadmium and zinc. M.S. thesis. Colorado State University, Fort Collins.

Griswold, D.L. and **J.H. Griswold**. 1996. *History of Leadville and Lake County, Colorado: From Mountain Solitude to Metropolis.* Volume 1. Colorado Historical Society in cooperation with the University Press of Colorado, Boulder, CO.

Groenendijk, D. 1999. Dynamics of metal adaptation in riverine chironomids. Ph.D. dissertation. University of Amsterdam, The Netherlands.

Hamilton, S.J., P.M. Mehrle, and **J.J. Jones**. 1987. Evaluation of metallothionein as a biological indicator of stress from cadmium in brook trout. *Transactions of the American Fisheries Society* **116**:551-560.

Harper, F.A., S.E. Smith, and **M.R. Macnair**. 1997. Where is the cost in copper tolerance in *Mimulus guttatus*? Testing the trade-off hypothesis. Functional *Ecology* **11**:764-774.

Heath, A.G. 1995. *Water Pollution and Fish Physiology*, 2nd edition. Lewis Publishers, Boca Raton, FL.

Holloway, G.J., R.M. Sibly, and **S.R. Povey**. 1990. Evolution in toxin-stressed environments. *Functional Ecology* **4**:289-294.

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Kiffney, P.M. and **W.H. Clements**. 1996. Size-dependent response of macroinvertebrates to metals in experimental streams. *Environmental Toxicology and Chemistry* **15**:1352-1356.

Klaverkamp, J.F. and **D.A. Duncan**. 1987. Acclimation to cadmium by white suckers: cadmium binding capacity and metal distribution in gill and liver cytosol. *Environmental Toxicology and Chemistry* **6**:275-289.

Klerks, P.L. and **J.S. Levinton**. 1989. Effects of heavy metals in a polluted aquatic ecosystem. In S.A. Levin, M.A. Harwell, J.R. Kelly, and K.D. Kimball, eds., *Ecotoxicology: Problems and Approaches*. Springer-Verlag, New York, NY.

Klerks, P.L. and **J.S. Levinton**. 1993. Evolution of resistance and changes in community composition in metal-polluted environments: a case study on Foundry Cove. In R. Dallinger and P.S. Rainbow, eds., *Ecotoxicology of Metals in Invertebrates*. SETAC Special Publications Series, Ann Arbor, MI. pp. 223-241.

Lewis, W.S. 1987. An evaluation of mining related metals pollution in the upper Arkansas River basin. M.S. thesis. Colorado School of Mines, Golden, CO.

Lloyd, R. 1960. The toxicity of zinc sulphate to rainbow trout. *Annals of Applied Biology* **48**:84-94.

Maltby, L. 1991. Pollution as a probe of life-history adaptation in *Asellus aquaticus* (Isopoda). *OIKOS* **61**:11-18.

Merritt, R.W. and **K.W. Cummins**. 1996. *An Introduction to the Aquatic Insects of North America*. Third Edition. Kendall/Hunt Publishing Company, Debuque, Iowa.

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Miller, M.P. and.**A.C. Hendricks.** 1996. Zinc resistance in *Chironomus riparius*: evidence for physiological and genetic components. *Journal of the North American Benthological Society* **15**:106-116.

Minshall, G.W. 1988. Stream ecosystem theory: a global perspective. *Journal of the North American Benthological Society* **7**:263-288.

Morihara, D.K. and **W.P. McCafferty**. 1979. The *Baetis* larvae of North America (Ephemeroptera: Baetidae). *Transactions of the American Entomological Society* **105**:139-221.

Mulvey, M. and **S.A. Diamond.** 1991. Genetic factors and tolerance acquisition in populations exposed to metals and metalloids. In M.C. Newman and A.W. McIntosh, eds., *Ecotoxicology of Metals: Current Concepts and Applications*. Lewis Publishers, Chelsea, MI.

Nelson, S.M. and **R.A. Roline.** 1996. Recovery of a stream macroinvertebrate community from mine drainage disturbance. *Hydrobiologia* **339**:73-84.

Nelson, S.M. and **R.A. Roline**. 1999. Relationships between metals and hyporheic invertebrate community structure in a river recovering from metals contamination. *Hydrobiologia* **397**:211-226.

Nevo, E., R. Ben-Shlomo, and **B. Lavie**. 1984. Mercury selection of allozymes in marine organisms: prediction and verification in nature. *Proceedings of the National Academy of Science USA* **81**:1258-1259.

Niederlehner, B.R. and **J. Cairns, Jr.** 1992. Community responses to cumulative toxic impact: effects of acclimation on zinc tolerance of *aufwuchs*. *Canadian Journal of Fisheries and Aquatic Sciences* **49**:2155-2163.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Oris, J.T. and **A.J. Bailer**. 1997. Equivalence of concentration-response relationships in aquatic toxicology studies: testing and implication for potency estimation. *Environmental Toxicology and Chemistry* **16**:2204-2209.

Petering, D.H., M. Goodrich, W. Hodgman, S. Krezoski, D. Weber, C.F. Shaw III, R. Spieler, and **L. Zettergren**. 1990. Metal-binding proteins and peptides for the detection of heavy metals in aquatic organisms. Chapter 14 In J.F. McCarthy and L.R. Shugart, eds., *Biomarkers of Environmental Contamination*. Lewis Publishers, Boca Raton, FL.

Posthuma, L., R.A. Verweij, B. Widianarko, and **C. Zonneveld.** 1993. Life history patterns in metal-adapted Collembola. *OIKOS* **67**:223-249.

Postma, J.F., S. Mol, H. Larsen, and **W. Admiraal**. 1995. Life-cycle changes and zinc shortage in cadmium-tolerant midges, *Chironomus riparius* (Diptera) reared in the absence of cadmium. *Environmental Toxicology and Chemistry* **14**:117-122.

Postma, J.F. and **D. Groenendijk.** 1999. Adaptation to metals in the midge *Chironomus riparius*: A case study in the River Dommel. Chapter 5 In V.E. Forbes, ed., *Genetics and Ecotoxicology*. Taylor & Francis, Ann Arbor, MI. pp. 79-101.

Robinson, C.T., L.M. Reed, and **G.W. Minshall**. 1992. Influence of flow regime on life history, production, and genetic structure of *Baetis tricaudatus* (Ephemeroptera) and *Hesperoperla pacifica* (Plecoptera). *Journal of the North American Benthological Society* **11**:278-289.

Roch, M. and **J.A. McCarter**. 1984. Hepatic metallothionein production and resistance to heavy metals by rainbow trout (*Salmo gairdneri*): II. held in a series of contaminated lakes. *Comparative Biochemistry and Physiology* **77C**, 77-82.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology* **22**:81-114.

Roline, R.A. 1988. The effects of heavy metals pollution of the upper Arkansas River on distribution of aquatic macroinvertebrates. *Hydrobiologia* **160**:3-8.

SAS Institute. 1985. SAS Procedures Guide for Personal Computers. Version 6 Edition. Cary, NC.

Sibly, R.M. and **P. Calow**. 1989. A life-cycle theory of responses to stress. Biological *Journal of the Linnaen Society* **37**:101-116.

Stokes, P.M., T.C. Hutchinson, and **K. Krauter**. 1973. Heavy metal tolerance in algae isolated from contaminated lakes near Sudbury, Ontario. *Canandian Journal of Botany* **51**:2155-2168.

Suzuki, K.T., H. Sunaga, Y. Aoki, S. Hatakayama, Y. Sugaya, Y. Sumi, and **T. Suzuki**. 1988. Binding of cadmium and copper in the mayfly *Baetis thermicus* larvae that inhabit a river polluted with heavy metals. *Comparative Biochemistry and Physiology* **91C**:487-492.

Suzuki, K.T., H. Sunaga, S. Hatakeyema, Y. Sumi, and **T. Suzuki**. 1989. Differential binding of cadmium and copper to the same protein in a heavy metal tolerant species of mayfly (*Baetis thermicus*) larvae. *Comparative Biochemistry and Physiology* **94C**:99-103.

Winge, D., J. Krasno, and **A.V. Colucci**. 1974. Cadmium accumulation in rat liver: correlation between metal and pathology. In W.G. Hoekstra, J.W. Suttie, H.E. Ganther, and W. Mertz, eds., *Trace Element Metabolism in Animals*, Volume 2. University Park Press, Baltimore, MD, pp. 500-502.

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Wilson, J.B. 1988. The cost of heavy-metal tolerance: an example. *Evolution* **42**:408-413.

Weis, J.S., P. Weiss, M. Heber, and **S. Vaidya**. 1981. Methylmercury tolerance of killifish (*Fundulus heteroclitus*) embryos from a polluted vs. a nonpolluted environment. *Marine Biology* **65**:283-287.

Wentsel, R., A. McIntosh, and **G. Atchinson**. 1978. Evidence of resistance to metals in larvae of the midge *Chironomus tentans* in a metal contaminated lake. *Bulletin of Environmental Contamination and Toxicology* **20**:451-455.

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Table 2.1. Concentrations of metals (in µg/L) in water in the Arkansas River (AR1, AR5, AR8) and in the Cache la Poudre River (PR3) at the time of mayfly collection for toxicity and metallothionein studies.

Test/Site	Cd	Cu	Zn
Static acute			
AR1	BD[a]	BD	32
AR8	BD	BD	46
Flow-through acute			
PR3	BD	BD	BD
AR1	BD	BD	20
AR5	BD	BD	124
Flow-through sublethal			
PR3	BD	BD	BD
AR1	BD	BD	33
AR8	BD	BD	258
Metallothionein			
PR3	BD	BD	BD
AR1	BD	BD	16
AR5	BD	BD	109

[a]BD=below detection for flame AAS.
Detection limits were 5 µg/L for Cd and Cu, and 10 µg/L for Zn.

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Table 2.2. Physicochemical characteristics of Arkansas River (AR 1, AR5, AR8) and Cache la Poudre River (PR3) field sites, and in experimental beakers or microcosms.

Test/Site	Temperature (°C)	D.O. (mg/L)	Conductivity (µmho)	pH	Hardness (mg/L as $CaCO_3$)
Static acute					
AR1	7	9.2	145	7.4	88
AR8	9	9.2	70	7.0	44
Laboratory	8.3 (±0.9)	9.4 (±0.4)	60 (±21.2)	7.2 (±0.5)	35.6 (±8.3)
Flow-through acute					
PR3	10.2	7.4	30	6.8	30
AR1	9.3	7.6	80	7.9	70
AR5	10.5	7.4	370	7.9	60
Laboratory	9.7 (±0.3)	8.4 (±0.4)	30 (±5.0)	6.7 (±0.3)	30.0 (±5.0)
Flow-through sublethal					
PR3	14.2	9.1	30	6.7	20
AR1	13.8	7.6	70	7.8	60
AR5	14.4	7.5	110	7.8	60
Laboratory	10.7 (±0.2)	7.0 (±0.7)	40 (±10.3)	6.7 (±0.1)	30.6 (±4.2)

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Table 2.3. Nominal (nom.) and measured (meas.) (±SD) concentrations of total metals (in µg/L) in water for the three toxicity tests.

Test/Trt.	Cd		Cu		Zn	
	Nom.	Meas.	Nom.	Meas.	Nom.	Meas.
Static acute[a]						
0 X	0.00	0.08 (±0.29)	0.00	12.08 (±4.96)	0.00	12.75 (±13.65)
6.25 X	6.25	2.67 (±1.56)	18.75	21.92 (±6.33)	1562.50	1339.75 (±736.33)
12.50 X	12.50	7.33 (±3.08)	37.50	43.08 (±11.72)	3125.00	2699.00 (±1191.83)
25.00 X	25.00	16.58 (±7.44)	75.00	69.75 (±24.28)	6250.00	6162.50 (±2056.70)
50.00 X	50.00	36.58 (±14.69)	150.00	98.71 (±38.87)	12500.00	12195.83 (±4524.84)
Flow-through acute[b]						
0 X	0.00	0.79 (±0.87)	0.00	2.75 (±1.21)	0.00	10.48 (±15.62)
12.50 X	5.51	5.62 (±2.39)	52.82	45.77 (±15.31)	477.70	392.87 (±121.48)
25.00 X	11.02	11.38 (±2.56)	105.65	87.98 (±22.41)	955.40	839.19 (±187.75)
Flow-through sublethal[c]						
0 X	0.00	1.79 (±4.29)	0.00	0.99 (±2.85)	0.00	4.03 (±5.03)
5.00 X	2.20	3.83 (±4.05)	21.13	19.41 (±7.18)	191.08	211.72 (±62.67)

[a]Measured n=12 (2 pops x 3 reps x 2 time pts.)
[b]Measured n=48 (3 pops x 4 reps x 4 time pts.)
[c]Measured n=90 (3 pops x 6 reps x 5 time pts.)
Detection limits were 5 µg/L for Cd and Cu, and 10 µg/L for Zn.

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Table 2.4. Fitted parameters (intercept and slope) from logistic regressions on concentration-mortality data and predicted 10, 50, and 90% lethal concentrations (95% CI) for the two populations (AR1 and AR8) of *B. tricaudatus* exposed to a mixture of metals in the static acute toxicity test.

Population	Intercept (s.e.)	Slope (s.e.)	LC10 (x) (LCL, UCL)	LC50 (x) (LCL, UCL)	LC90 (x) (LCL, UCL)
AR1	-3.203 (0.452)	0.1089 (0.0178)	9.23 (1.50, 14.2)	29.40 (24.80, 35.83)	49.57 (41.66, 63.93)
AR8	-2.437 (0.367)	0.0582 (0.0116)	4.12 (0, 12.9)	41.89 (34.04, 55.52)	79.66 (63.36, 115.30)

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Table 2.5. Percent total metals (in whole bodies) natively-bound to metallothionein, and percent total metallothionein natively-bound to metals in *B. tricaudatus* collected (n=4, 200 mayflies each) from three field sites.

	Site	Cd	Cu	Zn
% of total metal natively-bound to metallothionein				
	PR3	3.9	6.5	2.2
	AR1	18.5	8.6	0.1
	AR5	19.0	7.1	0.1
% of total metallothionein natively-bound to metal				
	PR3	0.3	6.4	10.9
	AR1	9.8	17.2	12.6
	AR5	22.5	29.2	24.2

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Figure 2.1. Map of collection sites on the Arkansas River in relation to the two predominant sources of metals to the system (California Gulch and Leadville Mine Drainage Tunnel). Insert shows location of the Arkansas River in relation to that of the Cache la Poudre River in Colorado.

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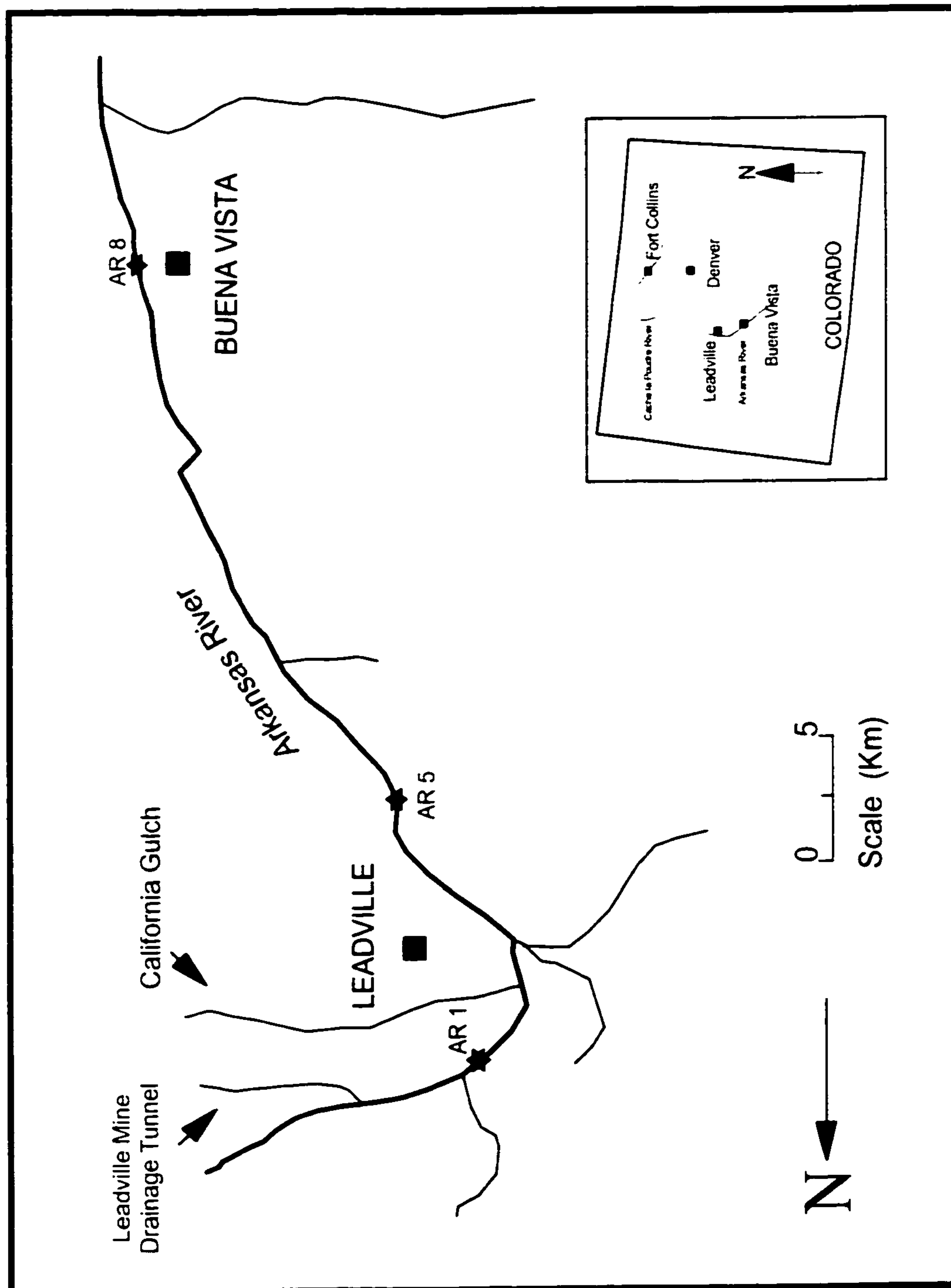

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Figure 2.2. Mean (n=3) % survival (±SD) in the static acute toxicity test. *B. tricaudatus* mayflies collected from sites AR1 and AR8 were exposed to a mixture of 0 to 50 X the (three year) average concentrations of Cd, Cu, and Zn measured at site AR5 for 96 h. Logistic regression with analysis of covariance showed slopes were significantly different between the two populations.

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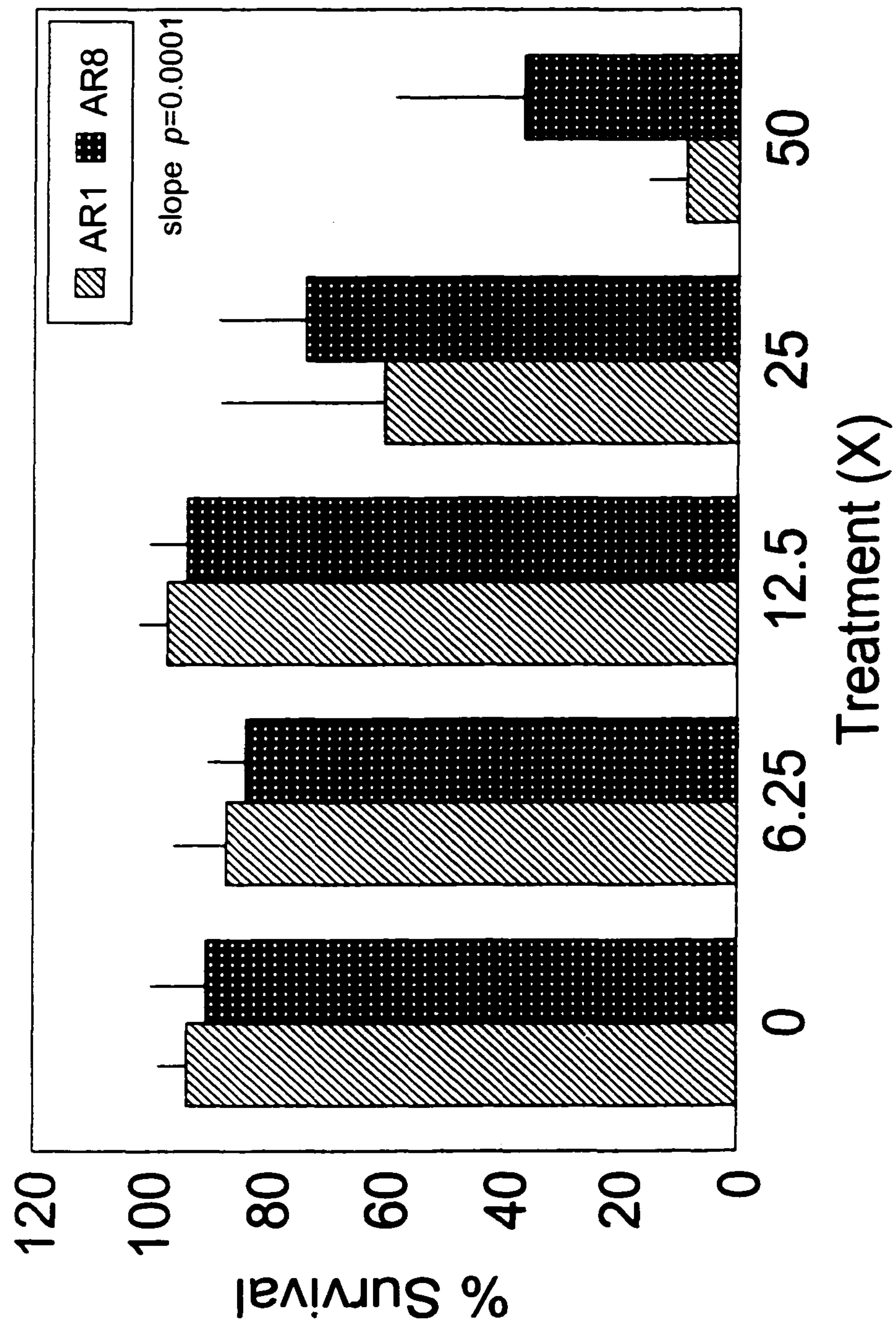

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Figure 2.3. Mean (n=4) % survival (±SD) in the flow-through acute toxicity test. *B. tricaudatus* mayflies collected from sites PR3, AR1, and AR5 were exposed to a mixture of 0, 12.5, or 25 X the chronic criterion values of Cd, Cu, and Zn in water for 7 d.

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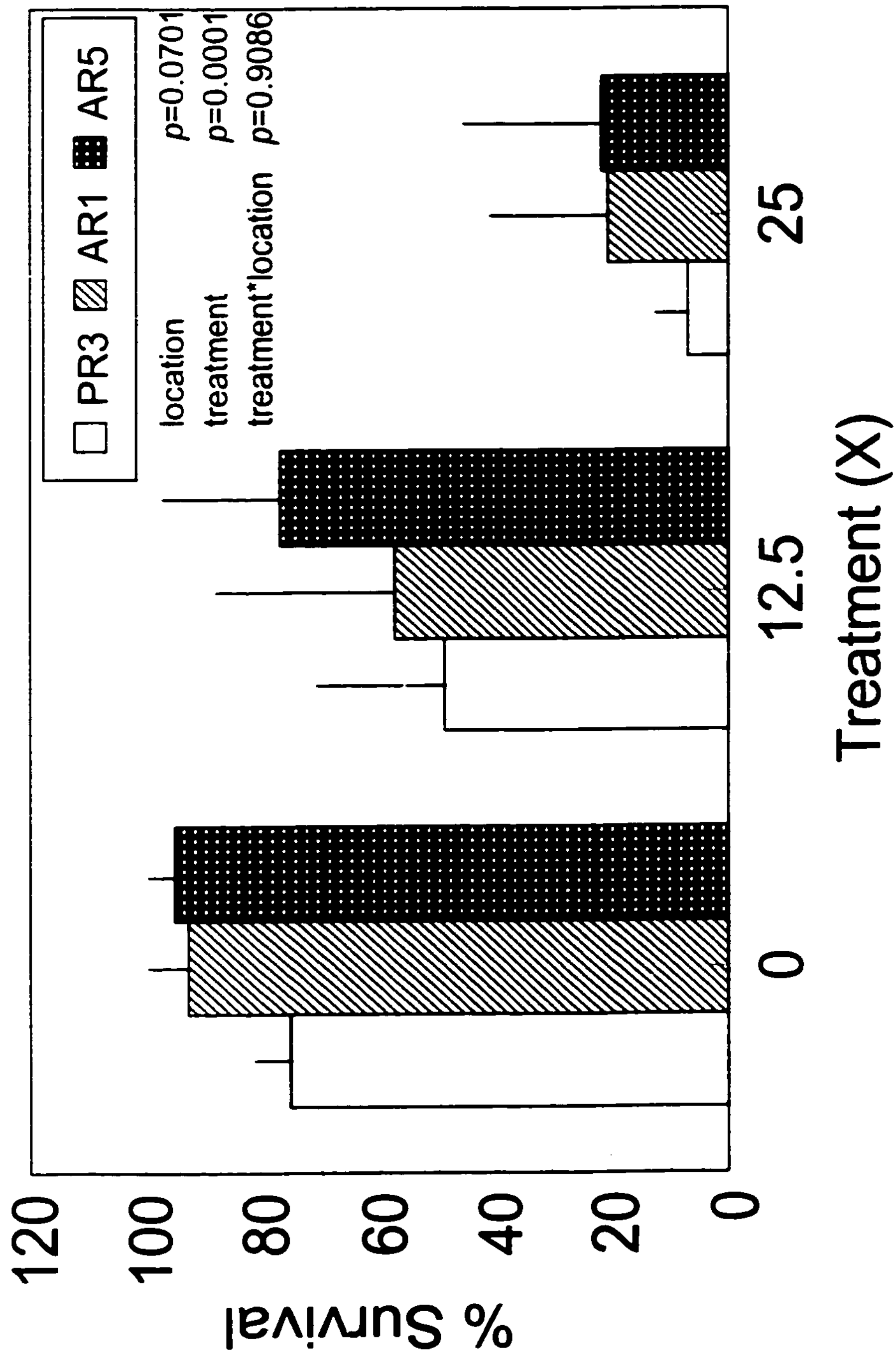

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Figure 2.4. Mean (n=4) % survival (±SD) in the flow-through sublethal toxicity test. *B. tricaudatus* mayflies collected from sites PR3, AR1, and AR5 were exposed to a mixture of 0 or 5 X the chronic criterion values of Cd, Cu, and Zn in water for 11 d.

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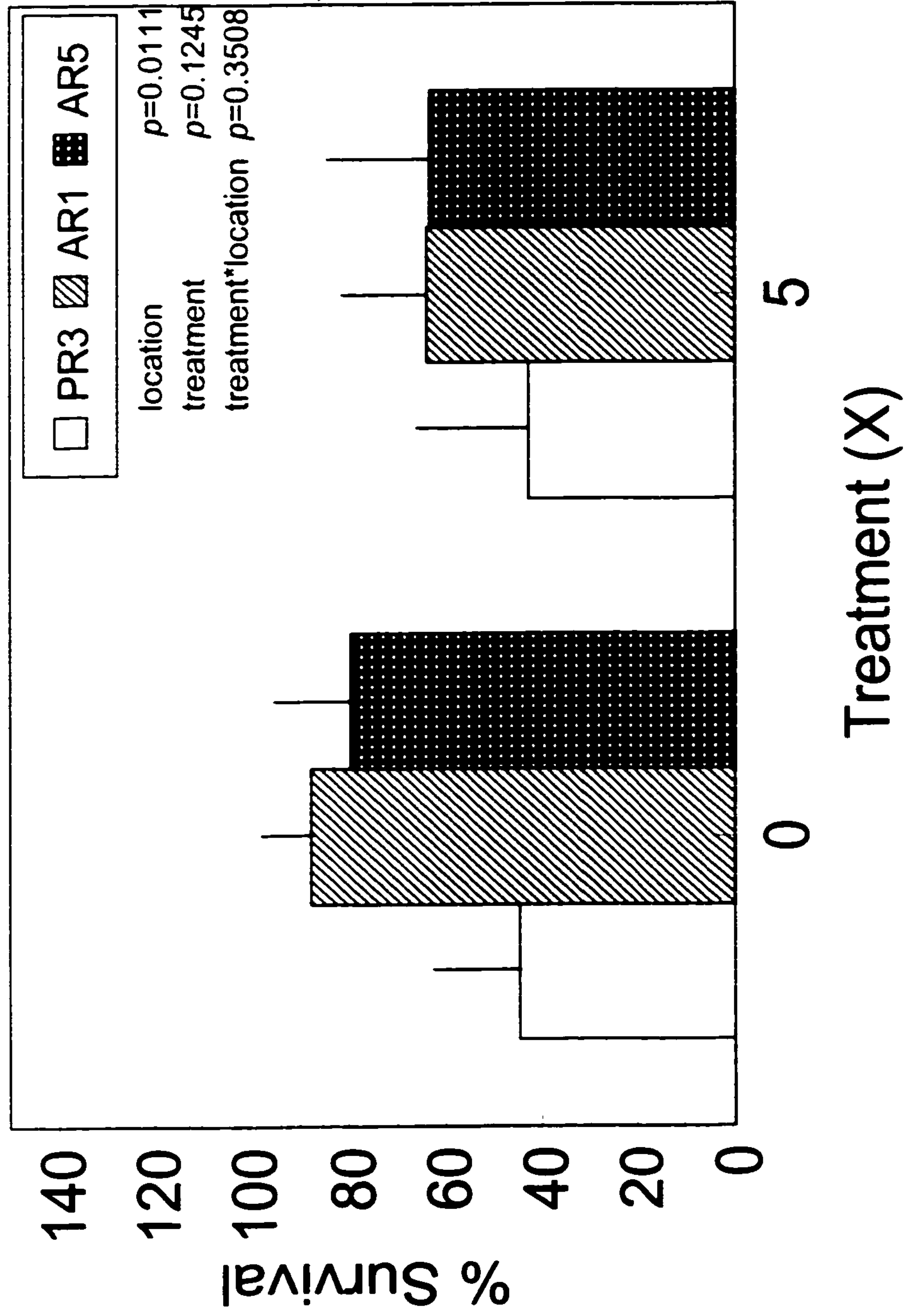

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Figure 2.5. Mean (n=6) bioaccumulation (±SD) of Cd, Cu, and Zn in the flow-through sublethal toxicity test. *B. tricaudatus* collected from sites PR3, AR1, and AR5 were exposed to a mixture of 0 or 5 X the chronic criterion values of Cd, Cu, and Zn in water for 11 d.

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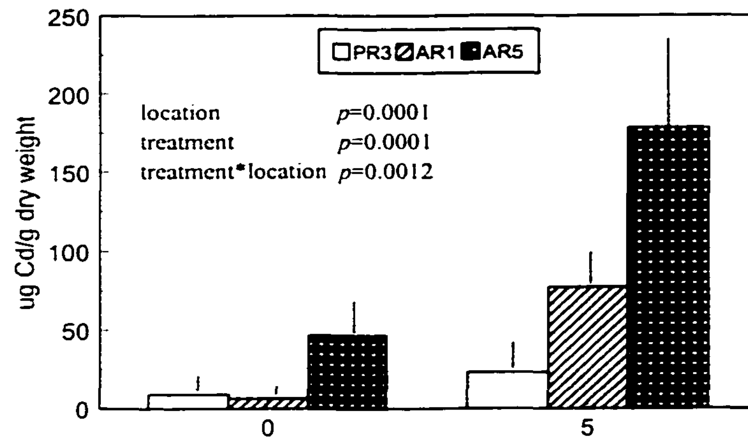

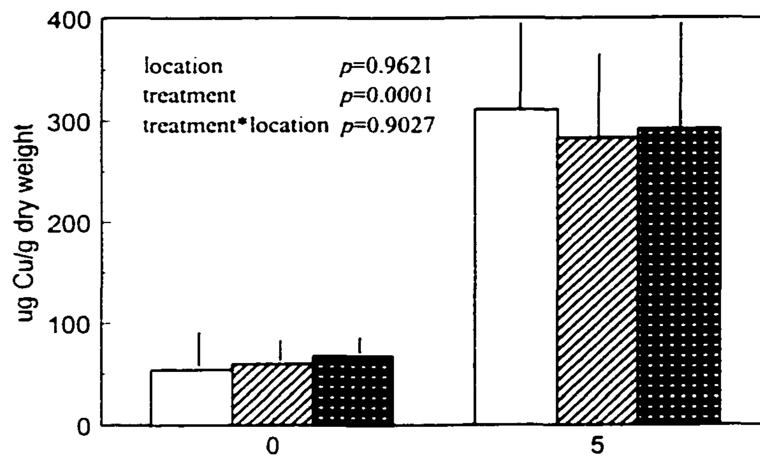

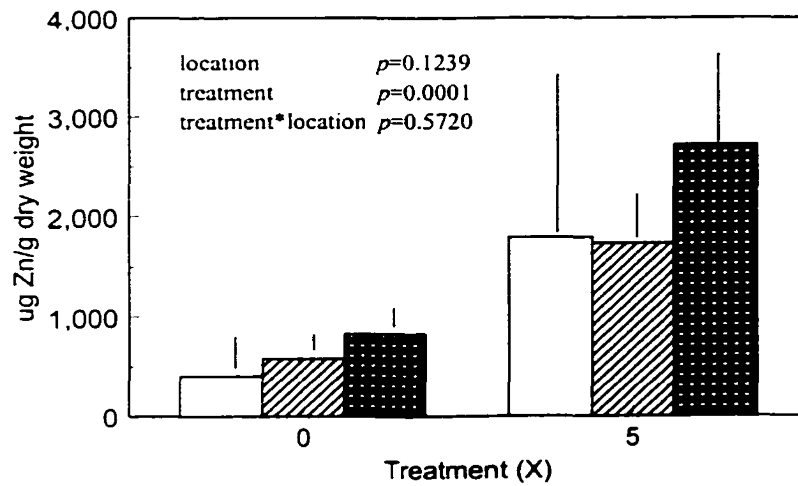

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Figure 2.6. Mean (n=6) growth rate (±SD) in the flow-through sublethal toxicity test. *B. tricaudatus* collected from sites PR3, AR1, and AR5 were exposed to a mixture of 0 or 5 X the chronic criterion values of Cd, Cu, and Zn in water for 11 d.

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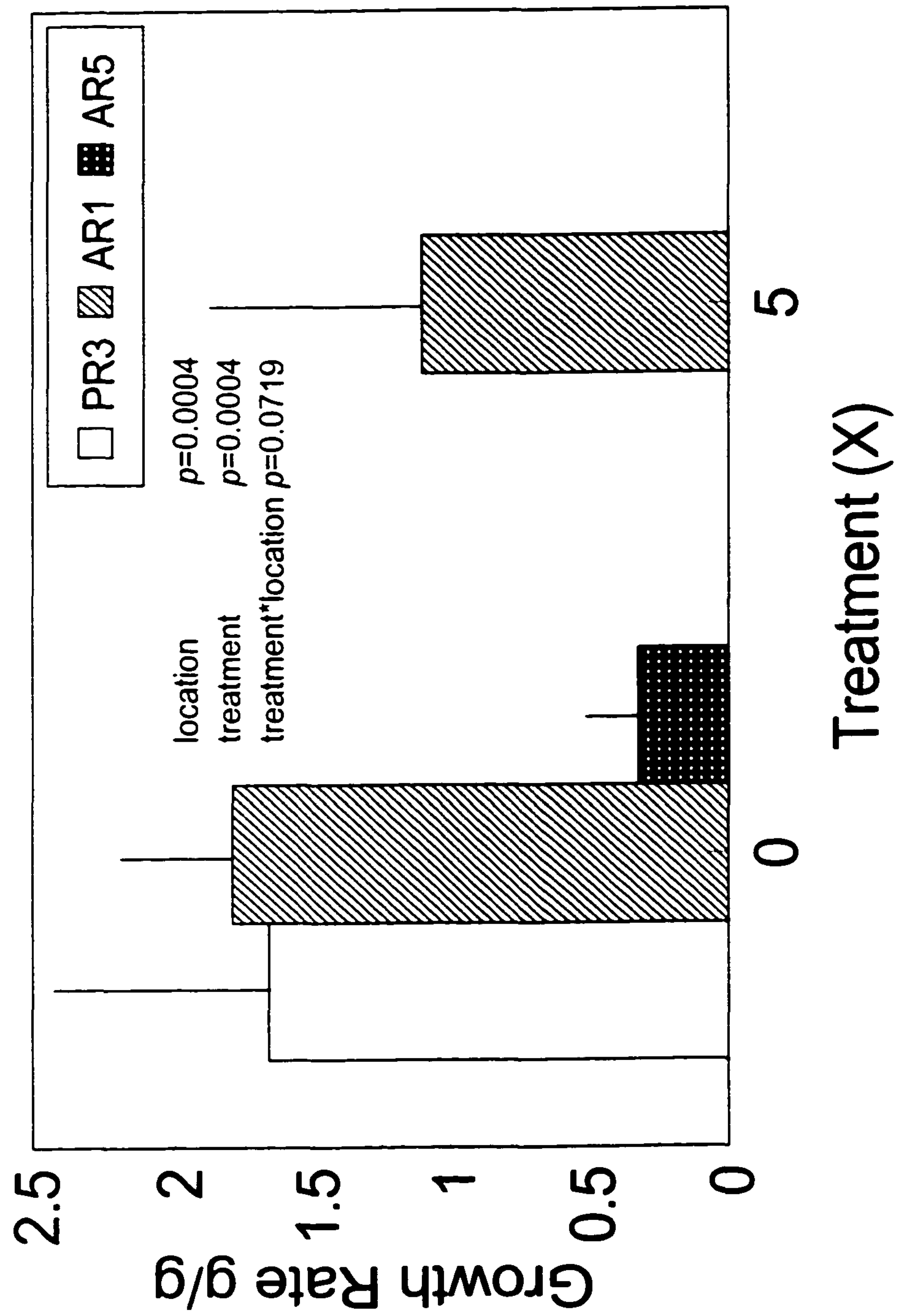

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Figure 2.7. Mean (n=4) concentrations (±SD) of total Cd, Cu, and Zn in mayflies collected from sites PR3, AR1, and AR5 for metallothionein analysis. Means with the same letter are not significantly different from each other (REGWQ multiple comparison test: $p<0.05$).

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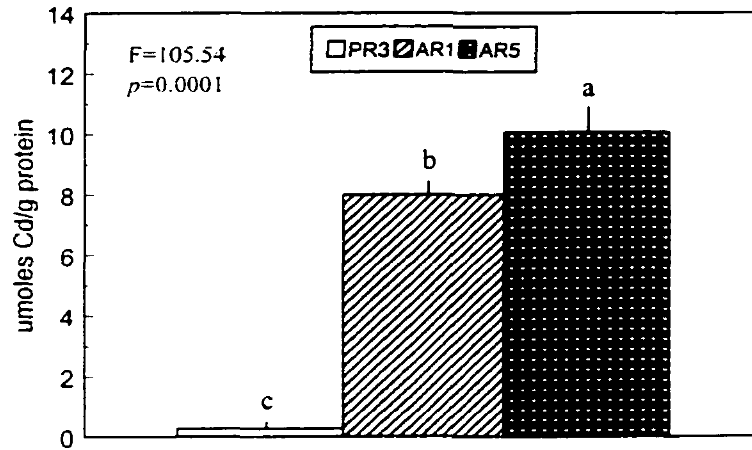

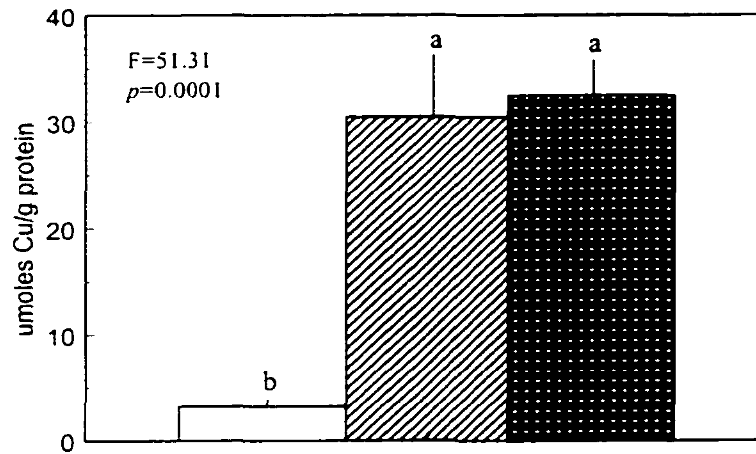

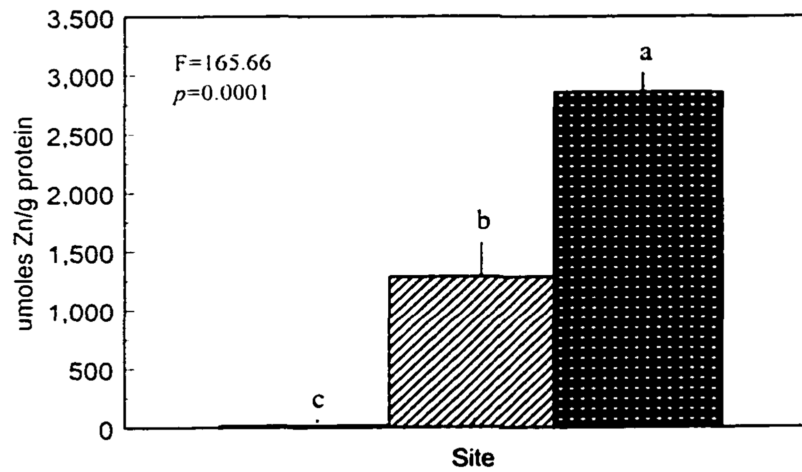

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Figure 2.8. Mean (n=4) concentrations (±SD) of Cd, Cu, and Zn natively-bound to metallothionein in mayflies collected from sites PR3, AR1, and AR5. Means with the same letter are not significantly different from each other (REGWQ multiple comparison test; $p<0.05$).

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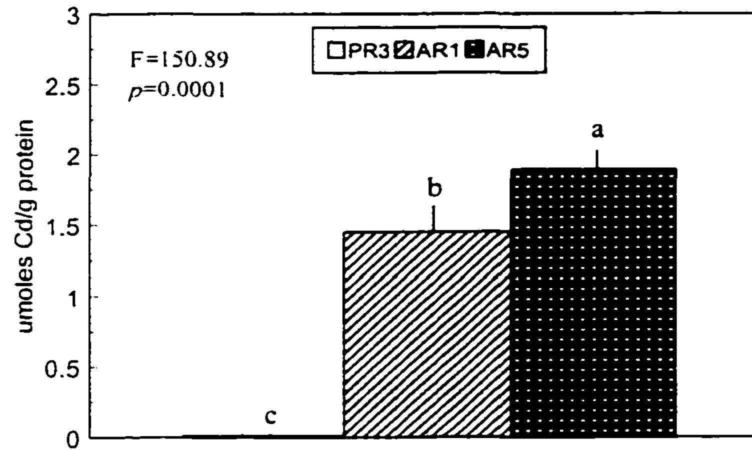

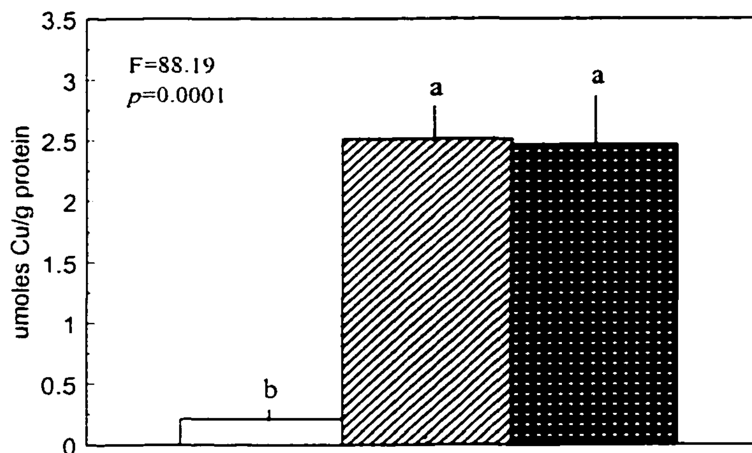

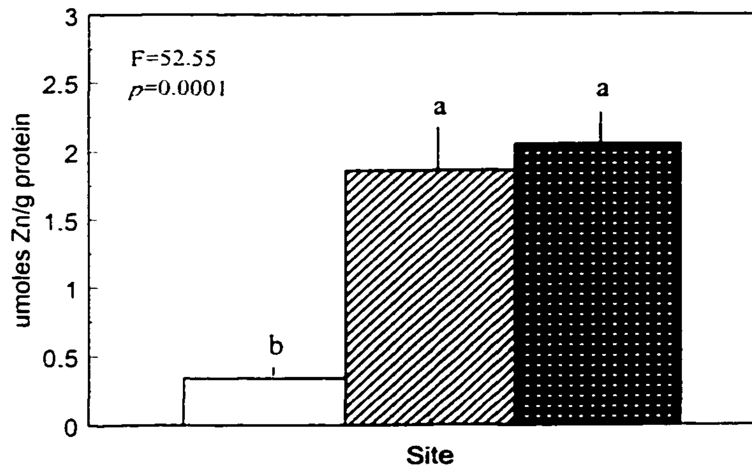

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Figure 2.9. Mean (n=4) concentrations (±SD) of total metallothionein in mayflies collected from sites PR3, AR1, and AR5. Means with the same letter are not significantly different from each other (REGWQ multiple comparison test; $p<0.05$).

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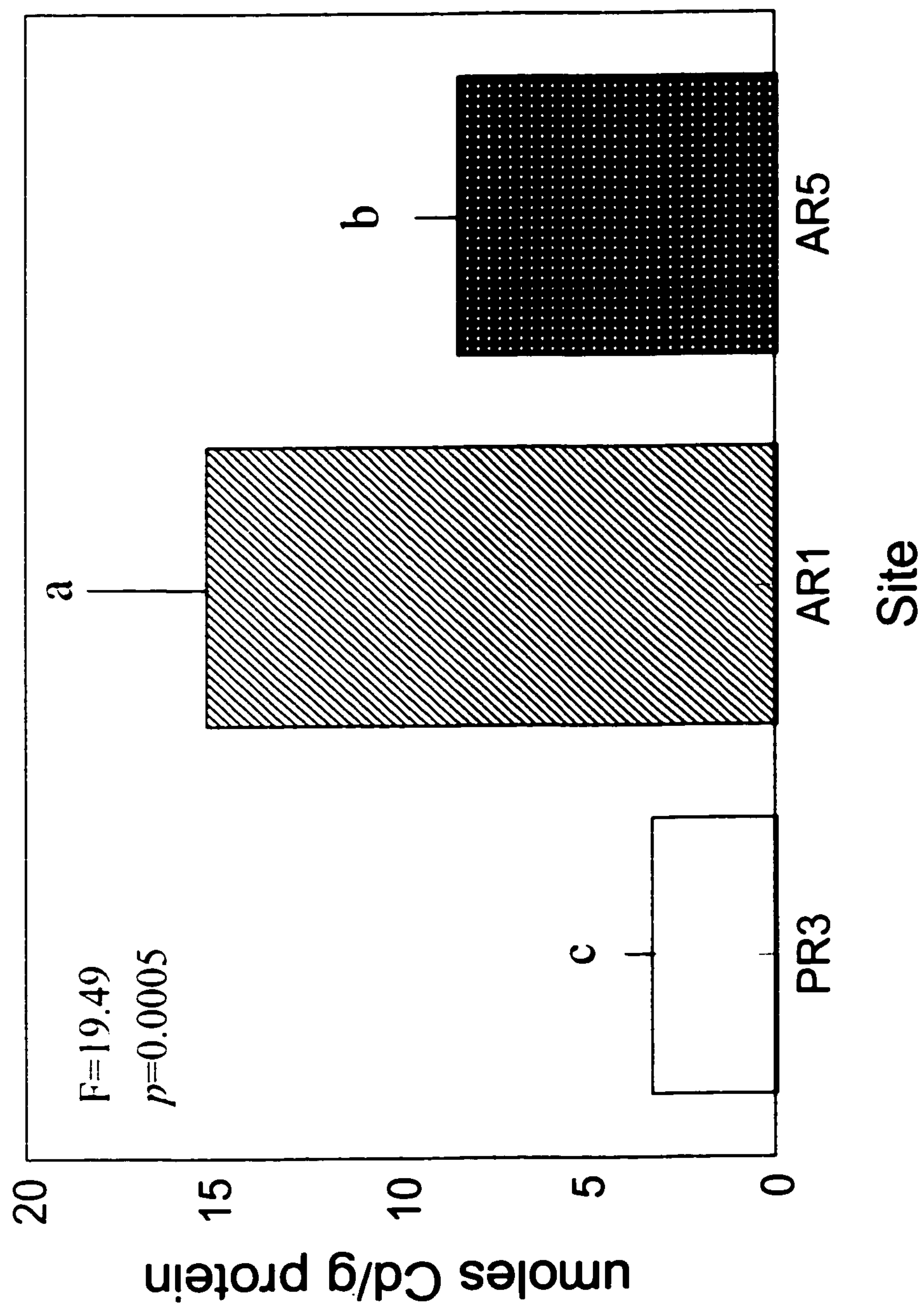

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CHAPTER 3

INFLUENCE OF PREVIOUS EXPOSURE TO METALS ON GENETIC DIVERSITY IN *BAETIS TRICAUDATUS* (EPHEMEROPTERA: BAETIDAE): A COMPARISON OF POPULATIONS

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ABSTRACT

The purpose of this study was to examine the effects of long-term exposure to heavy metals on genetic diversity in the mayfly *Baetis tricaudatus* (Ephemeroptera: Baetidae). Mayflies were collected (n=50) from each of seven sites on the Arkansas River at four different time points to allow both spatial and seasonal comparisons. One site was located above all metal inputs, four sites were located in moderately contaminated areas, and two sites were located in more heavily contaminated areas. Two of the collections took place immediately following spring runoff, and two took place in late summer. Species specific primers were designed for the NADH dehydrogenase subunit 1 (ND1) mitochondrial DNA gene. A 341 bp region of this gene was amplified by polymerase chain reaction (PCR) and analyzed by single strand conformation polymorphism (SSCP) analysis to determine haplotype. Seven different haplotypes were detected, one of which accounted for 78% of all individuals. The lowest diversity was measured at the most contaminated site for three of the time points. Diversity was generally greater in late summer. Correlation analyses conducted to determine if there was a relationship between haplotype diversity and metal concentration (in water, sediment, or periphyton) at a site yielded few significant results. However, haplotype diversity was significantly correlated with concentrations of Zn in water, and Cu and Zn in periphyton for one of the late summer time points. Periphyton is a significant food source for these mayflies. Because it bioaccumulates very high concentrations of metals, even in areas where concentrations of metals in water have decreased, the potential for continued long-term effects of metals on genetic diversity in the Arkansas River remain high.

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INTRODUCTION

Mining activities have had a major impact on watersheds in the Rocky Mountain Region since the discovery of gold in the mid-1800's. A recent analysis of randomly selected 3rd and 4th-order streams in Colorado indicated that approximately one-fourth of them were at least moderately polluted with heavy metals (Clements et al. 2000). It is estimated that 2,600 km of streams have been affected by heavy metals from over 5,000 abandoned waste dumps or tailings piles in this state alone (WQCD 1992).

The upper Arkansas River, located near Leadville, Colorado, has been highly impacted by wastes from mines and tailings piles in the Leadville Mining District. It receives these wastes predominantly through two tunnel systems: the Leadville Mine Drainage Tunnel and the Yak Tunnel. The Leadville Mine Drainage Tunnel discharges contaminated water into the Arkansas River just north of Leadville. The Yak Tunnel, a U.S. Environmental Protection Agency (EPA) Superfund site, discharges more highly contaminated water into California Gulch, which flows directly into the Arkansas River just southwest of Leadville (Figure 3.1). Concentrations of metals are generally orders of magnitude higher in California Gulch than in the Leadville Mine Drainage Tunnel. Acute toxicity (96-hour toxicity test with *Ceriodaphnia dubia*) has been measured >50 km downstream from California Gulch (Clements and Kiffney 1994) and metal levels have exceeded federal criteria for over 400 km downstream (Lewis 1987). Reduced species diversity and increased abundance of metal-tolerant macroinvertebrates have been reported for sites located in this downstream reach (Clements 1994). In 1992, two water treatment plants began treating the water draining the tunnels. Metals concentrations in the Arkansas River have decreased below the Leadville Mine Drainage Tunnel (Nelson

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and Roline 1996), but there has been little change in water quality below California Gulch. During high flow events, such as occur during spring runoff, water bypasses the treatment plant and flows directly into California Gulch and then the Arkansas River, carrying with it high concentrations of cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn).

Because placer mining of gold deposits began in California Gulch in 1859 (Griswold and Griswold 1996), it is likely that benthic macroinvertebrate populations in the Arkansas River have been exposed to heavy metals for over 130 years. Some populations may have even been entirely eliminated following periods of heavy mining activity. This study site provided an opportunity to investigate both ecotoxicological and molecular genetic effects of long-term exposure to heavy metals. Such complimentary studies allow examination of the gene pool that underlies tolerance/sensitivity responses observed in toxicity tests. Results of the ecotoxicological studies are presented elsewhere (Harrahy and Clements, in prep.). Here we will focus on the results of a population genetic study.

Anderson et al. (1994) called for a multidisciplinary approach to addressing ecological consequences of exposure to contaminants, and proposed a research framework that integrates ecotoxicology with population genetics to form a broader model for assessing and predicting effects of pollution. While considerable research has been conducted on the ecotoxicological effects of long-term exposure to contaminants (Antonovics 1971, Weis et al. 1981, Groenendijk 1999), ecotoxicologists have been slow to employ genetic markers to understand how contaminants interact with underlying genetic processes. Contaminants can cause changes in genetic structure through

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mutation, genetic drift, or genetic adaptation (Belfiore and Anderson 1998).

Mutations are caused through direct damage to the DNA molecule and can result in changes at a single locus (gene mutation) or at many loci (chromosomal aberration) (Hummel and Patarnello 1994). For example, heavy metals have been shown to increase DNA strand breakage in some organisms (Black et al. 1996, Husby and McBee 1999). Genetic drift refers to stochastic changes in allele frequencies unrelated to selection (Avers 1980), and is usually a result of small population size. Heavy metals may cause genetic drift by forming a barrier to gene flow and reducing the effective population size. In contrast, genetic adaptation refers to changes in allele frequencies due to selection (Belfiore and Anderson 1998). A number of studies have linked enhanced tolerance to heavy metals with specific allozyme genotypes (Nevo et al. 1984; Diamond et al. 1989; and Haegler et al. 1993). Schleuter et al. (1995) observed differential survival of allozymes for four enzyme loci in fathead minnows exposed to copper, and suggested that monitoring changes in allozyme frequencies in natural populations could be a useful biomarker.

The field of biomarker research is developing rapidly and recent advances in molecular biology have greatly enhanced our ability to study the genetics of natural populations. In particular, polymerase chain reaction (PCR)-based assays have increased our ability to detect differences in genetic variability within and among populations (Black and DuTeau 1997). Use of neutral genetic markers, such as mitochondrial DNA (mtDNA), allows us to take a phylogenetic approach to population genetics and examine the effects of historic factors such as population bottlenecks (Bickham and Smolen 1994). A phylogeny of genes that are neutral with respect to heavy metal tolerance can later be

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combined with a phylogeny of "metal-responsive" genes. Analysis of mtDNA is a powerful tool because the mitochondrial genome is primarily maternally inherited. Maternal transmission of mtDNA ensures that variation is generated by mutation only. In contrast, variation in nuclear DNA can be generated through both mutation and recombination. In addition, because mitochondria lack repair enzymes, mutations accumulate in mtDNA at a faster rate than in nuclear DNA (Murdoch and Hebert 1994). Thus, intraspecific variation is more frequently detectable (Simon et al. 1994).

One strategy to test for variability involves the use of PCR in combination with single strand conformation polymorphism (SSCP) analysis. SSCP analysis detects point mutations, which are manifest as band shifts on nondenaturing gels (Boge et al. 1994). Each variant is designated as a unique haplotype, which is defined as a mitochondrial genome with a unique sequence in the region examined by SSCP. Genetic diversity can then be analyzed using Shannon's Diversity Index (Norris et al. 1996).

Genetic diversity is important because it allows populations to respond to environmental changes (Chagnon and Guttman 1989). While evolution of tolerance mechanisms may allow populations to survive for the short-term, elimination of sensitive genotypes and retention of resistant genotypes may result in the narrowing of genetic diversity within populations, and therefore increase the susceptibility of these populations to further stress (Schlueter et al. 1995).

The purpose of this research was to examine the effects of long-term exposure to heavy metals on genetic diversity of the mayfly *Baetis tricaudatus* (Ephemeroptera: Baetidae). While mayflies are generally sensitive to metals, this particular species is commonly found in areas known to have relatively high concentrations of Cd, Cu, Pb,

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and Zn (Roline 1988, Clements 1994). Recent studies showed *B. tricaudatus* collected from these areas exhibited higher survival and growth, and bioaccumulated more Cd after exposure to metals in the laboratory than *B. tricaudatus* collected from unpolluted areas. In addition, the previously exposed mayflies had higher concentrations of the metal-binding protein metallothionein (Harrahy and Clements, in prep.). The present study was conducted to determine if there are underlying differences in diversity of the NADH dehydrogenase subunit 1 (ND1) mtDNA gene among these populations. A second marker (the 16S rDNA gene) was also examined, and the results of that study are presented elsewhere (DuTeau et al., in prep.).

Methods

Experimental animal

Baetis tricaudatus has a bivoltine life cycle in the study areas and emerges in the spring and fall. Considered to be a rapid colonizer (Robinson et al. 1992), it exhibits a relatively high degree of plasticity in its life history to allow for changing environmental conditions (Clifford 1982, Minshall 1988). *Baetis* larvae are classified trophically as collector-gatherers and scrapers (Merritt and Cummins 1996), and generally feed by grazing periphyton communities for diatoms and algae. *Baetis tricaudatus* is widely distributed in streams and rivers throughout the United States (Morihara and McCafferty 1979), making the methods and results of this study potentially applicable to other metal contaminated areas.

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Collection sites

The Arkansas River is located between the Sawatch and Mosquito mountain ranges in central Colorado. Its primary source of metals is California Gulch, in Leadville; however, other sources include the Leadville Mine Drainage Tunnel and several tributary streams. Metals of primary concern in the Arkansas River are Cd, Cu, Pb, and Zn. *Baetis tricaudatus* were collected (n=50) from each of seven sites on the Arkansas River at four different time points to allow both spatial and seasonal comparisons. One site (EF2) was located above all metal inputs and served as a reference site (Figure 1). Four sites (EF5, EF6, AR1, and AR2) were located below the Leadville Mine Drainage Tunnel, but above California Gulch. Mayflies collected from these sites represented populations with a moderately contaminated exposure history. And two sites (AR3 and AR6) were located below California Gulch. Mayflies collected from these sites represented populations with a more heavily contaminated exposure history. Two of the timepoints (June 1996 and July 1998) took place immediately following spring runoff, when concentrations of metals in water were expected to be highest, and two (August 1995 and August 1996) took place in late summer. Mayfly larvae were collected using kick nets and by hand and placed in vials containing 85% ethyl alcohol. Upon return to the laboratory, they were placed in a freezer (-20°C) for storage prior to DNA extraction.

Physicochemical measurements

Basic water chemistry parameters (temperature, hardness, pH, conductivity, and dissolved oxygen) and current velocity were measured at each site. It should be noted that all readings were taken close to shore, where the mayflies were actually collected and

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may not reflect the magnitude of the current velocity at the center of the stream. Water, sediment and periphyton samples (n=1 each) were collected from each field site, when mayflies were collected to measure concentrations of metals in each of these phases. Water for dissolved metals concentrations was collected with a syringe and passed through an Acrodisc (Gelman Sciences, Ann Arbor, MI) 0.45-µm filter. Total and dissolved water samples were acidified to pH 2 in the field. Sediment was collected in depositional areas in a 50-mL centrifuge tube. Periphyton was collected by scraping three whole rocks with a tooth brush and rinsing the contents into a 50-mL centrifuge tube. Sediment (approximately 2 g) and periphyton (approximately 0.5 g) samples were dried in an oven at 65°C until they reached a constant weight. They were then microwave digested in 70% nitric acid (HNO_3) and diluted to 30 mL. All samples were analyzed for Cd, Cu, and Zn by flame atomic absorption spectrophotometry.

Quality-control and quality-assurance procedures included analysis of National Institute of Standards & Technology (NIST, Gaithersburg, MD, USA) Buffalo River sediment (standard reference material 2704), Cd, Cu, and Zn standards, and HNO_3 blanks. Average recovery of Cd, Cu, and Zn from NIST Buffalo River sediment was 101, 87, and 114%, respectively. Metal standards were within 95% of nominal concentrations. Metals in HNO_3 blanks were consistently below detection (<5 µg/L Cd or Cu and <10 µg/L Zn). To minimize contamination, all labware was acid-washed and plastic materials were used wherever possible.

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DNA isolation

DNA was isolated from the head of each mayfly in August 1995 and the remainder of the body was saved in an individual vial for taxonomic purposes. For all other timepoints, each mayfly was examined under a dissecting microscope to ensure proper species identification prior to homogenization. DNA isolation followed the protocol outlined in Black and DuTeau (1997). Each mayfly was homogenized in 25 µL grinding buffer in an individual 1.5-mL microcentrifuge tube with a pestle, then briefly microfuged. The homogenate was incubated at 65°C for 30 min. While the tubes were still warm, 7 µL of 8M potassium acetate was added to each tube. Tubes were incubated on ice for 30 min, then microfuged at 14,000 *g* for 15 min. The supernatant was transferred to clean tubes. Ethanol (EtOH) (100 µL, 95%) was added to each tube and the tubes were left to incubate at room temperature to precipitate nucleic acids. After 5 min., tubes were microfuged at 14,000 *g* for 15 min. The EtOH was carefully removed so the DNA pellet remained in each tube. EtOH (100 µL, 70%) was added to each tube and the tubes were microfuged at 14,000 *g* for 5 min. The EtOH was removed again, then this step was repeated using 95% EtOH. After removing the EtOH, the DNA pellets were left to dry in the tubes before being resuspended in 200 µL TE (0.01 M Tris-HCl pH 8.0, 1M EDTA).

Primer design

A portion of the 12S mitochondrial ribosomal DNA (rDNA) gene was amplified by PCR using the forward and reverse primers 12S + 1 (5'-TACTATGTTACGACTTA-3') and 12S - 1 (5'-AAACTAGGATTAGATACCC-3') (Norris et al. 1996) in many of the

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mayflies collected. However, over time, amplification of the 12S gene proved unreliable. We chose to examine the protein coding NADH dehydrogenase subunit 1 (ND1) gene instead, and designed species specific primers for this mitochondrial gene region.

Using universal cytochrome b forward (5'-ACATGAATTGGAGCTCGA CCAGT-3') (CB-J-11545 in Simon et al. 1994) and 16S reverse (5'-ACATGATCTG AGTTCAAACCGG-3') (LR-N-12866 in Simon et al. 1994) primers we amplified a 1200 base pair (bp) fragment largely in the ND1 gene region, in individuals representative of each of the different 16S haplotypes detected in *B. tricaudatus* (DuTeau et al., in prep). PCR products were then digested with two restriction enzymes: *Alu*1 and *Msp*1. Digested products were run on an SSCP gel to allow visualization of the resulting fragments. Digestion of the 1200 bp fragment with *Alu*1 and *Msp*1 revealed three distinct patterns for each of the two restriction enzymes. The entire product was sequenced for representatives of each restriction fragment pattern. Sequencing was successful for two of the patterns. This allowed identification of sequences for variable regions. Sequences of different mayflies were aligned using SeqAid II, version 3.81 to look for conserved sequences in the forward and reverse directions flanking the variable region. This yielded 20- and 26-mer forward and reverse sequences, respectively. These sequences were then entered into the OLIGO program (Wojciech and Rychlik 1995) to determine optimum annealing temperature and to check for stability. Forward (11545-423; 5'-CTGGCGTACTCCGCCATGAA-3') and reverse (12866-739; 5'-CTACTCAGT GGATGGTCCTCTAACTC-3') primers were synthesized from these sequences by Operon Technologies, Inc.

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Polymerase chain reaction (PCR) amplification

A portion of the ND1 mitochondrial DNA (mtDNA) gene was amplified by PCR using the forward and reverse primers 11545-423 and 12866-739 for each individual mayfly. PCR reactions were performed in 25-μL volumes. Into each 0.2 mL microcentrifuge tube was added 22.25 μL ddH_2O, 2.5 μL reaction buffer (50 mM KCl. 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0), 0.25 μL dNTPs (2 mM each), and 0.045 μL each primer (500 μM). Mineral oil (20 μL) was layered on top of the contents of each tube. Tubes were briefly microfuged at 14,000 *g* and then exposed to ultraviolet light (260 nm) for 10 min to destroy any contaminating DNA prior to addition of template DNA (1.5 μL) through the oil. Tubes were heated to 95°C in a PTC-100 thermal cycler (M.J. Research, Inc., MA) for 5 min. The temperature was then reduced to 80°C while *Taq* polymerase was added to each tube. Amplification was completed using the following temperature program: 10 cycles of (1) 1 min at 92°C, (2) 1 min at 54°C, and (3) 2 min at 72°C, followed by 30 cycles of (1) 1 min at 92°C, (2) 35 sec at 60°C, and (3) 2 min at 72°C, then 7 min at 72°C. Tubes were then held at 4°C for up to 24 hours in the thermal cycler.

Negative controls containing no template DNA were run simultaneously and the entire reaction set was discarded if DNA appeared in the control. Amplified products were resolved by electrophoresis on 1.2% agarose gels, and visualized by staining with ethidium bromide.

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Single strand conformation polymorphism (SSCP) analysis

PCR products (1 µL) were mixed with loading buffer (9 µL; 10 mM NaOH, 95% formamide, 0.05% bromphenol blue and 0.05% xylene cyanol) in a 96-well plate. The PCR products were denatured by heating to 95°C for 5 min in a thermal cycler and then rapidly cooled by plunging into ice. This formed two types of single strand molecules: denatured single strands and renatured single strands formed by intrastrand base pairing. These products were then loaded (10 µL) onto vertical polyacrylamide gels prepared as outlined in Black and DuTeau (1997) using smaller (16 x 18 cm) glass plates (Hoefer product No. SE 6102, Pharmacia Biotech Inc., San Francisco, CA). A 1 kb ladder was loaded (0.25 µL, 1:5) on each end of the gel as a size marker for comparison. Once identified and sequenced, representatives of common haplotypes were also loaded on each gel for comparison. Electrophoresis was run for 4 to 5 hours at 20 mA and 5°C.

SSCP is based on the principle that electrophoretic mobility of a single stranded DNA molecule depends on both its size and shape. Mobility of a denatured single strand is determined by the size of the PCR product. Mobility of a renatured single is determined by its shape (conformation) and is sensitive to point mutations. Hayashi (1991) showed that SSCP could detect 99% of point mutations in DNA molecules 100-300 bp in length and 89% of point mutations in molecules of 300-450 bp in length.

Following electrophoresis, DNA was visualized by silver staining (Black and DuTeau 1997). Gel plates were scanned or photographed and scored. Each variant banding pattern was designated a unique haplotype (1, 2, 3, etc.). To ensure accurate identification, representatives of each tentative haplotype were sequenced.

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Sequence analysis

PCR products for at least two representatives of each haplotype (except where there was only one) were purified with the PCR Wizard kit (Promega Corporation, Madison, WI) and then sequenced (automated cycle sequencing) by MacroMolecular Resources (Colorado State University, Fort Collins, CO) or Davis Sequencing (University of California, Davis, CA). Forward and reverse sequences for each individual were aligned and edited using SeqAid II, version 3.81, to create a consensus sequence. Consensus sequences for each representative of a given haplotype were aligned to check for consistency (100% match). Representatives of different haplotypes were also aligned to check for redundancy.

To confirm amplification and analysis of the ND1 gene, the sequence of the most common haplotype was submitted to the National Center for Biotechnology Information's (NCBI's) GenBank to search for similar sequences. This search was performed using the Basic Local Alignment Search Tool (BLAST) program "blastx" to compare the nucleotide sequence to protein sequences in the database (Altschul et al. 1997). Over 100 sequences produced significant alignments. The most similar sequence was that of the ND1 gene in another insect, *Drosophila subobscura* (74% match; Barrio et al. 1994).

Analysis of diversity

The diversity of mitochondrial haplotypes at each site was calculated for each time point using Shannon's Index (Shannon and Weaver 1963):

$$\text{avg. } d = C / N\,(N \log_{10} N - \sum n_i \log_{10} n_i)$$

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where $C = 3.322$ (to convert base 10 log to base 2)

N = total number of individuals, and

n_i = total number of individuals of the ith haplotype

Diversity was not calculated if the total number of individuals analyzed at a site by PCR/SSCP was less than 15. Although somewhat arbitrary, this number seems to allow inclusion of some of the more rare haplotypes and is similar to minimum sample sizes used by others (Murdoch and Hebert 1994). The Shannon Index has two components, richness (number of haplotypes) and evenness (distribution of individuals among haplotypes) (Ward and Kondratieff 1992) and is the most frequently used index for calculating species diversity in benthic macroinvertebrate studies (Resh and McElravy 1993).

Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted to compare diversity among sites using the four timepoints as pseudoreplicates. A two-way ANOVA was conducted to examine the effects of both location and timepoint on diversity. Diversity values were square-root transformed to treat heterogeneity of variances. It was not possible to test for a significant location by timepoint interaction because of the lack of replication. (Calculation of diversity resulted in a single number at a site.)

Correlation analysis (SAS 1985) was conducted to determine if there was a relationship between haplotype diversity and metal concentration (in water, sediment, or periphyton) at a site. Separate analyses were conducted for each metal in each phase, and for each time point. Data for the two late summer timepoints (August 1995 and August

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1996) and for the two early summer (immediately following runoff) timepoints (June 1996 and July 1998) were combined for additional analyses. Because correlation analysis assumes a linear relationship, haplotype diversity data were log or square-root transformed as appropriate to give the most linear scatter plot of concentration versus diversity possible. For each analysis, Pearson's correlation coefficient (r) was calculated and the relationship was considered significant if the p-value was less than 0.1.

RESULTS

Physicochemical measurements

Dissolved oxygen was at or near saturation (7 to 8.5 mg/L for this elevation) at all sites each time mayflies were collected. Temperature varied with elevation and time of day, but did not differ significantly between early and late summer (Table 3.1). Stream current velocity was greatest immediately following runoff in June 1996. Water hardness ranged from 30 (AR1, June 1996) to 134 mg $CaCO_3$/L (AR3, August 1996), with an overall mean of 62 mg $CaCO_3$/L. Metal bioavailability generally decreases with increasing water hardness, but no general pattern in water hardness by site, season or year was discernable. pH ranged from 6.2 to 8.3 and conductivity ranged from 70 to 290 µmohs/sec. No general trends were observed for either of these variables.

Zn was detected in total and dissolved water samples at all sites at every timepoint (Figure 3.2). Concentrations of Cd and Cu were always below detection (<5 µg/L) by flame atomic absorption spectrophotometry. Concentrations of Zn were greatest at AR3 (located just downstream of where California Gulch enters the Arkansas River) and lowest at upstream sites. Concentrations of Zn were always less than the chronic

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criterion value of 59 μg/L at sites above California Gulch, and always greater than the chronic criterion value at all sites below.

Metals concentrations in sediment were higher downstream than upstream of California Gulch (Figure 3.3). However, they were generally higher further (18.25 km) downstream from California Gulch than immediately (0.25 km) downstream, probably due to sediment transport processes. Concentrations of Zn were generally two orders of magnitude greater than concentrations of Cu or Cd.

Metals in periphyton were more patchily distributed among locations (Figure 3.4). Highest concentrations of Cu and Zn were found at AR3, but the highest concentration of Cd was measured just downstream of the Leadville Mine Drainage Tunnel at EF6, in August of 1995. Concentrations of metals in periphyton were greater than concentrations in sediment, and concentrations of metals in sediment and periphyton were both orders of magnitude greater than concentrations in water.

PCR/SSCP analysis

A total of 1,400 mayflies were collected from the seven sites over the four timepoints. Twenty-five of these mayflies were identified in the laboratory as species other than *B. tricaudatus* (mostly *Diphetor hageni*). PCR amplification and SSCP analysis were successful for 1,043 of the remaining 1,375 *B. tricaudatus*. The majority of individuals for which PCR amplification was not possible were collected in August 1996. It may be that sequences in the ND1 gene region studied were different enough in these individuals that the primers designed for this study could not anneal to them and inhibited amplification.

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Seven different haplotypes were detected (Figure 3.5). Haplotype 2 was the most common overall, accounting for 78.43% of the individuals analyzed. Haplotypes 6, 1, and 5 accounted for 10.35, 8.44, and 2.01%, respectively. The remaining haplotypes (3, 4, and 7) were very rare and accounted for less than 1% each. Haplotype 2 was the most common haplotype at every site except for AR6 (August 1995) and EF2 and EF6 (August 1996). In each of these cases, haplotype 1 was more common (Table 3.2).

Haplotypes 2 and 6 accounted for 99% of the individuals analyzed in early summer, immediately following runoff (June 1996 and July 1998). These were also the only haplotypes ever detected at site AR3, located just downstream of California Gulch (Figure 3.6).

Sequence analysis

PCR products from 35 individuals representing 13 different haplotypes tentatively identified by banding patterns on polyacrylamide gels were sequenced to ensure accurate identification. Several tentative haplotypes were determined to have the same DNA sequences. For example, tentative haplotypes 2 and 8 were determined to be identical, so haplotype 8 was renamed 2. We were unable to sequence haplotype 4 which was observed in only one individual, but sequences for the remaining six final haplotypes are presented in an appendix to this chapter (Appendix 3.1). Alignment of these sequences revealed that haplotypes differed by as little as one base pair (haplotypes 6 and 7).

DNA sequences were translated to protein sequences (Appendix 3.2). Alignment of these protein sequences revealed that haplotypes 3, 6, and 7 all code for the same amino acid sequence. Haplotype 2 has only one amino acid different from haplotypes 3,

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6, and 7, while haplotypes 1 and 5 each have eleven to nineteen amino acids different from all other haplotypes (Table 3.3).

Diversity analysis

Haplotype diversity was calculated at each site where at least 15 individuals were analyzed by PCR/SSCP, using Shannon's Index, for each time point (Table 3.4). This excluded sites EF5 and AR2 (August 1996 only), and AR6 (August 1995 only). Diversity ranged from 0.00 at site AR3 (August 1996) to 2.10 at site AR6 (June 1996). The lowest diversity for three out of the four timepoints (August 1995, June 1996, and August 1996) was measured at site AR3 (located just downstream of California Gulch). The lowest diversity in July 1998 was measured at sites AR1 and AR2 (0.27 each). With the exception of site AR6, June 1996, diversity values greater than one were measured only during the late summer timepoints. ANOVA indicated no significant differences in diversity among sites when timepoints were treated as replicates (p=0.2595). Two-way ANOVA indicated no significant site (p=0.2721) or timepoint (p=0.4664) effects on diversity as well.

Correlation of genetic diversity with metals

Only five out of 43 correlation analyses yielded significant results (Table 3.5). Haplotype diversity was significantly correlated (p<0.10) with concentrations of Zn in water, and Cu and Zn in periphyton in August 1996. When data for the two late summer timepoints were combined, haplotype diversity remained significantly correlated with concentrations of Zn in water and Zn in periphyton. Pearson's correlation coefficient (r)

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for these five analyses ranged from -0.75 to -0.93, indicating that in these instances, the concentration of a metal (in water or periphyton) explained 56 to 86% of the diversity in haplotypes.

DISCUSSION

In general, genetic diversity in *Baetis tricaudatus* was not correlated with concentrations of heavy metals in the Arkansas River. One possible explanation for this lack of association is that the metals simply did not have an effect (either through genetic drift or selection) on these mayfly populations. However, given the magnitude of historic concentrations of metals in the Arkansas River downstream of California Gulch, and the results of biomonitoring (Roline et al. 1988, Clements 1994) and toxicity (Clements and Kiffney 1994, Harrahy et al., in prep.) studies, this explanation seems improbable.

It is more likely that metals created a "bottleneck", historically removing all *B. tricaudatus* or greatly reducing their population size in the most contaminated reach of the river, when metals concentrations were highest. Such a bottleneck usually results in genetic change through genetic drift (Belfiore and Anderson 1998). However, *B. tricaudatus* is a rapid colonizer (Robinson et al. 1992) and makes up a large proportion of the invertebrate drift in streams (Kohler 1985, Peckarsky and Cowan 1995). In a test of the colonization cycle hypothesis, a stable isotope tracer experiment revealed that approximately one-third to one-half of a *Baetis* nymph population drifted at least 2.1 km downstream over summer, and approximately the same proportion flew 1.6 to 1.9 km upstream from where they emerged (Hershey et al 1993). Such continued immigration may reduce the effects of a population bottleneck on genetic drift in *B. tricaudatus*.

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Populations at sites EF2 and AR6 may serve as examples of the potential influence of colonization, relative to metals, on genetic diversity. Site EF2 was considered a reference site for this study, but haplotype diversity was highest here only for the July 1998 timepoint. Because it is located high upstream at an approximate elevation of 3,048 m, and there are few tributary streams nearby, there may have been less immigration from outside populations, resulting in lower diversity. In contrast, site AR6, which had higher concentrations of metals than site EF2, had the highest haplotype diversity for two of the timepoints (August 1995 and June 1996). This site is located just upstream of Lake Creek which contributes a large volume of water to the Arkansas River. It is also located just upstream of Spring Creek. Both of these tributaries have relatively low concentrations of metals (Nuckols, unpublished data) and colonization from these areas may have contributed to the higher diversity at site AR6. Studies conducted to examine dispersal and gene flow in *Baetis* spp. (Schmidt et al. 1995, Bunn and Hughes 1997) have shown that upstream adult flight is a major mechanism of dispersal for these mayflies.

Another possible explanation for the generally low correlation between haplotype diversity and concentrations of metals is the amount of variation in the ND1 gene region studied was too low to allow greater detection of differences among individuals. Haplotype diversity for the 341 bp region of the ND1 gene studied was generally low (<1) throughout the study reach at all time points (with a maximum of 2.10). Only seven different haplotypes were detected. We initially chose to examine this gene because it codes for a protein, and therefore expected to find more diversity than in a gene that codes for a ribosome. Because of the nature of the amino acid code, substitutions in the

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third and some first codon positions may not result in changes in amino acids. These positions therefore are less constrained and may evolve at a higher rate (Simon et al. 1994). In the present study, three different haplotypes were determined to code for the same amino acids. However, in a companion study (DuTeau et al., in prep.) that examined the 16S ribosomal mitochondrial DNA gene in these same individual mayflies, more haplotypes (12) were detected and higher diversity was measured at some of these sites on the Arkansas River. One possible reason for this difference is that only 340 bp were examined for the ND1 gene while 406 bp were examined for the 16S gene. In looking at a smaller region, we may not have been able to detect as much variation.

Alternatively, haplotype diversity may have been low throughout the study reach because of the presence of metals at all sites. Even the reference site (EF2) had relatively high concentrations of metals in sediment and periphyton (when compared to concentrations in the unpolluted Cache la Poudre River; Harrahy, unpublished data). This likely reflects the highly mineralized geology in the Leadville area. Murdoch and Hebert (1994) showed that historical environmental degradation in the Great Lakes may have reduced populations of brown bullhead (*Ameiurus nebulosus*) in the past, resulting in decreased present-day mitochondrial DNA diversity. A population that has undergone a severe reduction in size due to contamination may lose much of its mitochondrial DNA variability. Loss of mitochondrial DNA diversity has been observed in *Nitocra lacustris* (Copepoda) near offshore oil and gas production platforms, and also in cultures exposed to phenanthrene in the laboratory (Street and Montagna 1996, Street et al. 1998). However, it is unknown how diversity of the ND1 gene in *B. tricaudatus* in the Arkansas River compares to diversity of this gene in a true reference site such as the Cache la

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Poudre River.

It should be noted that sample size may have affected our ability to accurately measure haplotype diversity. Numbers of individuals for which PCR amplification was successful were generally lower for the August 1996 timepoint and may indicate the presence of novel haplotypes, which if detected, would have increased the diversity. The same may be true at site AR6 for the July 1998 timepoint. Shannon's diversity index is based on the assumptions that (1) the sample collected is a random sample from an infinitely large population, and (2) all species (haplotypes) present in the 'community' are represented in the sample (Ward and Kondratieff 1992). Since one or both of these assumptions may not have been met for some samples, caution should be taken in interpreting the diversity and correlation analyses results.

Having stated that, there were some exceptions to the overall lack of association between diversity and concentrations of metals. Haplotype diversity was significantly correlated with concentrations of Zn in water, and Cu and Zn in periphyton in August 1996. During this late-summer timepoint, genetic diversity was higher upstream of California Gulch, and concentrations of metals were lower than during any other timepoint. Because periphyton accumulates metals to such high concentrations and provides an important food source for *Baetis*, it may be a more important exposure pathway than water or sediment.

In this study, concentrations of metals in periphyton were patchily distributed among sites. If one assumes that the same patchiness of concentrations in periphyton exists within a site, particularly at the more moderately contaminated sites, it may be possible for more sensitive haplotypes to coexist with more resistant haplotypes, resulting

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in increased diversity. The intermediate disturbance hypothesis (Connell 1978), which was originally used to explain high species diversity in the tropics, states that under an intermediate disturbance regime, habitat becomes a mosaic of patches where some resident species persist, along with colonizing species which exploit the disturbed areas. Such a hypothesis, if applied to haplotypes rather than species, may help explain patterns in diversity observed for the August 1995 timepoint when diversity was greatest at site AR1, a moderately contaminated site.

Such a hypothesis assumes that certain haplotypes for the ND1 gene, or for some other gene that is linked to the ND1 gene, give a selective advantage to mayflies living in the presence of metals. Haplotypes 2 and 6 were the most common haplotypes in general, but their frequency increased at sites where metals concentrations were high. They were the only two haplotypes ever detected at site AR3, located just downstream of California Gulch. When concentrations of metals in periphyton at site EF6 were lowest (August 1996) there was a shift from haplotype 2 being the most common to haplotype 1. Differences among these haplotypes (meaning differences in sequences in the region of the ND1 gene examined) may translate to subtle differences in protein structure and function. While the mitochondrial ND1 gene was assumed to be a neutral marker in designing this study, it should be noted that this gene codes for a protein which functions in glycolysis and ATP production (Darnell et al. 1986, Eto et al. 1999). It is conceivable that differences in protein structure may result in differential effects of metals on metabolism and energy.

Alternatively, differences in haplotype frequencies may be related by linkage to metal-responsive genes, such as the one that codes for metallothionein (a metal-binding

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protein). A recent study (Harrahy et al., in prep.) showed that concentrations of metallothionein were higher in *B. tricaudatus* collected from a more moderately polluted site on the Arkansas River than from either a reference site or a more heavily contaminated site. Other studies have demonstrated differential survival of allozyme genotypes in the presence of metals (Nevo et al. 1984, Diamond et al. 1989, Schlueter et al. 1995).

Whether a result of population collapse or elimination of sensitive genotypes and retention of resistant genotypes, decreases in genetic diversity may lead to increases in susceptibility of these populations to further stress. For example, while *B. tricaudatus* collected from the Arkansas River were more tolerant to metals than *B. tricaudatus* collected from the unpolluted Cache la Poudre River, they were also more sensitive to acidification (Courtney and Clements 2000). The effects of long-term exposure to metals on survival, growth and metallothionein concentrations in *B. tricaudatus* collected from various sites on the Arkansas River has been investigated (Harrahy et al., in prep.). The present study was designed to complement that work and provide information on genetic structure underlying differences observed in the ecotoxicological endpoints. Unfortunately, the primers designed in this study did not work for all individuals collected, including those collected from the more heavily contaminated site (AR5) and the outside reference site (PR3) studied in the toxicity and metallothionein experiments (Harrahy, unpublished data). Continuation of this project would require designing more universal primers to allow examination of less similar haplotypes, but may provide more detailed information on the influence of previous exposure to metals on genetic diversity.

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While concentrations of metals in overlying water have decreased in the Arkansas River below the Leadville Mine Drainage Tunnel following construction of a water treatment plant (Nelson and Roline 1996), there has been little change in concentrations of metals below California Gulch. In addition, concentrations of metals in sediment and periphyton remain high at downstream locations. Periphyton, a significant food source for many macroinvertebrates, was shown to bioaccumulate metals to concentrations that are orders of magnitude greater than in the overlying water. Since haplotype diversity was significantly correlated with concentrations of Cu and Zn in periphyton, the potential for continued long-term effects of metals on genetic diversity remain high.

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LITERATURE CITED

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and **D.J. Lipman**. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**:3389-3402.

Anderson, S. et al. 1994. Genetic and molecular ecotoxicology: A research framework. *Environmental Health Persp*ectives **102**:3-8.

Antonovics, J., A.D. Bradshaw, and **R.G. Turner**. 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* **7**:1-85.

Avers, C.J. 1980. *Genetics*. D. Van Nostrand and Company, NY.

Barrio, E., A. Latorre, and **A. Moya**. 1994. Phylogeny of the *Drosophila obscura* species group deduced from mitochondrial DNA sequences. *Journal of Molecular Evolution* **39**:478-488.

Belfiore, N.M. and **S.L. Anderson**. 1998. Genetic patterns as a tool for monitoring and assessment of environmental impacts: the example of genetic ecotoxicology. *Environmental Monitoring and Assessment* **51**:465-479.

Bickham, J.W. and **M.J. Smolen**. 1994. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. *Environmental Health Perspectives* **102**:25-28.

Black, M.C., J.R. Ferrell, R.C. Horning, and **L.K. Martin, Jr.** 1996. DNA strand breakage in freshwater mussels (*Anodonta grandis*) exposed to lead in the laboratory and field. *Environmental Toxicology and Chemistry* **15**:802-808.

Black, W.C. IV and **N.M. DuTeau**. 1997. RAPD-PCR and SSCP analysis for insect population genetic studies. Chapter 31 In: *Molecular Biology of Insect Disease*

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Vectors: A Methods Manual. J.M. Crampton, C.B. Beard and C. Louis, (eds.) Chapman and Hall, New York, NY. p. 361-373.

Boge, A., R. Gerstmeier, and **R. Einspanier**. 1994. Molecular polymorphism as a tool for differentiating ground beetles (*Carabus* species): application of ubiquitin PCR/SSCP analysis. *Insect Molecular Biology* **3**:267-271.

Bunn, S.E. and **J.M. Hughes**. 1997. Dispersal and recruitment in streams: evidence from genetic studies. *Journal of the North American Benthological Society* **16**:338-346.

Chagnon, N.L. and **S.I. Guttman**. 1989. Differential survivorship of allozyme genotypes in mosquitofish populations exposed to copper or cadmium. *Environmental Toxicology and Chemistry* **8**:319-326.

Clements, W.H. 1994. Benthic community responses to heavy metals in the upper Arkansas River basin, Colorado. *Journal of the North American Benthological Society* **13**:30-44.

Clements, W.H., D.M. Carlisle, J.M. Lazorchak, and **P.C. Johnson**. 2000. Heavy metals structure benthic communities in Colorado mountain streams. Ecological Applications 10:626-638.

Clements, W.H. and **P.M. Kiffney**. 1994. Integrated laboratory and field approach for assessing impacts of heavy metals at the Arkansas River, Colorado. *Environmental Toxicology and Chemistry* **13**:397-404.

Clifford, H.F. 1982. Life cycles of mayflies (Ephemeroptera), with special reference to voltinism. *Quaestiones Entomologicae* **18**:15-90.

Connell, J.H. 1978. Diversity in tropical rain forests and coral reefs. *Science* **199**:1302-1310.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Courtney, L.A. and **W.H. Clements**. 2000. Sensitivity to acidic pH in benthic invertebrate assemblages with different histories of exposure to metals. *Journal of the North American Benthological Society* **19**:112-127.

Darnell, J., H. Lodish, and **D. Baltimore**. 1986. *Molecular Cell Biology*. Scientific American Books, Inc., New York, NY.

Diamond, S.A., M.C. Newman, M. Mulvey, P.M. Dixon, and **D. Martinson**. 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute exposure to inorganic mercury. *Environmental Toxicology and Chemistry* **8**:613-622.

Eto, K., Y. Tsubamoto, Y. Terauchi, T. Sugiyama, T. Kishimoto, N. Takahashi, N. Yamauchi, N. Kubota, S. Murayama, T. Aizawa, Y. Akanuma, S. Aizawa, H. Kasai, Y. Yazaki, and **T. Kadowaki**. 1999. Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. *Science* **283**:981-985.

Griswold, D.L. and **J.H. Griswold**. 1996. *History of Leadville and Lake County, Colorado: From Mountain Solitude to Metropolis*. Volume 1. Colorado Historical Society, in cooperation with the University Press of Colorado, Boulder, CO.

Groenendijk, D. 1999. Dynamics of metal adaptation in riverine chironomids. Ph.D. dissertation. University of Amsterdam, The Netherlands.

Haegler, M.G., M.C. Newman, M. Mulvey, and **P.M. Dixon**. 1993. Allozyme genotype in mosquitofish, *Gambusia holbrooki*, during mercury exposure: temporal stability, concentration effects and field verification. *Environmental Toxicology and Chemistry* **12**:385-395.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Hayashi, K. 1991. PCR-SSCP: A simple and sensitive method for detection of mutations in genomic DNA. *PCR Methods and Applications* **1**:34-38.

Hershey, A.E., J. Pastor, B.J. Peterson, and **G.W. Kling**. 1993. Stable isotopes resolve the drift paradox for *Baetis* mayflies in an Arctic River. *Ecology* **74**:2315-2325.

Hummel, H. and **T. Patarnello**. 1994. Genetic effects of pollutants on marine and estuarine invertebrates. In: *Genetics and Evolution of Aquatic Organisms*. A.R. Beaumont (ed.), Chapman & Hall, London.

Husby, M.P. and **K. McBee**. 1999. Nuclear DNA content variation and double-strand DNA breakage in white-footed mice (*Peromyscus leucopus*) collected from abandoned strip mines, Oklahoma, USA. *Environmental Toxicology and Chemistry* **18**:926-931.

Kohler, S.L. 1985. Identification of stream drift mechanisms: an experimental and observational approach. *Ecology* **66**:1749-1761.

Lewis, W.S. 1987. An evaluation of mining related metals pollution in the upper Arkansas River basin. M.S. thesis, Colorado School of Mines. Golden, CO.

Merritt, R.W. and **K.W. Cummins**. 1996. *An Introduction to the Aquatic Insects of North America*. Third Edition. Kendall/Hunt Publishing Company, Debuque, Iowa.

Minshall, G.W. 1988. Stream ecosystem theory: a global perspective. *Journal of the North American Benthological Society* **7**:263-288.

Morihara, D.K. and W.P. McCafferty. 1979. The *Baetis* larvae of North America (Ephemeroptera: Baetidae). *Transactions of the American Entomological Society* **105**:139-221.

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Murdoch, M.H. and **P.D.N. Hebert**. 1994. Mitochondrial DNA diversity of brown bullhead from contaminated and relatively pristine sites in the Great Lakes. *Environmental Toxicology and Chemistry* **13**:1281-1289.

Nelson, S.M. and **R.A. Roline**. 1996. Recovery of a stream macroinvertebrate community from mine drainage disturbance. *Hydrobiologia* **339**:73-84.

Nevo, E., R. Ben-Shlomo, and **B. Lavie**. 1984. Mercury selection of allozymes in marine organisms: prediction and verification in nature. *Proceedings of the National Academy of Sciences USA* **81**:1258-1259.

Norris, D.E., J.S.H. Klompen, J.E. Keirans, and **W.C. Black IV**. 1996. Population genetics of *Ixodes scapularis* (Acari: Ixodidae) based on mitochondrial 16S and 12S genes. *Journal of Medical Entomology* **33**:78-89.

Peckarsky, B.L. and **C.A. Cowan**. 1995. Microhabitat and activity periodicity of predatory stoneflies and their mayfly prey in a western Colorado stream. *OIKOS* **74**:513-521.

Resh, V.H. and **E.P. McElravy**. 1993. Contemporary quantitative approaches to biomonitoring using benthic macroinvertebrates. In: *Freshwater Biomonitoring and Benthic Macroinvertebrates*. D.M. Rosenberg and V.H. Resh (eds.). Chapman and Hall, New York, NY.

Robinson, C.T., L.M. Reed, and **G.W. Minshall**. 1992. Influence of flow regime on life history, production, and genetic structure of *Baetis tricaudatus* (Ephemeroptera) and *Hesperoperla pacifica* (Plecoptera). *Journal of the North American Benthological Society* **11**:278-289.

Roline, R.A. 1988. The effects of heavy metals pollution of the upper Arkansas River on

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distribution of aquatic macroinvertebrates. *Hydrobiologia* **160**:3-8.

SAS Institute. 1985. *SAS Procedures Guide for Personal Computers*. Version 6 Edition. Cary, NC.

Schlueter, M.A., S.I. Guttman, J.T. Oris, and **A.J. Bailer**. 1995. Survival of copper-exposed juvenile fathead minnows (*Pimephales promelas*) differs among allozyme genotypes. *Environmental Toxicology and Chemistry* **14**:1727-1734.

Schmidt, S.K., J.M. Hughes, and **S.E. Bunn.** 1995. Gene flow among conspecific populations of *Baetis* sp. (Ephemeroptera): adult flight and larval drift. *Journal of the North American Benthological Society* **14**:147-157.

Shannon, C.E. and **W. Weaver.** 1963. *The Mathematical Theory of Communication.* University of Illinois Press, Urbana.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and **P. Flook**. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* **87**:651-701.

Street, G.T. and **P.A. Montagna**. 1996. Loss of genetic diversity in harpacticoida near offshore platforms. *Marine Biology* **126**:271-282.

Street, G.T., G.R. Lotufo, P.A. Montagna, and **J.W. Fleeger**. 1998. Reduced genetic diversity in a meiobenthic copepod exposed to a xenobiotic. *Journal of Experimental Marine Biology and Ecology* **222**:93-111.

Ward, J.V. and **B.C. Kondratieff**. 1992. *An Illustrated Guide to the Mountain Stream Insects of Colorado*. University Press of Colorado, Niwot, CO.

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Weis, J.S., P. Weiss, M. Heber, and **S. Vaida.** 1981. Methylmercury tolerance of killifish (*Fundulus heteroclitus*) embryos from a polluted vs. a nonpolluted environment. *Marine Biology* **65**:283-287.

WQCD. 1992. *Water Quality in Colorado.* Water Quality Control Division, Colorado Department of Health.

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APPENDIX

Appendix 3.1. Sequences of six haplotypes of a 341 bp region of the mitochondrial NADH dehydrogenase subunit 1 (ND1) gene in *B. tricaudatus* mayflies amplified by PCR and identified by SSCP analysis. Sequences were generated through automated cycle sequencing by MacroMolecular Resources (Colorado State University, Fort Collins, CO) or Davis Sequencing (University of California, Davis, CA).

Haplotype 1
CTGGCGTACTCCGCCATGAAAATCAAAGCAAATCCCCCACTGCTGTACTCGA
TATTAAACCCGGACACGAGTTCGGACTCGCCCTCTGCCAAATCGAAAGGGGT
TCGGTTAGTTTCTGCCAGGCTGGATGCCAATCACATTAGTCCTAAAGGGGGA
GCCACTATGATAAATCTATACCCTGTTTGGGCGGCTGAGAATTCATTTAAACT
GAAACTTCCAATTAAAATCAGGAAGGATAATAGGATTAGGGATAGTCTGACT
TCATAAGAAATGGTTTGGGCAACTCTTCGCAAACCCCCTAATACCGCGTATTT
TGAGTTAGAGGACCATCCACTGAGTAG

Haplotype 2
CTGGCGTACTCCGCCATGAAGATTAGCGCGAATCCCCCACTTCTATACTCGAT
ATTGAACCCGGAAACTAATTCTGACTCCCCTTCAGCGAGGTCGAAGGGGGTG
CGGTTGGTCTCTGCTAGACTAGAAGCCATCCACATAAGAGCCAAGGGTGGGG
CTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTG
AAACTACCTACTAGAATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTT
CATATGAGATGGTTTGAGCTACGCTTCGGAGTCCCCCAAGTATGGAGTACTTC
GAGTTAGAGGACCATCCACTGAGTAG

Haplotype 9
CTGGCGTACTCCGCCATGAAGATTAGCGCGAAGCCCCCACTTCTATACTCGAT
ATTGAACCCGGAAACTAATTCTGATTCCCCTTCAGCGAGGTCGAAGGGGGTG
CGGTTGGTCTCTGCTAGACTAGAAGCCAACCACATAAGAGCCAAGGGTGGGG
CTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTG
AAACTACCTACTAGAATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTT
CATATGAGATGGTTTGAGCTACGCTTCGGAGTCCCCCGAGTATGGAGTACTTC
GAGTTAGAGGACCATCCACTGAGTAG

Haplotype 13
CTGGCGTACTCCGCCATGAAGATTAGGGCGAAACCCCCACTACTGTACTCGA
CATTAAACCCCGATACTAGTTCTGACTCCCCCTCAGCTAAGTCAAATGGGGTG
CGGTTGGTCTCCGCCAGGCTTGACGCCAACCATATGAGGGCCAGAGGTGGGG
CTACTATAAAAAAACTACAAGTGTTTTGGGCTTCTATTAGTTCGTTTAAGTTA
AATCTGCCCACTAGAATTAAAAAGGAGATTAAAATCAAGGCTAGTCTGACTT
CATACGAAATTGTTTGTGCCACACTACGAAGGCCACCCAACATAGAATATTTT
GAGTTAGAGGACCATCCACTGAGTAG

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Haplotype 14

CTGGCGTACTCCGCCATGAAGATTAGCGCGAATCCCCCACTTCTATACTCGAT
ATTGAACCCGGAAACTAATTCTGACTCCCCTTCAGCGAGGTCGAAGGGGGTG
CGGTTGGTCTCTGCTAGACTAGAAGCCAACCACATAAGAGCCAAGGGTGGGG
CTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTG
AAACTACCTACTAGAATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTT
CATATGAGATGGTTTGAGCTACGCTTCGGAGTCCCCCAAGTATGGAGTACTTC
GAGTTAGAGGACCATCCACTGAGTAG

Haplotype 17

CTGGCGTACTCCGCCATGAAGATTAGCGCGAATCCCCCACTTCTATACTCGAT
ATTGAACCCGGAAACTAATTCTGACTCCCCTTCAGCGAGGTCGAAGGGGGTG
CGGTTGGTCTCTGCTAGACTAGAAGCCAACCACATAAGAGCCAAGGGTGGGG
CTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTG
AAACTACCTACTAGAATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTT
CATATGAGATGGTTTGAGCTACGCTTCGGAGCCCCCCAAGTATGGAGTACTTC
GAGTTAGAGGACCATCCACTGAGTAG

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Appendix 3.2. DNA sequences of, and protein coded by, a 341 bp region of the NADH dehydrogenase subunit 1 mitochondrial DNA gene for six different haplotypes detected in *Baetis tricaudatus* mayflies. Forward and reverse primer sequences are underlined.

```
Haplotype 1    CTGGCGTACTCCGCCATGAAAATCAAAGCAAATCCCCCACTGCTGTACTCGATATTAAACCCGGACACGAGTTCG
                L  L  S  G  W  S  S  N  S  K  Y  A  V  L  G  G  L  R  R  V  A  Q  T  I  S
Haplotype 2    CTGGCGTACTCCGCCATGAAGATTAGCGCGAATCCCCCACTTCTATACTCGATATTGAACCCGGAAACTAATTCT
                L  L  S  G  W  S  S  N  S  K  Y  S  I  L  G  G  L  R  S  V  A  Q  T  I  S
Haplotype 3    CTGGCGTACTCCGCCATGAAGATTAGCGCGAAGCCCCCACTTCTATACTCGATATTGAACCCGGAAACTAATTCT
                L  L  S  G  W  S  S  N  S  K  Y  S  I  L  G  G  L  R  S  V  A  Q  T  I  S
Haplotype 5    CTGGCGTACTCCGCCATGAAGATTAGGGCGAAACCCCCACTACTGTACTCGACATTAAACCCCGATACTAGTTCT
                L  L  S  G  W  S  S  N  S  K  Y  S  M  L  G  G  L  R  S  V  A  Q  T  I  S
Haplotype 6    CTGGCGTACTCCGCCATGAAGATTAGCGCGAATCCCCCACTTCTATACTCGATATTGAACCCGGAAACTAATTCT
                L  L  S  G  W  S  S  N  S  K  Y  S  I  L  G  G  L  R  S  V  A  Q  T  I  S
Haplotype 7    CTGGCGTACTCCGCCATGAAGATTAGCGCGAATCCCCCACTTCTATACTCGATATTGAACCCGGAAACTAATTCT
                L  L  S  G  W  S  S  N  S  K  Y  S  I  L  G  G  L  R  S  V  A  Q  T  I  S
```


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```
Haplotype 1   GACTCGCCCTCTGCCAAATCGAAAGGGGTTCGGTTAGTTTCTGCCAGGCTGGATGCCAATCACATTAGTCCTAAA
               Y  E  V  R  L  S  L  I  L  L  S  F  L  I  L  I  G  S  F  S  L  N  E  F  S
Haplotype 2   GACTCCCCTTCAGCGAGGTCGAAGGGGGTGCGGTTGGTCTCTGCTAGACTAGAAGCCATCCACATAAGAGCCAAG
               Y  E  V  S  L  A  L  I  L  I  S  F  L  I  L  V  G  S  F  N  L  N  E  L  V
Haplotype 3   GATTCCCCTTCAGCGAGGTCGAAGGGGGTGCGGTTGGTCTCTGCTAGACTAGAAGCCAACCACATAAGAGCCAAG
               Y  E  V  S  L  A  L  I  L  I  S  F  L  I  L  V  G  S  F  N  L  N  E  L  V
Haplotype 5   GACTCCCCCTCAGCTAAGTCAAATGGGGTGCGGTTGGTCTCCGCCAGGCTTGACGCCAACCATATGAGGGCCAGA
               Y  E  V  R  L  A  L  I  L  I  S  F  L  I  L  V  G  R  F  N  L  N  E  L  I
Haplotype 6   GACTCCCCTTCAGCGAGGTCGAAGGGGGTGCGGTTGGTCTCTGCTAGACTAGAAGCCAACCACATAAGAGCCAAG
               Y  E  V  S  L  A  L  I  L  I  S  F  L  I  L  V  G  S  F  N  L  N  E  L  V
Haplotype 7   GACTCCCCTTCAGCGAGGTCGAAGGGGGTGCGGTTGGTCTCTGCTAGACTAGAAGCCAACCACATAAGAGCCAAG
               Y  E  V  S  L  A  L  I  L  I  S  F  L  I  L  V  G  S  F  N  L  N  E  L  V
```


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Haplotype 1	GGGGGAGCCACTATGATAAATCTATACCCTGTTTGGGCGGCTGAGAATTCATTTAAACTGAAACTTCCAATTAAA
	A A Q T G Y R F I I V A P P L G L M < L A S S L A
Haplotype 2	GGTGGGGCTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTGAAACTACCTACTAGA
	E T Q T G S R F F M V A P P L A L M W M A S S L A
Haplotype 3	GGTGGGGCTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTGAAACTACCTACTAGA
	E T Q T G S R F F M V A P P L A L M W L A S S L A
Haplotype 5	GGTGGGGCTACTATAAAAAAACTACAAGTGTTTTGGGCTTCTATTAGTTCGTTTAAGTTAAATCTGCCCACTAGA
	E A Q N T C S F F I V A P P L A L I W L A S S L A
Haplotype 6	GGTGGGGCTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTGAAACTACCTACTAGA
	E T Q T G S R F F M V A P P L A L M W L A S S L A
Haplotype 7	GGTGGGGCTACCATAAAAAATCTACTCCCAGTCTGGGTCTCCACCAGCTCATTTAAGTTGAAACTACCTACTAGA
	E T Q T G S R F F M V A P P L A L M W L A S S L A

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```
Haplotype 1   ATCAGGAAGGATAATAGGATTAGGGATAGTCTGACTTCATAAGAAATGGTTTGGGCAACTCTTCGCAAACCCCCT
               E  T  N  R  T  P  F  D  L  A  E  G  E  S  E  L  V  S  G  F  N  I  E  Y  S
Haplotype 2   ATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTTCATATGAGATGGTTTGAGCTACGCTTCGGAGTCCCCCA
               E  T  N  R  T  P  F  D  L  A  E  G  E  S  E  L  V  S  G  F  N  I  E  Y  R
Haplotype 3   ATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTTCATATGAGATGGTTTGAGCTACGCTTCGGAGTCCCCCG
               E  T  N  R  T  P  F  D  L  A  E  G  E  S  E  L  V  S  G  F  N  I  E  Y  R
Haplotype 5   ATTAAAAAGGAGATTAAAATCAAGGCTAGTCTGACTTCATACGAAATTGTTTGTGCCACACTACGAAGGCCACCC
               E  T  N  R  T  P  F  D  L  A  E  G  E  S  E  L  V  S  G  F  N  V  E  Y  S
Haplotype 6   ATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTTCATATGAGATGGTTTGAGCTACGCTTCGGAGTCCCCCA
               E  T  N  R  T  P  F  D  L  A  E  G  E  S  E  L  V  S  G  F  N  I  E  Y  R
Haplotype 7   ATTAAAAATGAAATTAAAATTAGCGCCAAACTTACTTCATATGAGATGGTTTGAGCTACGCTTCGGAGCCCCCCA
               E  T  N  R  T  P  F  D  L  A  E  G  E  S  E  L  V  S  G  F  N  I  E  Y  R
```

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```
Haplotype 1    AATACCGCGTATTTTGAGTTAGAGGACCATCCACTGAGTAG
                 S  G  G  F  A  L  I  F  M  A  E  Y  A  ?
Haplotype 2    AGTATGGAGTACTTCGAGTTAGAGGACCATCCACTGAGTAG
                 S  G  G  F  A  L  I  F  M  A  E  Y  A  ?
Haplotype 3    AGTATGGAGTACTTCGAGTTAGAGGACCATCCACTGAGTAG
                 S  G  G  F  A  L  I  F  M  A  E  Y  A  ?
Haplotype 5    AACATAGAATATTTTGAGTTAGAGGACCATCCACTGAGTAG
                 S  G  G  F  A  L  I  F  M  A  E  Y  A  ?
Haplotype 6    AGTATGGAGTACTTCGAGTTAGAGGACCATCCACTGAGTAG
                 S  G  G  F  A  L  I  F  M  A  E  Y  A  ?
Haplotype 7    AGTATGGAGTACTTCGAGTTAGAGGACCATCCACTGAGTAG
                 S  G  G  F  A  L  I  F  M  A  E  Y  A  ?
```

Table 3.1. Physicochemical characteristics of Arkansas River water measured at each time and site of *B. tricaudatus* mayfly collection.

Timepoint/Site	Temperature (°C)	Current (cm/sec)	Hardness (mg $CaCO_3$/L)	pH	Conductivity (μmohs/sec)
August 1995					
EF2	7	69	54	6.8	80
EF5	8	80	70	6.4	110
EF6	10	114	70	—	110
AR1	11	75	50	—	90
AR2	12	95	56	—	90
AR3	12	117	68	—	125
AR6	10	78	60	6.8	105
June 1996					
EF2	6	88	46	8.1	100
EF5	7	124	47	8.0	110
EF6	9	106	56	7.9	120
AR1	11	108	30	6.8	70
AR2	12	119	38	6.7	70
AR3	13	108	44	6.9	80
AR6	13	97	40	6.9	80
August 1996					
EF2	8	58	76	6.4	130
EF5	9	60	98	6.2	200
EF6	10	54	58	—	210
AR1	12	56	84	6.3	170
AR2	14	49	86	8.1	140
AR3	15	37	134	8.1	290
AR6	17	66	98	8.3	240
July 1998					
EF2	9	84	56	8.0	105
EF5	11	90	64	8.2	112
EF6	12	80	66	8.1	135
AR1	14	96	50	8.1	108
AR2	15	94	52	8.1	108
AR3	15	86	58	8.1	110
AR6	15	60	38	7.9	86

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Table 3.2. Number of individual *B. tricaudatus* of a given haplotype at each site on the Arkansas River, for each of four time points. Total number of individuals analyzed by PCR/SSCP is also presented.

Timepoint/Site	Haplotype							Total # Analyzed
	1	2	3	4	5	6	7	
August 1995								
EF2	0	43	0	0	0	7	0	50
EF5	3	39	0	0	0	7	0	49
EF6	2	32	0	0	0	2	0	36
AR1	17	19	1	1	1	1	0	40
AR2	0	40	0	0	1	4	3	48
AR3	0	46	0	0	0	4	0	50
AR6	28	3	0	0	1	8	0	40
June 1996								
EF2	0	43	0	0	0	4	0	47
EF5	0	39	0	0	0	7	0	46
EF6	0	37	0	0	0	12	0	49
AR1	0	44	0	0	0	6	0	50
AR2	0	47	0	0	0	3	0	50
AR3	0	47	0	0	0	1	0	48
AR6	1	6	3	0	0	5	0	15
August 1996								
EF2	14	5	0	0	4	0	0	23
EF5	3	3	0	0	4	0	0	10
EF6	18	7	0	0	10	0	0	35
AR1	0	25	0	0	0	2	0	27
AR2	0	5	0	0	0	0	0	5
AR3	0	24	0	0	0	0	0	24
AR6	0	22	0	0	0	0	0	22

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July 1998								
EF2	0	35	0	0	0	14	0	49
EF5	0	42	0	0	0	7	0	49
EF6	2	39	0	0	0	4	0	45
AR1	0	41	0	0	0	2	0	43
AR2	0	42	0	0	0	2	0	44
AR3	0	42	0	0	0	5	0	47
AR6	0	1	0	0	0	1	0	2

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Table 3.3. Number of nucleotides (out of 341)/**amino acids** (out of 113) different between two haplotypes (Hap.) for a region of the NADH dehydrogenase subunit 1 mitochondrial DNA gene examined by PCR/SSCP analysis in *B. tricaudatus*.

	Hap. 1	Hap. 2	Hap. 3	Hap. 5	Hap. 6	Hap. 7
Hap. 1	-	119/**19**	122/**18**	120/**19**	120/**18**	120/**18**
Hap. 2	-	-	4/**1**	95/**12**	1/**1**	2/**1**
Hap. 3	-	-	-	95/**11**	3/**0**	4/**0**
Hap. 5	-	-	-	-	94/**11**	94/**11**
Hap. 6	-	-	-	-	-	1/**0**
Hap. 7	-	-	-	-	-	-

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Table 3.4. Shannon's diversity of haplotypes for *B. tricaudatus* collected from seven sites on the Arkansas River at four different time points. Diversity was not calculated if total number of individuals analyzed was less than 15.

Timepoint	EF2	EF5	EF6	AR1	AR2	AR3	AR6
August 1995	0.58	0.91	0.61	1.57	0.88	0.40	1.24
June 1996	0.42	0.61	0.80	0.53	0.33	0.15	2.10
August 1996	1.35	—	1.47	0.38	—	0.00	0.22
July 1998	0.86	0.59	0.69	0.27	0.27	0.49	—

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Table 3.5. Results of correlation analyses conducted to determine if there was a relationship between genetic diversity and concentration of metal (in water, sediment, or periphyton). Each time point was analyzed separately. In addition, the two late summer time points (August 1995 and August 1996) and the two early summer (immediately following runoff; June 1996 and July 1998) were combined and analyzed. Pearson correlation coefficients (*r*) and *p*-values are presented.

Timepoint(s)	Water		Sediment			Periphyton		
	Cd	Zn	Cd	Cu	Zn	Cd	Cu	Zn
August 1995								
r	-0.040	-0.295	0.004	-0.241	-0.026	-0.372	-0.549	-0.517
p	0.932	0.520	0.993	0.602	0.956	0.411	0.202	0.234
June 1996								
r	---	0.248	0.573	0.296	0.155	-0.197	-0.203	-0.316
p	---	0.592	0.179	0.519	0.740	0.672	0.662	0.490
August 1996								
r	---	**-0.840**	-0.547	-0.348	0.473	-0.781	**-0.931**	**-0.911**
p	---	**0.074**	0.340	0.565	0.420	0.118	**0.022**	**0.031**
July 1998								
r	---	-0.263	-0.356	0.534	-0.046	0.586	0.476	0.387
p	---	0.614	0.488	0.275	0.931	0.221	0.339	0.449
August 1995 and August 1996								
r	---	**-0.912**	-0.267	-0.317	0.238	-0.427	-0.263	**-0.754**
p	---	**0.0001**	0.402	0.315	0.457	0.166	0.409	**0.005**
June 1996 and July 1998								
r	---	0.131	0.471	0.226	0.151	-0.053	-0.073	-0.114
p	---	0.670	0.104	0.458	0.622	0.863	0.812	0.711

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Figure 3.1. Map showing location of sample sites where *Baetis tricaudatus* mayflies were collected for the population genetic study.

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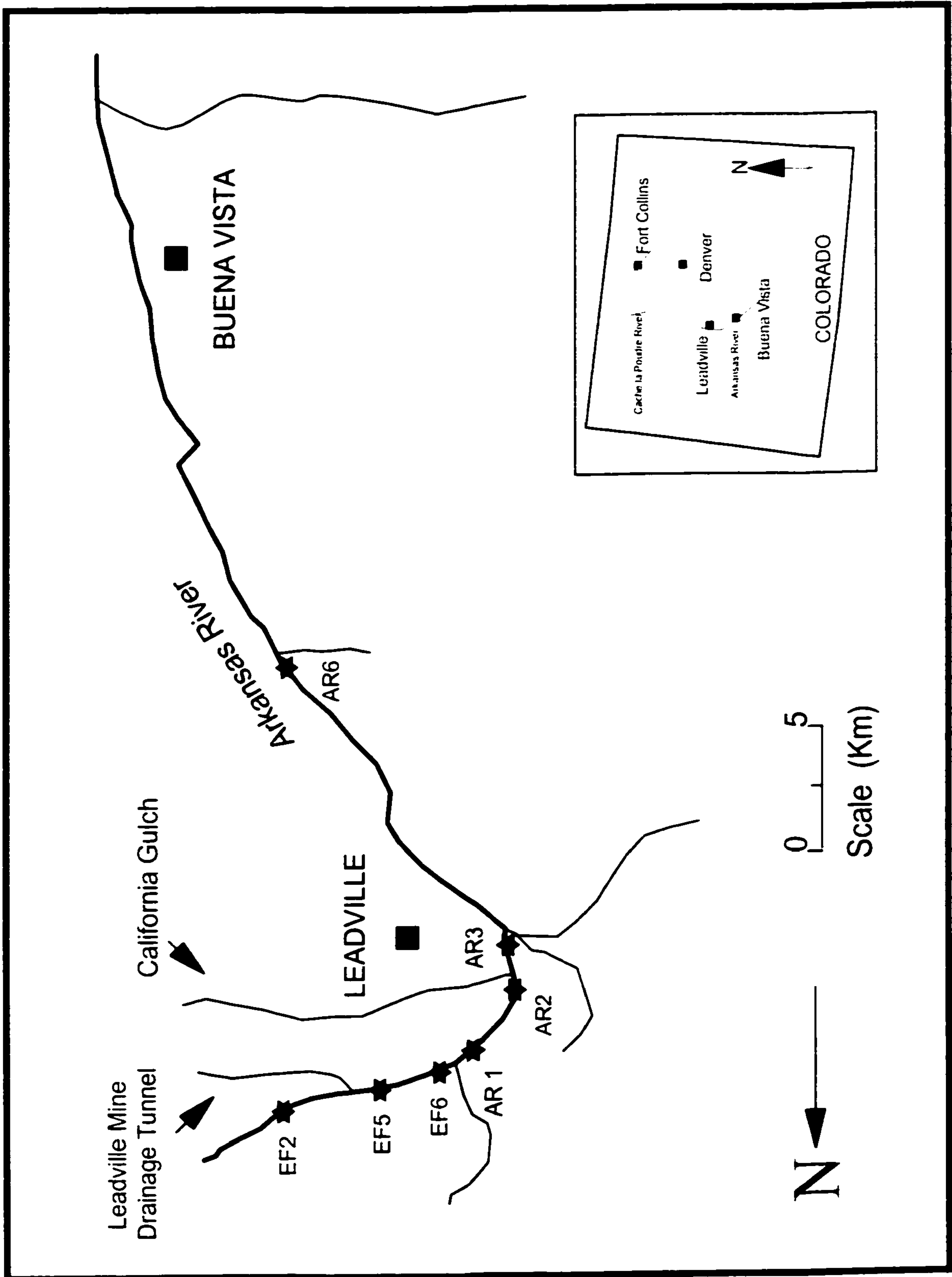

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Figure 3.2. Concentrations of Zn measured in water at each site on the Arkansas River for each of the four time points. Concentrations of Cd and Cu were always below detection (<5 μg/L) and are not presented.

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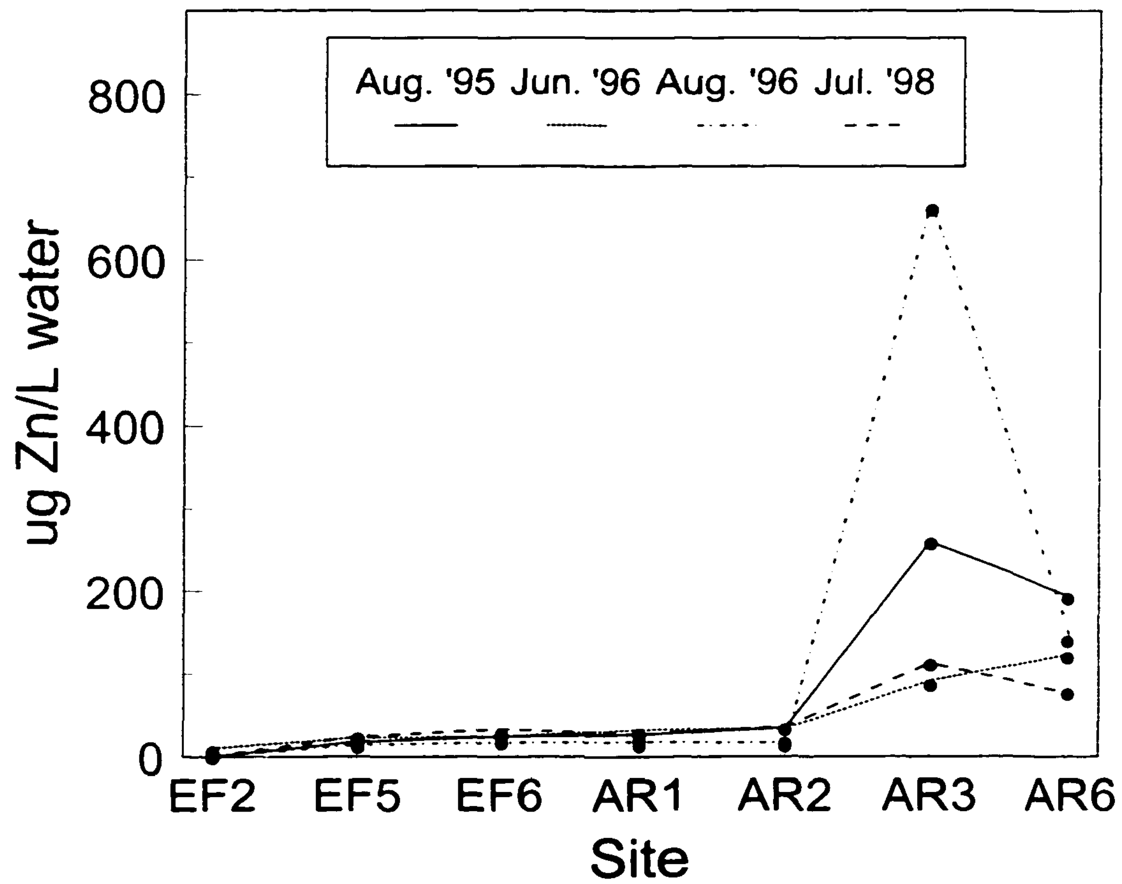

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Figure 3.3. Concentrations of metals (Cd, Cu and Zn) measured in sediment at each site on the Arkansas River for each of the four time points.

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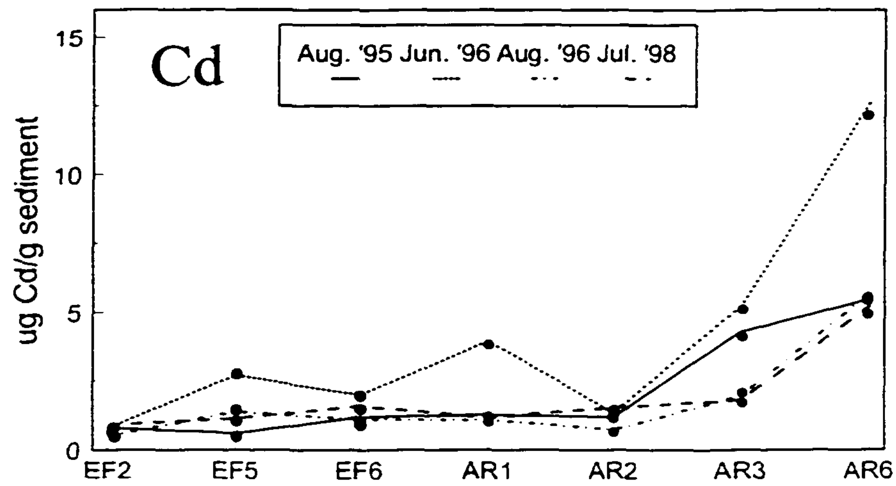

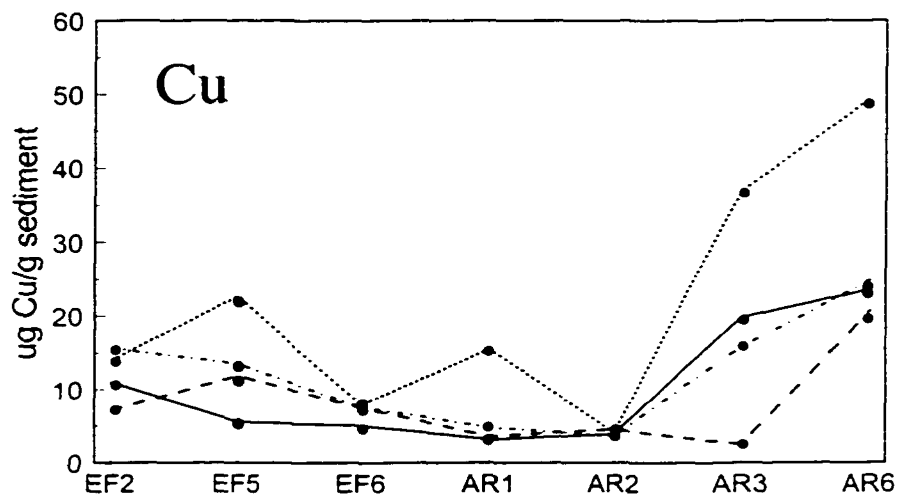

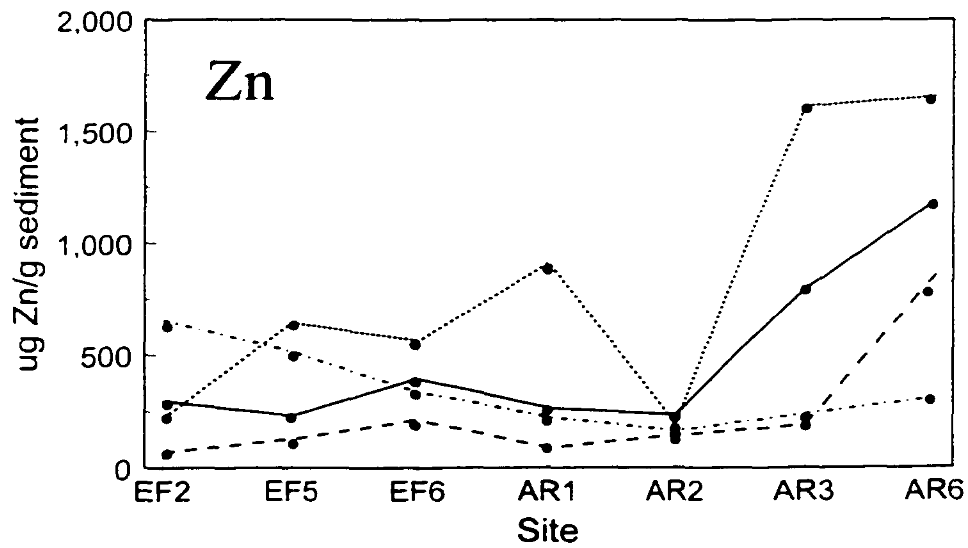

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Figure 3.4. Concentrations of metals (Cd, Cu and Zn) measured in periphyton at each site on the Arkansas River for each of the four time points.

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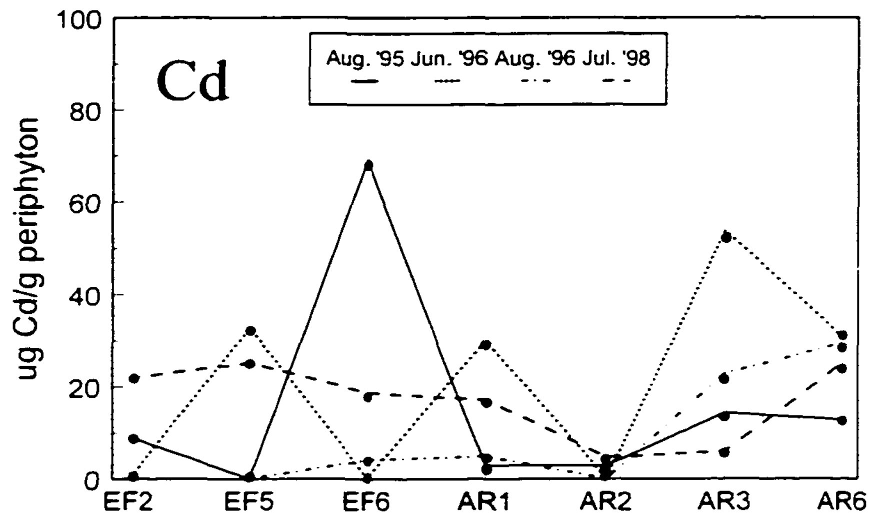

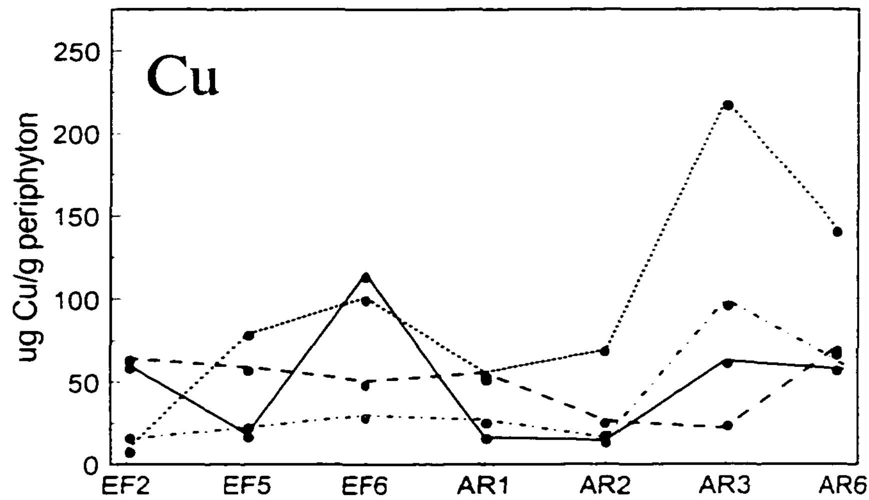

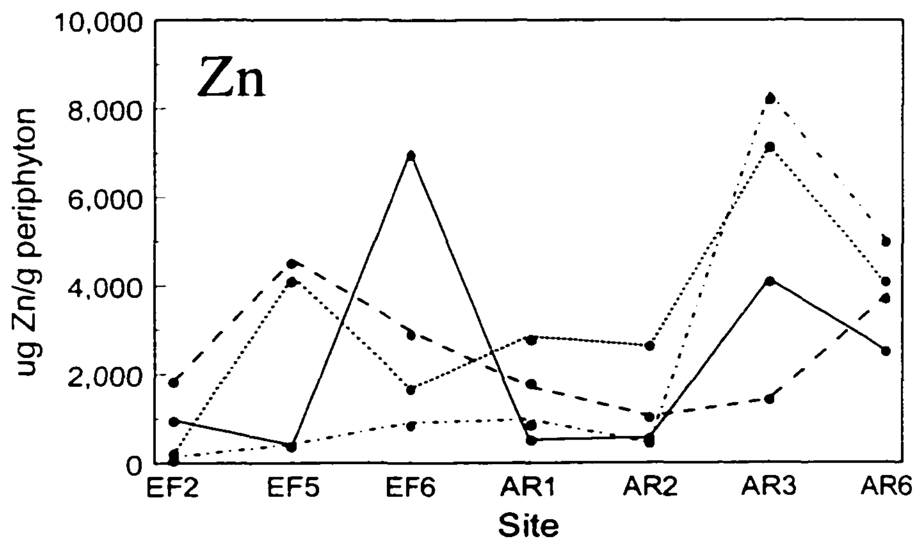

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Figure 3.5. Silver stained SSCP gel showing the seven different haplotypes detected by amplifying 341 bp of the ND1 mitochondrial DNA gene. RSS indicates the renatured single stranded DNA and DSS indicates the denatured single stranded DNA.

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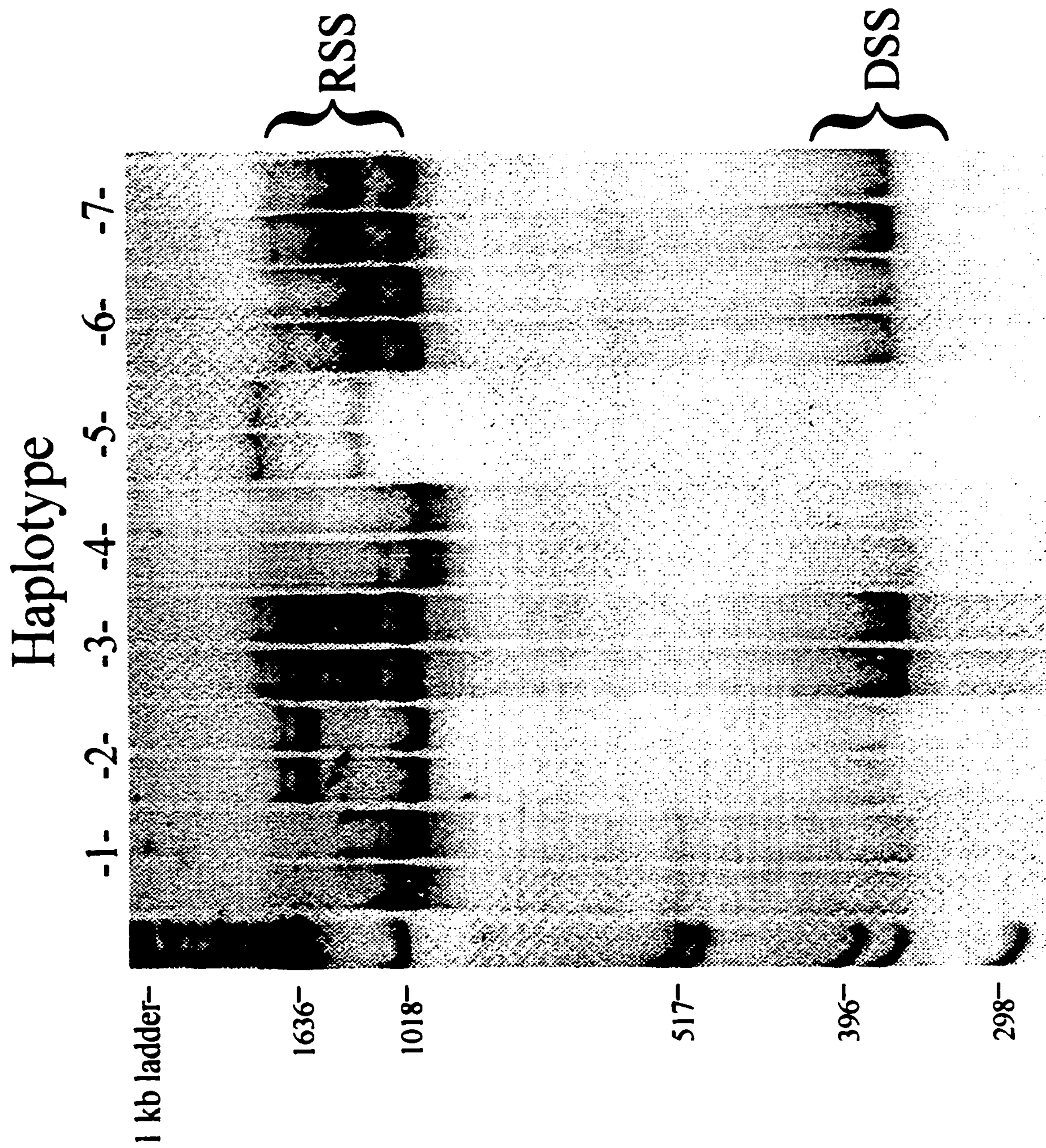

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Figure 3.6. Number of individual *B. tricaudatus* of a given haplotype (x-axis) at each site (y-axis) for each of the four timepoints. Stacked bars do not sum to 50 at sites where PCR amplification/SSCP analysis was not successful for all 50 individuals collected.

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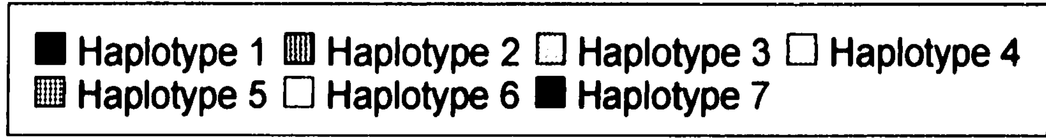

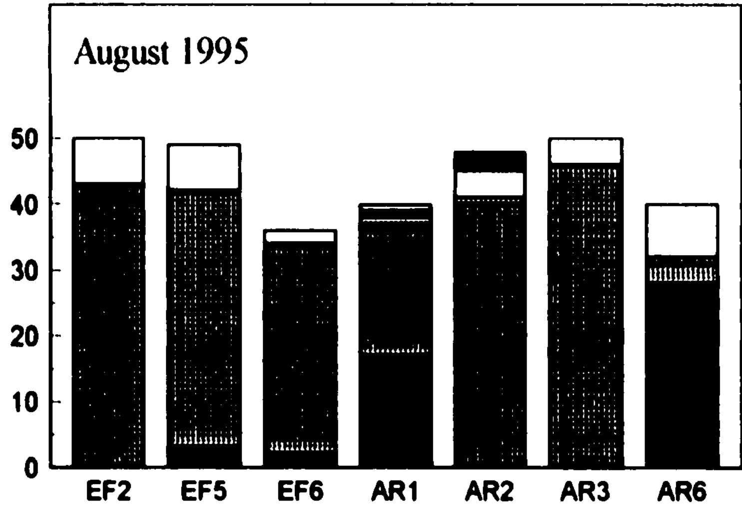

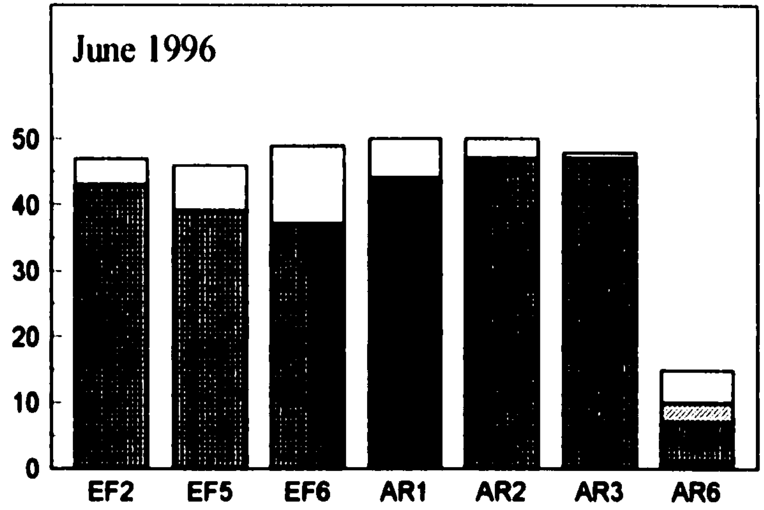

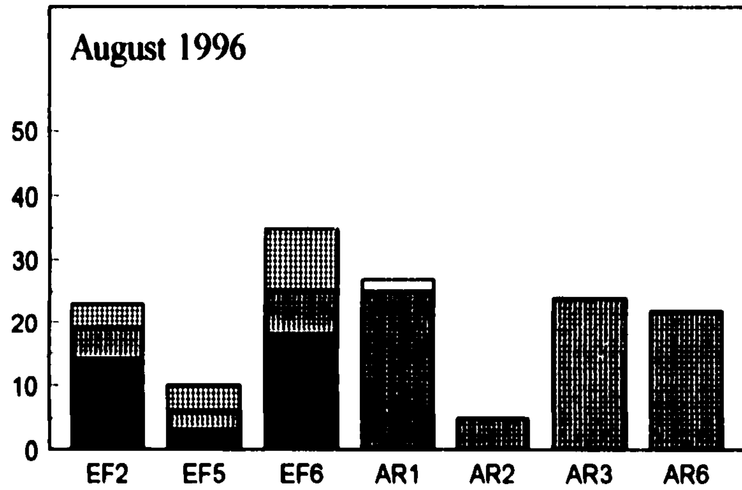

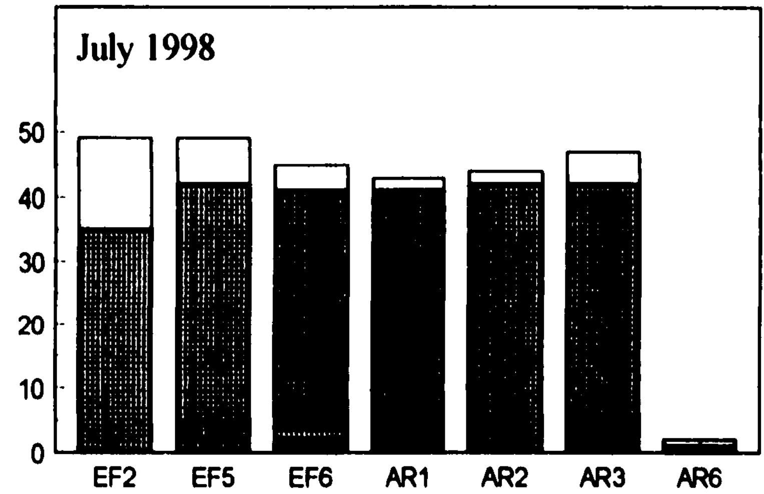

APPENDIX 1

MAP SHOWING LOCATIONS OF ALL SAMPLE SITES WHERE *BAETIS TRICAUDATUS* MAYFLIES WERE COLLECTED FOR THE POPULATION GENETIC STUDY

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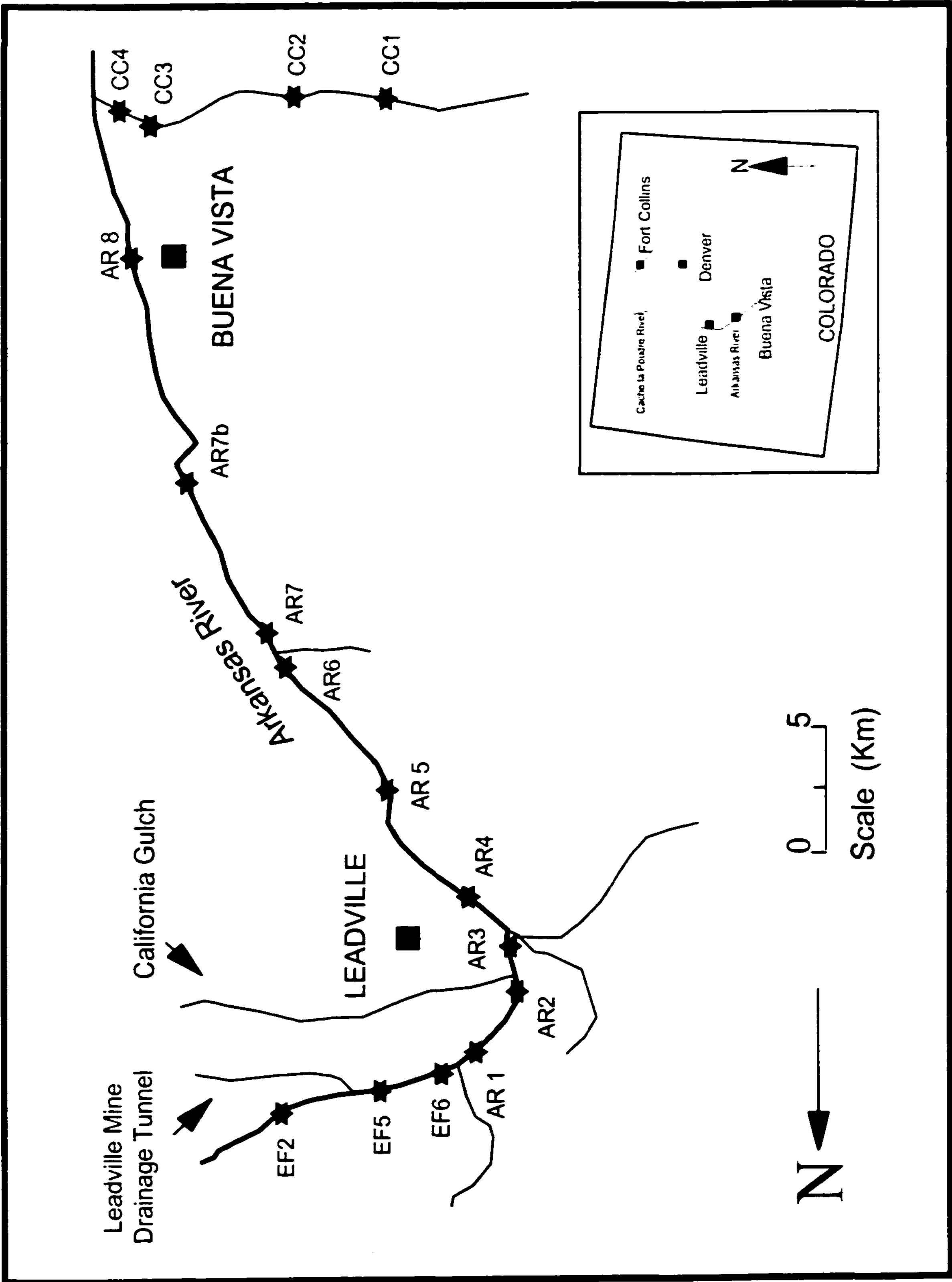

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APPENDIX 2

TABLES SHOWING HAPLOTYPES DETECTED BY PCR/SSCP

IN *BAETIS TRICAUDATUS* MAYFLIES COLLECTED FROM

18 SITES OVER FOUR TIME POINTS IN THE

POPULATION GENETIC STUDY

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Table A.1. Haplotypes detected in individual (Ind.) *B. tricaudatus* mayflies collected from 18 sites (EF2 through PR3) for timepoint A (August 1995). A region of the NADH dehydrogenase subunit 1 gene was amplified by polymerase chain reaction (PCR) and analyzed by single strand conformation polymorphism (SSCP) analysis. An empty cell indicates PCR/SSCP was not successful for that individual. NBt = Non-*B. tricaudatus* individual. "-" = not collected.

Ind.	EF2	EF5	EF6	AR1	AR2	AR3	AR5	AR6	AR7	AR7 b	AR8	CC1	CC2	CC3	CC4	PR1	PR2	PR3
1	14	2	2	1	2	2		2				14	14	2	14			
2	2	14	2	2	2	2		2				14	14	14	14			2
3	2	2	2	NBt	17	2						2	2	14	14			2
4	2	2	2	NBt	2	2					NBt	14	2	NBt	2			2
5	2	2	2	1	2	2		14				14	2	14	14			2
6	2	2	2	2	14	2		14				14	2	14	14			
7	2	2	NBt	2	2	2		1				14	14	NBt	14			
8	14	2	2	NBt	17	2		14				14	14	NBt	14			
9	2	2	NBt	NBt	2	2		14				2	14		2			
10	2	2	NBt	2	2	2		14				14		NBt				
11	2	2	NBt	NBt	2	2		14				14	14	17	14			
12	2	2	NBt	NBt	NBt	2		14				14	14	14				
13	2	2	NBt	2	2	2						2	14	NBt	2			
14	2	2	2	NBt		2		14				14	14	14				

15	2	2	2	2	2	14						14	14	NBt	2			
16	2	2	1	NBt	2	14						14	2		14			
17	14	14	2	NBt	2	2		1				14	2	14	14			
18	2	14	2	2	13	2						14	NBt	14	14			
19	2	2	2	1	2	2						NBt	2	14	14			
20	2	2	14	NBt	2	2		13	1			14	14	NBt	NBt			
21	2	2	NBt	1	2	2		1				14	14	NBt	14			
22	2	14	NBt	1	2	2		1				14	2	NBt	14			
23	2	2	2	1	2	2		1				2	2	NBt	14			
24	2	2	14	1	2	2	NBt	1				14	14	NBt				
25	2	2	NBt	13	2	2		1			NBt	14	14		14			
26	2	2	2	2	2	2		1					14	NBt	14			
27	2	1	2		2	2						14	14	NBt	NBt			
28	2	2	2	9	2	2		1				14	14	NBt	14			
29	14	2	2	1	2	2						14	2	NBt	14			
30	2	14	2	1	2	2		1				17	2	14	2			
31	14	2	2	1	17	2		1				14	14	14	2			
32	14	2	2	2	2	2		2				14	14	14	14			
33	2	2	2	NBt	14	2		1				14	14	14	14			

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34	2	2	2	NBt	NBt	2		1				2	14	14	14			
35	2	14	2	NBt	2	2		1				14	14	14	14			
36	2	2	2	2	2	2		1				2	14	14	14			
37	2	14	NBt	2	NBt	2		1	14			14	17	14	14			
38	14	2	2	1	2	2		1				14	14	NBt	14			
39	2	NBt	2	10	2	14		1				14	14	14	14			
40	2	2	NBt	NBt	2	2		1				14	14	14	14			
41	2	2	2	NBt	2	2		1				14	14	14	14			
42	2	2	2		2	14		1				2	14	NBt	14			
43	2	2	2	1	2	2		1				14	14	14	2			
44	2	2	2	1	2	2		1				14	14	14	14			
45	2	2	NBt	14	2	2		1				14	14	14	14			
46	2	1	NBt	2	2	2		1			-	14	14	NBt	14			
47	2	2	NBt	2	2	2		1			-	14	14	NBt	14			
48	2	2	2	2	2	2		1		NBt	-	14	14	17	14			
49	2	2	1	2	14	2		1			-	14	14	14	14			
50	2	1	2	1	2	2					-	14	14	14	14			
51	-	-	-	2	14	-	-	-	-	-	-	-	-	-	-	-	-	-
52	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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53	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Table A.2. Haplotypes detected in individual (Ind.) *B. tricaudatus* mayflies collected from 18 sites (EF2 through PR3) for timepoint B (June 1996). A region of the NADH dehydrogenase subunit 1 gene was amplified by polymerase chain reaction (PCR) and analyzed by single strand conformation polymorphism (SSCP) analysis. An empty cell indicates PCR/SSCP was not successful for that individual.

Ind.	EF2	EF5	EF6	AR1	AR2	AR3	AR5	AR6	AR7	AR7 b	AR8	CC1	CC2	CC3	CC4	PR1	PR2	PR3
1	2	2	2	2	2			14				14	14		14	2		
2	2	14	14	2	2							14	14	14	14	2		
3	2	2	2	2	2	2						2	2	14	14	2		2
4	14	2	2	2	2	2	2					14	14	14	2	2		
5	2	2	2	2	2	2		9				14	14	14	14			
6		2	2	2	2	2						14	14	14		14		
7	2	2	2	2	2	2		2				14	14	14				
8	2	2	14	2	2	2						14	14		14			
9	2	2	2	2	2	2						2	2	14	14	14		
10	2	2	2	2	14	2						14	14	14				
11	2	14	14	2	2	2						14	14	2	14	14		
12	2	2	2	2	2	2						14	14	14	14	2		
13	2	2	2	2	2	2			17			14	14	14		14		
14	2	2	2	2	14	2		14	2			14	14	14		14		

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15	2	2	2	2	2	2	2		2				14	14	14	14		
16	2	2	2	2	2	2						2	14	14	2			
17	2		2	2	2	2			2			2	2	14	2			
18	2	2	2	2	2	2						14	14	14	14			
19	2	2	2	2	2	2		14				17	17	14	13	14		
20	2	2	2	14	2	2			2			1	1	14	13			
21	2	2	2	2	14	2		9	2			14	14	14	14	14		
22	2	2	2	2	2	2			2			14	14	14				
23	2	2	2	14	2	2		2	2				14	17	14			
24	2	2	2	2	2	2		1				14	14	14	13			
25		2	2	14	2	2						14	14	14				
26		2	2	2	2	2		9	2			14	14	14	14			
27	2	2	2	2	2	2		2				14	14	2				
28	2	2	2	2	2	2			2			14	14	14				
29	2	2	2	14	2	2			2				14		14			
30	2	2	2	2	2	2						14	14	14		14		
31	2	14	14	2	2	2							14	14				
32	2	14	14	2	2	2							14					
33	14		2	2	2	2			2			14	14	14				

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34	2		14	2	2	2		14	2			14	14	14				
35	2	2	2	2	2	14			2			14	14	17				
36	2	2	2	2	2	2						14	14	14				
37	2	14	14	2	2	2							14	14				
38	2	2	14	2	2	2						14	2			14		
39	2	2	2	2	2	2							14	17	13			
40	2	2	2	2	2	2			2				14	14				
41	2	14	14	2	2	2		14	2				14	14				
42	2	2	14	2	2	2	2					2	2	2				
43	2	2	2	2	2	2						14	14	14	14			
44	2	2	2	2	2	2						14	14	14				
45	2	2	2	2	2	2			2				14					
46	14		14	2	2	2						14	14	17				
47	2	2	2	14	2	2		2				17	17	14	14	14		
48	2	14	14	14	2	2						14	14	2				
49	14	2		2	2	2		2				2	2	13				
50	2	2	2	2	2	2		2					14	14				

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Table A.3. Haplotypes detected in individual (Ind.) *B. tricaudatus* mayflies collected from 18 sites (EF2 through PR3) for timepoint C (August 1996). A region of the NADH dehydrogenase subunit 1 gene was amplified by polymerase chain reaction (PCR) and analyzed by single strand conformation polymorphism (SSCP) analysis. An empty cell indicates PCR/SSCP was not successful for that individual.

Ind.	EF2	EF5	EF6	AR1	AR2	AR3	AR5	AR6	AR7	AR7 b	AR8	CC1	CC2	CC3	CC4	PR1	PR2	PR3
1	2			2			2	2				2	14	14	14			
2		2	1	2	2	2	2	2				17	2	14	14		2	
3				14			2					14		14	14			
4	2	2	1			2	2	2					14				2	
5							2	2				14		14				
6		2				2						2	17	14	14			
7	2		1	2								14	2					
8	1	1	13	2		2	2	2				2	2					
9	1	1	1				2					2	17		14			
10			1				2						14					
11			1				2	2				14	2	17				
12				14									2					
13		1	2										2	17				
14	1	13	13				2						14		14			

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15		13											14		14			
16		13				2						14						
17	1		1		2	2	2						14		14			
18			2			2	2					14						
19	1		1			2	2		2			2	14					
20						2	2		2			14	14					
21		13		2		2	1					14	17					
22				2		2	2		2			2	2					
23	1		2			2	2					14	14					
24			1			2						14						
25	1		13		2	2	2					14	14					
26			13	2								2						
27			1				2											
28			1			2	2					14						
29	1		1	2		2	2	2				17	14					
30			1			2		2										
31			1	2		2	2	2				14	14					
32			1					2	2									
33	13		1	2		2	2	2				14		14				

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34			1	2			2	2				14	14					
35	1		1	2			2	2				2	2					
36	1		2			2		2					14					
37			13	2		2		2				14	14					
38	2		13	2				2				14	14					
39	13		13	2		2	2	2				2	14					
40	1		13	2				2				14						
41	1			2				2					14					
42	2		13	2				2				14						
43	1		13	2				2				14	14					
44				2	2			2										
45	1			2								14	14	14				
46			2	2								14	14					
47					2													
48	13			2			2							14				
49			2	2		2	2							14				
50	13		2	2		2						14						

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Table A.4. Haplotypes detected in individual (Ind.) *B. tricaudatus* mayflies collected from 19 sites (EF2 through PR3) for timepoint D (July 1998). A region of the NADH dehydrogenase subunit 1 gene was amplified by polymerase chain reaction (PCR) and analyzed by single strand conformation polymorphism (SSCP) analysis. An empty cell indicates PCR/SSCP was not successful for that individual.

Ind.	EF2	EF5	EF6	AR1	AR2	AR3	AR4	AR5	AR6	AR7	AR7 b	AR8	CC1	CC2	CC3	CC4	PR1	PR2	PR3
1	2	2	2	2	14	2	2						2	14			2	2	
2	2	14	2	2	2	2	2						14	17					14
3	14	2		2		2	14						2	2	14		14		
4	2	14		2	2	2	2						14	14					
5	2	2	2	2	2	2	2						14	14	2				
6	2	2	2	2	2	2	2	14										2	
7	2	2	1	2	2	2	2						14					14	
8	2	2	2	2	2	2	2						14	17				14	
9	2	2	2	2	14	2	2						14	14					
10	14	2	2	2	2	2	2						14	14			14		
11	2	14	2	2	2	2	2						14	2	14	14			
12	14		2	2	2	2	2						14	14		14	2		
13		2	2	2	2	2	2						14	14	14				
14	2	2	2	2	2	2	2						14	14	2	2			

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15	2	2			2	2	2						2	14	14	14			
16	14	2	2	14		2	2						14	14	17				
17	2	2	2	2	2	2	2						14	14	14		14		
18	2	2	2	2	2	2	2							14	14				
19	2	2	2			14	2						14	14		14			
20	2	2	1	2	2	2	2						14	2	2				
21	14	2	2	2	2	2	2						14	14	2				
22	14	2	2	2	2	2	2						14	2	14	14	14		
23	14	2	2	2	2	2	2						14	14		14			
24	2	2		2		2	2						14	14					
25	14	2	2	2	2	2	2						14	14	2	14			
26	14	2	14	2	2	2	2						17	14	14	17			
27	2	14	2		2	2	2						14	14	17	14			
28	2	2	14	2	2	2	2	2	14				14	2	14	14			
29	14	2	2	2	2	2	2						14	14		14	14		
30	2	2		2	2	2	2						14	14		14			
31	2	2	2	2	2	2	2	2					14	14	14				
32	2	2	2	2	2	2	2		2				2	14	14	14			
33	2	2	2	2	2	14	2						14	14	2				

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34	2	2	2	2	2	2	2						2	14		2			
35	2	2	2	2	2	2	2						14	14	2	14			
36	2	2	2		2	2	2						2	14	14	14			
37	2	2	2	2	2	14	2						14	14	14	14		2	
38	2	2	2		2		2						14	14		2	14		
39	2	2	14	2	2		2						14	2	14	2			
40	2	2	14	14	2	14	2						14	14	17	14			
41	14	2	2	2	2	14	2						14	2		14			
42	2	2	2	2	2	2	2						14	2		14			
43	2	2	2	2	2		2						2	14		2			
44	2	2	2	2	2	2	2						14	14	17	14			
45	14	2	2	2	2	2	2						14		2	14			
46	2	14	2	2	2	2	2						14	14	14				
47	14	2	2	2	2	2	2						14	14	14	14			
48	14	14	2	2	2	2	2						14	14	14				
49	2	2	2			2	2						14	14		14			
50	2	14	2			2	2						14	14	14				

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