ARKANSAS RIVER RESEARCH STUDY

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By

Stephen F. Brinkman, Patrick H. Davies, Daria Hansen,
and Nicole Vieira

Colorado Division of Wildlife

STATE OF COLORADO

Bill Owens, Governor

COLORADO DEPARTMENT OF NATURAL RESOURCES

Russell George, Executive Director

COLORADO DIVISION OF WILDLIFE

Bruce McCloskey, Director

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AQUATIC RESEARCH STAFF

Mark S. Jones, General Professional VI, Aquatic Wildlife Research Leader
Richard Anderson, General Professional IV, F-289 Riverine Fish/Flow Investigations
Arturo Avalos, Technician III, Research Hatchery
Stephen Brinkman, General Professional IV, F-243, Water Pollution Studies
Patrick Martinez, General Professional V, F-242, Coldwater Reservoir Ecology & GOCO - Westslope Warmwater
R. Barry Nehring, General Professional V, F-237, Stream Fisheries Investigations
Phil Schler, Hatchery Technician IV, Research Hatchery
George Schisler, General Professional IV, F-394, Salmonid Disease Investigations
Kevin Rogers, General Professional IV, GOCO - Boreal Toad and Colorado Cutthroat Studies
Kevin Thompson, General Professional IV, F-427, Whirling Disease Habitat Interactions
Rod Van Velson, General Professional IV, F-161, Stream Habitat Investigations and Assistance
Harry Vermillion, Scientific Programmer/Analyst, F-239, Aquatic Data Analysis
Nicole Vieira, Physical Scientist III, Water Quality Issues
Rosemary Black, Program Assistant I

Paula Nichols, Federal Aid Coordinator
Jackie Boss, Librarian

TABLE OF CONTENTS

The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Division of Wildlife policy by the Director or the Wildlife Commission.

LIST OF TABLES

LIST OF FIGURES

FISH POPULATIONS AND WATER QUALITY IN THE UPPER ARKANSAS RIVER BASIN 1994-2005

INTRODUCTION

A chemical and biological monitoring program was initiated in the upper Arkansas River basin in 1994. The program's primary objective was to measure water quality and biological community following the cleanup of point and nonpoint sources of metals in the upper Arkansas River.

OBJECTIVES

The objective of this report is to summarize results of an 11 year chemical and biological monitoring study documenting effects of heavy metals loadings to the upper Arkansas River drainage. Specifically, annual, seasonal and spatial changes in hydrology, water chemistry, and biotic responses at sites along a 30 mile stretch of the river, both above and below a major source of metals input (California Gulch). We document changes in these parameters for 1 year before, and 10 years after remediation activities reduced the metals loadings in California Gulch.

METHODS and MATERIALS

The year 2005 represented the final year of a chemical and biological monitoring study initiated in 1994. Data were collected over an area that starts at the headwaters of the Arkansas River and extends downsteream to Granite Colorado, a distance of approximately 30 miles (Figure 1). Stations include several sites along the East Fork of the Arkansas River (Figure 2a), the mainstem Arkansas River downstream to Granite, CO (Figure 2b), and selected tributaries (Figure 2c). Stations on the mainstem were positioned closely enough to evaluate effects of tributaries on brown trout population parameters and water quality.

Site Descriptions

Station EF0

The EF0 sampling site is located downstream from Climax CO. Water samples and water quality data are collected however this is not an electrofishing station. Ice and snow limit access to this site for much of the year.

Station EF1

EF1 is located upstream of Hwy 91 just north of Leadville. Water quality data and fish population estimates are collected at this site.

Station LMDT

The Leadville Mine Drainage Tunnel (LMDT) is treated for metal removal by the Bureau of Reclamation. LMDT flows into the East Fork of the Arkansas River between Colorado Hwy 91 (EF1 station) and the confluence with Evans Gulch. Water quality data are collected at this site.

Station EF2

East Fork Station EF2 is located upstream of the confluence with Evans Gulch, below the LMDT. EF2 is not a fish sampling station.

Station EG3

Station EG3 is located in Evans Gulch upstream of its confluence with the East Fork. The upper reaches of Evans Gulch were subjected to extensive mining in the past. Evans Gulch is an intermittent stream near the confluence with the East Fork but permanently flows farther upstream.

Station EF3

Station EF3 is located on the East Fork immediately downstream from Highway 24 crossing, at the USGS gage. This is the last sampling station on the East Fork before the confluence with Tennessee Creek that forms the mainstem of the Arkansas River. Water quality data and fish population estimates are collected at this site.

Station TC7

Station TC7 is located at the old USGS gage below St Kevins Gulch. St. Kevins Gulch is a significant source of metals to Tennessee Creek as a result of historic mining in the area. Water quality data and fish population estimates are collected at this site.

Station AR1/AR1a

AR1 is the uppermost station on the mainstem of the Arkansas River and is located just downstream of the confluence to the East Fork and Tennessee Creek at the USGS gauge station. To insure complete mixing, effective May, 2000, the water quality sampling station (identified as AR1a) was relocated one-half mile downstream of AR1 to County Road 4. AR1 serves as a reference site although it receives inputs of zinc, cadmium, and other metals from Tennessee Creek. Water quality data and fish population estimates are collected at this site.

Station AR2

AR2 is located immediately upstream of California Gulch on Edith Seppi's property at highway 300. The two sides of the river were designated AR2 East and AR2 West. California Gulch comes into the Arkansas River approximately 150 feet downstream from AR2 East.

Station CG4

Station CG4 (designated as CG6 by most other agencies) is located on Edith Seppi's property downstream of highway 300 just upstream of the confluence with the Arkansas River. California Gulch is, by far, the most significant source of metals to the Upper Arkansas River.

Station AR3a

AR3a is located about one-fourth mile downstream of California Gulch on Harry Beck's property. Water quality data and fish population estimates are collected at this site.

Station LF22

Station LF22 is in the Lake Fork of the Arkansas River and located on the Ledbetter property downstream of County Rd 11/Forest Route 160. Halfmoon Creek merges with the Lake Fork downstream from this station. The Lake Fork flows into the mainstem of the Arkansas River just upstream from Station AR4. Water quality data and fish population estimates are collected at this site.

Station AR-4

AR4 is located on Dr. Bernard Smith's property upstream of County Rd. 44 and below the confluence of the Lake Fork. Water quality data and fish population estimates are collected at this site.

Station IG2

Iowa Gulch station IG2 is located upstream of its confluence with the Arkansas River at U.S. Highway 24 culvert. Iowa Gulch flows intermittently at this site.

Station AR5

AR5 is located between U.S. Highway 24 bridge and Empire Gulch. Iowa Gulch is the largest tributary between AR4 and AR5. Water quality data and fish population estimates are collected at this site.

Station AR-6a

AR6a is located approximately a half mile upstream of County Rd. 55 at Kobe. With the acquisition of the Hayden Ranch by the City of Aurora, this reach of the Arkansas River became opened to public fishing. Consequently, this station was added as a new fish sampling station. Water quality data are not collected at this site but values are expected to be similar to station AR6 which is located a half mile down stream.

Station AR6

Station AR6 is located immediately downstream of County Rd. 55. Water quality at this station is considered to be representative of station AR6a about ½ mile upstream. Water quality data and fish population estimates are collected at this site.

Station AR7

Station AR7 is located downstream of the confluence of the Arkansas River with Lake Creek at the USGS gauge at Granite, CO. Metal concentrations in Lake Creek below twin Lakes reservoir is typically low in metals and hardness is < 20 mg/L (Davies et al. 2002). Lake Creek greatly increases the discharge of the Arkansas River and significantly dilutes metals concentrations relative to upstream stations. Water quality data and fish population estimates are collected at this site.

Water Quality and Metal Concentrations

The Aquatic Toxicology Research Group of the Colorado Division of Wildlife investigated the effects of heavy metals on the fish communities of the Upper Arkansas River Basin. Water quality data and water samples for metal analysis were collected from stations along the East Fork of the Arkansas River, selected tributaries, and the mainstem Arkansas River downstream to Granite, CO. Water quality parameters (pH, alkalinity, hardness, conductivity, temperature, and dissolved oxygen) were measured on site. Alkalinity and EDTA hardness were determined according to Standard Methods (APHA 1998). A Thermo Orion 635 meter measured pH, conductivity, and temperature. The meter was calibrated with 4.00, 7.00 and 10.00 pH buffers and two conductivity standards each sampling day. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. Water quality parameters collected between October 2001 and September 2005 are presented in Appendix A. Site water for metals analyses was immediately passed through a 0.45 µm filter (Acrodisc), collected in 60 ml high density polyethylene bottles (Nalgene), and immediately preserved with Ultrex® triple distilled nitric acid (JT Baker) to pH <2. Field splits and blanks were collected on >20% of samples.

Concentrations of metals were determined using an axial inductively coupled argon plasma spectrometer (Thermo Jarrell Ash), equipped with an ultrasonic nebulizer (CETAC). Each water sample was analyzed for aluminum (Al), cadmium (Cd), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), lead (Pb), selenium (Se), and zinc (Zn). Water quality values and metal concentrations have been previously reported (Davies et al. 1997, Davies et al. 2000, Davies et al. 2002, Brinkman et al. 2006). Sampling efforts focused on the months during spring runoff. Previous investigations have shown that metal concentrations in the upper Arkansas River are greatest during high discharge (Davies et al. 1997, Davies et al. 2000, EPA 2004). For the purposes of this report, spring runoff period is defined between April and July. Peak runoff in the Upper Arkansas River can occur between the end of April and end of June, depending on snowpack and spring temperatures.

Zinc, and to a lesser extent cadmium, are the primary toxicants of concern to aquatic life in the Upper Arkansas River, based on frequency and magnitude of exceedences of water quality criteria (Davies et al. 1997, Davies et al. 2000, EPA 2004). Exceedences of copper, iron and aluminum aquatic life criteria have also occurred, however, those exceedences were relatively infrequent and small in magnitude. Consequently, zinc and cadmium were the focus of previous reports. Spatial and temporal patterns of zinc concentrations in the Upper Arkansas River are closely paralleled by cadmium, although cadmium is present at much lower levels (Brinkman et al. 2006). Therefore this report will focus on the behavior of zinc over the 11 year time frame of this study. Trends identified with zinc can be applied to cadmium with reasonable accuracy.

Fish Surveys

Annual fish community surveys of the Upper Arkansas River Basin were initiated in 1997 in cooperation with aquatic biologists from the Division and Wildlife and from the consulting firm of Chadwick and Associates representing Resurrection Mining Company. Sites were selected based on representiveness of habitat and proximity to water sampling locations. Four to seven hundred feet of stream were electroshocked and population estimates based on the two pass removal method (Seber and LeCren 1967). An additional pass was conducted if

sampling efficiency is less than 65% on the first pass. Surveys were conducted during the fall of 1994, 1997, 1999, and 2001 through 2005. In 1998 and 2000, sampling occurred in the spring, shortly after ice-off. After 2000, both parties agreed to discontinue spring in favor of annual fall sampling. At low water temperatures, juvenile trout burrow deep into the interstitial spaces in the substrate (Campbell and Neuner 1985, Griffith and Smith 1993, Heggenes et al. 1993, Griffith and Smith 1995, Meyer and Griffith 1997) making them difficult to capture, potentially biasing density estimates.

The fall sampling was scheduled in mid to late August, after high runoff flows but prior to spawning migrations. Fish were identified to species and individual lengths (mm) and weights (g) measured and recorded. Species composition, brown trout length frequency distribution, and estimates of population density (#/ha), and biomass (Kg/ha) are reported for each sampling location. Estimates for salmonds were based on ≥1+ age class. In nearly all instances, 1+ or older were classified as fish ≥10 cm in length, based on length-frequency distributions. Population estimates were based on the measured area of the station at the time of the sampling. In some cases, this may slightly affect our estimates because the width of some sites varied with water levels. An extreme example occurred during the record drought in 2002 when stream widths were 10-20% less than average. In instances where the number of individuals of a species were small (e.g. rainbow trout), estimates were based on the combined number of individuals collected on both passes, divided by the area.

Between the years 2002-2005, 1+ age class brown trout collected by electrofishing were marked with fluorescent color visual implant elastomer (VIE) tag. These marks were used to confirm known age brown trout with estimated scale-ages, to determine longevity, and to assess potential movement among sampling sites. Individuals were marked behind the right eye in 2002, behind the left eye in 2003, on the right posterior opercle in 2004 and the left posterior opercle in 2005. Fluorescent orange was used to mark fish collected from the East Fork, fluorescent green marked fish from Tennessee Creek, fluorescent red marked Arkansas River fish above California gulch, and fluorescent yellow marked fish below California Gulch.

Results of fish surveys have been previously reported (Davies et al. 1997, Davies et al. 2000, Davies et al. 2002, Brinkman et al. 2006).

Macroinvertebrate Surveys

Annual surveys for benthic macroinvertebrates were conducted in spring (May) and fall (October) between 1994 -2005 at sites AR1, AR3a, AR5, AR8 and a site in the East Fork (EF3) by other research groups (Dr. Will Clements, Colorado State University, Chadwick Ecological Consultants, Inc.). Study details and results for these surveys are published elsewhere (Clements 1994; Clements and Kiffney 1995) or are not yet available (Chadwick Ecological Consultants, Inc.). Here, we summarize available information on community trends at the aforementioned stations.

Brown Trout Toxicity Studies

Acute and chronic toxicity tests were conducted to study the effects of cadmium and zinc exposure on brown trout embryos, larvae, and fry. Multiple tests at different water hardness levels were conducted in order to quantify the effect of water hardness on toxicity of these metals to brown trout. Results of the cadmium and zinc toxicity tests are summarized in Appendices A and B, respectively.

Experiments were conducted to study the effect of acclimation of brown trout to metals. Brown trout embryos, larvae and fry were exposed to sublethal levels of zinc and copper singly and mixtures of zinc and cadmium and zinc and copper. Sublethal exposure was found to increase tolerance relative to naïve unexposed brown trout. Once returned to clean water, exposed brown trout lost their tolerance. Results of the tests are reported in Appendix C.

RESULTS and DISCUSSION

The eleven year time span of our monitoring project was sufficiently long to study seasonal, spatial and inter-annual changes in hydrology, water chemistry, metals contamination and responses of aquatic biota. Data were collected in years with exceptionally high spring runoff as well as during drought years. Runoff during the early years of the study, 1995-1997, was much greater than average. Runoff since 2000 has been well below average. In 2002, Colorado experienced the greatest drought on record. In the mid 1990s, shortly after the start of the study, remedial activities were undertaken in California Gulch and its tributaries in an attempt to reduce metal loading to the Arkansas River. Several mine tailings piles were removed or capped and revegetated. Therefore, we were able to capture abiotic and biotic responses to remediation activities at metals-impacted sites, and could compare these responses to changes observed at reference sites.

Water Hardness - Spatial Effects

Water quality in the mainstem of the Upper Arkansas River is greatly affected by its tributaries (Figure 3). Mean water hardness varies from a high of 99 mg/L at EF3 to a low of 42 mg/L at AR7 and AR8. Average hardness decreases due to dilution from tributaries with low hardness. These tributaries include Tennessee Creek (TC7), the Lake Fork (LF22), and Lake Creek (LC1). In contrast, hardness increases as a result of high hardness present in the Leadville Mine Drainage Tunnel (LMDT), California Gulch (CG4), and Iowa Gulch (IG2). Tributaries that are low in hardness have a much greater discharge than tributaries with the high hardness water. Consequently, the overall trend is decreasing hardness as one travels downstream in this reach of the Arkansas River. At first, this pattern may seem counterintuitive, since hardness typically increases from headwaters to lower elevation rivers. However, the spatial scale of this study (approximately 30 miles) is too small to be influenced by coarser elevational patterns in water chemistry.

Water Hardness – Seasonal Effects

Water hardness varies seasonally in the Arkansas River and tributaries. The mechanism for the strong seasonality pattern is dilution from the melting of mountain snow pack that occurs during the spring. The Arkansas River at Leadville (AR1) will be used an example for what occurs at other

stations. A hydrograph of mean monthly discharge at station AR1 illustrates the increase in discharge during May, June, and July (Figure 4). As discharge increases, mean monthly hardness decreases from 110 mg/L during low flows in the winter to 44 mg/L during springtime high flows. The relationship between discharge and hardness was relatively strong (Figure 5). Individual hardness measurements at AR1 during spring run off are frequently as low as 30 mg/L. A similar relationship is observed farther downstream at Granite (AR7) (Figure 6). The effect of seasonal discharge on hardness at AR7 is moderated by tributaries that flow into the Arkansas River between AR1 and AR7, in particular Lake Creek. As a result, the range of hardness observed at AR7 is less than the range at AR1.

Metal Concentrations – Spatial Effects

Tributaries to the mainstem Arkansas River exert two effects on metal concentrations. Some tributaries serve as source of metals and other tributaries provide dilution which acts to reduce metal concentrations. The effect of the tributaries is largely the same for both zinc and cadmium (Figure 7). Tennessee Creek (TC7) increases metal concentrations between the East Fork (EF3) and the Arkansas River at Leadville (AR1). California Gulch (CG4) greatly increases metal concentrations between stations AR1 and AR3a. Dilution flows from the Lake Fork (LF22) significantly reduce metal concentrations at station AR4. A similar though less dramatic dilution resulted from inputs from Lake Creek (LC1). Figure 7 clearly demonstrates that California Gulch is the overwhelming source of metals to the upper Arkansas River.

Metal Concentrations – Seasonal Effects

Metal concentrations vary seasonally in the upper Arkansas River. A box and whisker plot of the Arkansas River at Leadville (AR1) demonstrate that zinc concentrations tend to be much higher in April, May and June than during other months (Figure 8). Concentrations during these months are also more variable. A similar seasonal pattern was also apparent at the mouth of California Gulch (CG4) (Figure 9). At CG4, zinc concentrations were highest and most variable during April, May, and June. At station AR3a downstream from California Gulch, zinc concentration did not exhibit a strong seasonal pattern (Figure 10). Maximum zinc concentrations were observed in April, May and June but average zinc concentrations were highest September to December, perhaps because upstream dilutions flows decrease following spring runoff. The Arkansas River stations between the Lake Fork and Lake Creek exhibit a similar seasonal pattern of zinc concentrations as AR1 and CG4 (Figure 11). Data from the three stations in this stream reach were combined due to their similarity. Lastly, at the lowermost Arkansas River station at Granite (AR7), metal concentrations are high in May, April and June, followed by a sharp decrease in July and August (Figure 12). At AR7, concentrations appear to gradually increase after August through November, similar to the pattern observed at AR3a. This is possibly due to metal inputs in Lake Creek which joins the Arkansas River above this station. Although not shown, other metals especially cadmium, displayed similar seasonal relationships.

It is widely believed that surface runoff from melting snowpack in April, May, and June, flows over and through mine tailings leading to the introduction of metals into receiving streams. The high inputs of metals from California Gulch during the months of April, May, and June were largely responsible for the seasonality of metals concentrations at downstream stations. A similar seasonal pattern was observed at AR1 which is unaffected by California Gulch. However, AR1 receives metal

inputs from St Kevins gulch in the Tennessee Creek drainage. Mechanisms that act to introduce metals in California Gulch are also likely to operate at the historical mining sites in St Kevins Gulch.

Metal Concentrations – Effects of Stream Discharge

During the course of this project, the upper Arkansas River basin experienced above average precipitation in the mid 1990s as well as drought conditions during the late 1990 and early 2000s. Daily stream discharge in the Arkansas River at Leadville (AR1) reflects the variability of conditions experienced by the upper Arkansas River basin 1994-2005 (Figure 13). The average peak discharge for the period of record at this site (1967-2004) is 684 cfs. Peak discharge during 1995, 1996, and 1997 were well above average. In 2000 and 2003, peak discharge was near average. The years 1994, 1998, 1999, 2001, 2002, 2004, and 2005 were well below average. In 2002, stream discharge was the lowest on record for the Arkansas River. Average stream discharge at AR1 during spring runoff roughly parallels peak discharge, although there are some differences (Figure 14). Average spring runoff was higher than average during 1995, 1996, and 1997. The years 1994, 1998 and 2000 to 2005 were below the average spring discharge of 72 cfs for the period of record (31 years). Since 1997, the only year with above average discharge was 1999. Stream discharge during spring runoff was below average in 2000 and 2003 although the peak discharge during those years was above average. In contrast, 1999 was above average in terms of mean discharge although the peak was below average. At Granite (AR7), the most downstream station, the pattern of wet and dry years was similar to AR1 (Figure 15). The effect of drought during 2002 is particularly apparent. Trans-basin diversions augment flows in the Arkansas River at Granite, primarily through Lake Creek. Variable stream discharge and spring snowpack during the project provides an opportunity to evaluate the influence of discharge on metal concentrations.

If surface runoff leads to the introduction of metals into the Arkansas River, it seems plausible that increased stream discharge would be associated with increased metal concentrations and loading. An evaluation of such a relationship is limited by the availability of relevant daily discharge data on the Upper Arkansas River. The USGS operates stream gauges on the East Fork at US Highway 24 site # 07079300 (EF3), Arkansas River at Leadville site # 07081200 (AR1), Arkansas River below Empire Gulch near Malta site #07083710 (AR5), and Arkansas River at Granite site #07086000 (AR7). A flume and staff gauge exists at the mouth of California Gulch (CG4). Discharge at station AR3a is expected to be similar to discharge at AR1 due to its proximity and because the input of water from California Gulch is small relative to the Arkansas River. Metal concentrations are uniformly low at EF3, and the gauge at AR5 has been operating only since 2004. Consequently we will focus on stations AR1, CG4, AR3a, and AR7.

At AR1, dissolved zinc concentrations were not related to stream discharge (Figure 16). Although non-linear regression analysis suggested that the relationship was significant ($p<0.001$), the model's fit was very poor ($r^2=0.09$). The weak association was due to high zinc variability at lower flows. Years with high runoff were not associated with high zinc concentrations in general. Average Zn concentrations at AR1 were also unrelated to mean discharge April-July and peak discharge (Figures 17a and 17b, respectively).

Zinc concentrations were much more strongly related to discharge at the mouth of California Gulch (CG4) (Figure 18). A quadratic regression model provided the best fit for the data ($p<0.0001$; $r^2=0.70$).

AR3a was similar to AR1 in that the association of discharge with individual zinc concentrations was very weak ($p<0.001$; $r^2 = 0.06$), and that large increases of discharge are associated with very small changes in zinc (Figure 19). However, average spring zinc concentrations at AR3a were positively associated with mean discharge ($p<0.001$; $r^2=0.70$) (Figure 20a). Spring zinc concentrations were also associated with peak discharges ($p<0.003$; $r^2=0.62$) (Figure 20b). At the Arkansas River at Granite (AR7), individual zinc concentrations were not related to discharge at the time of sample collection ($p>0.08$) (Figure 21). Like AR3a, however, mean spring zinc concentrations at AR7 were related to average discharge ($p<0.016$; $r^2=0.59$) and also peak discharge ($p<0.026$; $r^2=0.53$) (Figures 22a and 22b, respectively).

In summary, individual zinc concentrations in the mainstem Arkansas River are independent of, or very weakly related to stream discharge at the time of sample collection. This is not the case for California Gulch which shows a strong relationship with discharge. A previous study conducted on the Arkansas River 1990-1993 found weak or non-significant correlations of dissolved zinc and cadmium with stream discharge (Clark 1996). In contrast, for stations downstream from California Gulch, years with higher average discharges and peak discharges are associated with higher Zn concentrations.

Metal Concentrations – Effect of California Gulch Remediation

Concentrations of metals in California Gulch have decreased greatly following remediation activities in 1995-1997. Average spring zinc concentrations in California Gulch have steadily decreased from a high of 26,000 µg/L in 1995 to a low of around 2,500 µg/L in 2004 and 2005 (Figure 23). Maximum observed zinc concentrations exceeded 40,000 µg/L in 1994 and 1995 but were only 8,000 to 10,000 µg/L in 2004 and 2005. Variability of zinc concentrations has decreased since remediation. The Arkansas River stations downstream from California Gulch have experienced similar declines (Figures 24, 25, 26, 27, 28). Major tributaries between stations AR3a and AR4 (the Lake Fork) and between AR6 and AR7 (Lake Creek) significantly reduce zinc concentrations through dilution. Concentrations of zinc at stations AR4, AR5, and AR6 (Figures 25, 26, and 27 respectively) were very similar as there are no major tributaries between these stations. A few general observations can be made after combining Zn concentration data from downstream stations into a single figure (Figure 29). Zinc concentrations at AR1 remained relatively low during the term of the study. However, zinc concentrations at downstream stations in 1994 were much lower than 1995-1997. Lower concentrations in 1994 may be due to low runoff relative to the following three years. Zinc concentrations in 1995-1997 were higher than other years. These years had much higher than average runoff and were during the time reclamation activities were taking place. Starting in 1998, Zn concentrations began a steep decline and with the exception of 2003, zinc concentrations appear to have leveled off since 2001. Although not presented, cadmium concentrations exhibited trends over time that were very similar to zinc (Brinkman et al. 2006).

Aquatic Vertebrates

Brown trout represent the overwhelming proportion of fish species found in the Arkansas River between Climax and Granite. Brook trout were found in upper reaches (EF1, EF3, TC7, AR1, AR3a, AR4) but were limited to single individuals at lower stations (AR5, AR6a, AR6, AR7) during fall sampling (Brinkman et al. 2006). Brook trout comprisd a greater percentage of the fish community at AR3a than nearby stations such as AR1, AR4, or LF22. Since 1994, the proportion of brook trout have declined at several stations particularly AR3a, EF1, and TC7, possibly due to the influence of whirling disease which impact other salmonid to a greater degree than brown trout.

Other salmonids sampled during the study include rainbow trout and various strains of native cutthroat trout. These species are consistently sampled in stations downstream of the confluence with the Lake Fork, although their density was low. Rainbow and cutthroat were infrequently sampled at stations above the confluence with the Lake Fork. These species likely immigrated from nearby waters and their presence is not due to natural reproduction (Greg Policky CDOW, personal communication). Longnose and white suckers were also collected. Individuals were limited to young of the year and were found primarily at downstream stations AR6 and AR6a.

Brown Trout – Population Responses

Brown trout population density at stations unimpacted by California Gulch (i.e. upstream stations and tributaries) were generally similar to each other and did not exhibit any strong trends with time (Figure 30). Brown trout density at stations downstream from California Gulch increased significantly in the years following remediation activities in 1995-1997 (Figure 31). The greatest increase occurred at AR3a immediately downstream from the confluence with California Gulch. Brown trout density tended to be highest at both impacted and unimpacted stations in 2002 during the extreme drought.

Length-frequency distributions are presented for each sampling station for each year sampled (Figures 32-42). Examination of histograms generally support conclusions based on brown trout density. Specifically, size structure was similar at unimpacted stations (EF1, EF3, TC7, AR1, and LF22) and did not exhibit any strong trend with time. In contrast, numbers of brown trout in stations downstream from California Gulch (AR3a, AR4, AR5, AR6, AR6a, and AR7) increased following remediation activities in 1995-1997. Brown trout young-of-the-year (3-9 cm) and 1+ age class (ca. 10-20 cm) tended to be present in particularly high numbers at most stations in 2002 during the drought.

Brown Trout – Effects of Stream Discharge

Brown trout recruitment success was inversely related to spring runoff discharge in the South Platte River (Nehring and Anderson 1985) as well as in 11 other Colorado Streams (Nehring and Anderson 1993). In the Arkansas River, brown trout density and growth were negatively related to stream discharge (Nehring 1986, Anderson and Krieger 1994). However, the study sites in those investigations (Salida and Wellsville) were far downstream from the study area of this report.

Mean and peak discharges from USGS gauges at EF3, AR1, and AR7 were compared to estimated brown trout densities (individuals >10 cm in length) from 1994 – 2005. Comparisons of discharge and brown trout density were made at Tennessee Creek (TC7) and Arkansas River below California Gulch (AR3a). Discharge at TC7 was estimated by subtracting measured discharge at EF3 from AR1. Discharge at AR1 was used as an estimate for discharge at AR3a. Regression results and parameters are summarized in (Table 1).

Densities of brown trout were not related to either mean or peak discharge at EF3 (Figure 43a and 43b). An outlier, which represents the drought year of 2002, falls outside an otherwise decreasing trend in densities with increases in flow. In contrast, densities of brown trout were significantly related to mean discharge ($p<0.04$) but not peak discharge ($p>0.09$) in Tennessee Creek (TC7) (Figure 44a and 44b). In the mainstem Arkansas River near Leadville (AR1), brown trout densities were inversely related to mean discharge but not peak discharge (Figure 45a and 45b). Similarly, brown trout density at Arkansas River below California Gulch (AR3a) was inversely related to AR1 discharge (Figure 46a). However, density was unrelated to peak discharge (Figure 46b). At the Arkansas River at Granite (AR7), the most downstream station of the project, brown trout densities were unrelated to mean or peak discharge (Figure 47a and Figure 47b). The most profound example of stream discharge affecting trout density occurred in 2002. A region-wide drought resulted in the lowest stream discharge for the period of record. Brown trout densities were also highest during this year for nearly all stations (EF3 was an exception). Some of the increased density can be attributed to reduced stream width due to low water levels at the time of electro-shocking.

In summary, stations in the upper portion of the study area but not the lowest station displayed a negative trend of brown trout density with mean discharge. The negative trend was significant at all upper stations except for EF3. The best fit model was a power function at two stations (TC7 and AR1) and exponential at AR3a. Power functions are most often the best model for recruitment and discharge (Nehring 1986). Between 50% and 70% of the observed variation in brown trout density at these stations could be explained by mean discharge. In contrast, peak discharge was not significantly related to brown trout density.

Brown Trout – Effect of Zinc Concentrations

Toxicity tests conducted by Colorado Division of Wildlife aquatic toxicology laboratory have confirmed the ameliorating effect of water hardness on the toxicity of zinc to trout. Comparison of laboratory toxicity test results to exposures in field situations are complicated by fluctuations in both zinc concentrations and water hardness over time and space. In order to effectively examine the effect of zinc concentrations on brown trout populations, it is helpful to adjust or normalize zinc concentrations using water hardness. A common approach for normalizing metal concentrations is to use hazard quotients (HQ). Hazard quotients are calculated by taking the ratio of a measured toxicant concentration and a toxicity reference value. A $HQ> 1$ indicates a measured concentration of a toxicant exceeds a laboratory toxicity reference value. For the purpose of these discussions, HQs are calculated using the following procedure:

1. Brown trout acute and chronic criteria are calculated based on measured hardness:

Brown trout acute criterion = $e^{(0.9634*\ln(\text{hardness})+1.986)}$
Brown trout chronic criterion = $e^{(0.9634*\ln(\text{hardness})+1.763)}$

Acute and chronic brown trout HQ are then calculated by dividing measured concentration by the criterion:

Brown Trout HQ(acute)=HQac= (measured dissolved Zn conc.)/(Brown trout acute criterion)

Brown Trout HQ(chronic)=HQch=(measured dissolved Zn conc.)/(Brown trout chronic criterion)

Where hardness is expressed as mg $CaCO_3$/L

The details and derivation of brown trout zinc criteria can be found in Appendix B.

Brown trout densities from stations downstream of California Gulch were plotted against maximum and mean springtime HQ. Maximum HQs are based on acute criteria and represent potential toxic effects from short term exposures. Mean HQs were calculated using chronic criteria and averaged over the spring runoff months (April-July). Mean chronic HQs represent potential effects from a longer term, time–averaged exposure to zinc.

Brown trout densities at AR3a decreased as mean and maximum HQs increased (Figure 48a and 48b, respectively). Brown trout densities decline as mean chronic HQ values increase. Unexpectedly, this decline is evident at chronic HQ values less than one, a level deemed to be safe for brown trout. Brown trout densities also declined with increasing maximum acute HQs. The decline of brown trout density occurred at acute HQ values greater than one. There are insufficient data points to evaluate whether there is a trend of brown trout densities at HQ values less than one. Densities and HQ values from stations between the Lake Fork and Lake Creek were combined for convenience. Water quality and zinc concentrations among these stations are very similar, although fish habitat varies considerably. Many of the features and trends observed at AR3a are present at stations downstream from the confluence with the Lake Fork (Figure 49a and 49b). Specifically, densities decrease as chronic and acute HQs increase. However, the Lake Fork influences the Arkansas River in a number of ways that alters the HQs. Firstly, the difference between maximum and mean HQs are much smaller at downstream stations relative to AR3a, reflecting a moderating influence by the Lake Fork that buffers extreme fluctuations in zinc concentrations. Secondly, HQs are generally lower. Apparently, beneficial dilution of zinc by flow from Lake Fork overrides the increased potential for toxicity from reduced hardness levels. Brown trout densities at AR4, AR5, AR6 and AR6a all appeared to increase in response to decreases of zinc acute and chronic HQ values below one.

At AR7, the most downstream station, brown trout densities do not appear related to either mean or maximum HQs (Figure 50a and 50b). In general, HQ values at this station were low and not expected to adversely affect brown trout.

For stations between California Gulch and Lake Creek, brown trout densities increased with decreased HQ values even as HQ values decreased below one. This finding was surprising in that an

HQ value equal to one is predicted to be safe for brown trout and adverse effects from zinc are not expected at HQ<1. A possible explanation is that laboratory-derived toxicity values underestimate toxicity for instream exposures. Underestimating toxicity could result from a significantly higher calcium-magnesium ratio of laboratory water relative to the Arkansas River. The molar ratio of calcium to magnesium was 6.8 for the CDOW laboratory water and about 2.1 for the Arkansas River. The mitigating effect of water hardness on toxicity of zinc is generally attributed to the calcium and not magnesium ion (Alsop and Wood 1999, Alsop et al. 1999, DeSchamphelaere and Janssen 2004). Consequently, laboratory tests results by the CDOW could underpredict toxicity in the Arkansas River. However, toxicity tests conducted by EPA using Arkansas River water have been in general agreement with tests conducted concurrently by CDOW. Another possible explanation for the observed trend at HQs<1 is that other factors at these sites are increasing brown trout density (e.g. reduced flows). Brown trout may be responding to favorably low flows which also lower HQ values.

Macroinvertebrates – Temporal and Spatial Variation in Community Indices

Macroinvertebrate trends for the years 1994-2004 were available for a station in the East Fork (EF3), AR1, AR3a, and two downstream stations (AR5 and AR8). Samples collected in 2005 are still being processed by the CSU Ecotoxicology laboratory. Stations upstream of California Gulch show similar trends in taxa richness and abundance, where both metrics showed an increasing trend from 1994 to 2004 (Figures 51 and 52). Taxa richness was similar between sites and ranged from 20-40 taxa across the ten year period. Macroinvertebrate abundances were higher at the site in the mainstem of the Arkansas (AR1). The number of individuals in the mayfly Family Heptageniidae, which is considered a metals sensitive group of organisms (Nelson and Roline 1993, Kiffney and Clements 1994, Clements 1994), was variable at both upstream sites. A decreasing trend was observed at EF3 between 2000-2004, while stronger population fluctutations were observed during the same years at AR1. Reasons behind declines are unclear. Lower than average discharge occurred in 2001-2002 and 2004, which may impact negatively algal food resources required by these organisms. On the other hand, metals concentrations increased slightly in 2003 which may have elicited avoidance behavior (e.g. drift). Hepatgeniid genera collected at all sites included *Rithrogena*, *Epeorus*, and *Cinygmula*.

Patterns in taxa richness and abundance at AR3a showed a stronger increase over time compared to upstream sites, especially after the year 1999 (Figure 53). This pattern corresponds to marked decreases in zinc and cadmium concentrations at this site between 1999 and 2004 (Figure 24). By 1999, taxa richness was similar to richness observed at upstream sites (between 20 and 40 taxa), and abundances exceeded the upstream stations (>1000 organisms). The number of heptageniid mayflies showed the most dramatic temporal patterns, where abundances increased three-fold by fall of 1999. Except for a brief decline in fall 2002 through spring 2003 and a spike in spring 2004, heptageniid abundances remained fairly stable after 1999. Divergence in heptageniids abundance in 2002 and 2003 was similar to the pattern observed at AR1, and may be a result of both decreased flow followed by an increase in metals in 2003.

Taxa richness at lower stations (AR5 and AR8) ranged between 20 and 40 taxa in most years, and, like upstream stations, showed a slight increasing trend between 1994-2004 (Figures 54 and 55). Total abundance of organisms ranged widely in the ten year period at AR5, but the range of individuals (400-1500) was similar to the range observed at AR3a. Total abundances at AR8 were higher, but showed a different temporal pattern. Abundances were fairly stable

between 1994-1998, and then began to vary widely between 1999-2002, before returning to stable densities in 2003-2004. The period of high, variable densities largely corresponds with years with lower than average flow (Figure 15). Total abundances of heptageniid mayflies at AR5 ranged from 25-75 individuals in most years, although abundances dropped fairly low in most of the fall samples. Heptageniids abundances were much higher at station AR8 than at AR5, but similar to nearly all stations, dropped dramatically in 2002. Since metal concentrations were not unusually high in this year, the heptageniid response was likely associated with changing environmental conditions such as reduced flow during a drought year.

CONCLUSIONS

The spatial and temporal gradient of metal concentrations and corresponding fish population estimates enables some inferences to be made regarding effects of metals on the fish community of the Arkansas River.

Hardness and metal concentrations in the upper Arkansas River fluctuated considerably by season and stream reach. These fluctuations exert strong influences on the toxicity of metals to aquatic organisms in the Arkansas River. Water hardness greatly affects the toxicity of metals to aquatic organisms. As hardness decreases, the toxicity of metals increases. Zinc and cadmium, the primary metals of concern for the upper Arkansas River, are among the metals whose toxicity is influenced by hardness. Spatial and seasonal effects can alter potential toxicity in both positive and negative ways. Dilution of metal concentrations by tributaries may also reduce hardness levels. The result is lower metal concentrations but also increased potential for metal toxicity. Water quality conditions are often expressed using averages in order to reduce large quantities of data into workable amounts. Use of averages can be misleading when evaluating toxicity of metals in a system that experiences wide ranging conditions. Acute responses of aquatic biota will be based on adverse extremes and not average conditions. For metals in the Arkansas River, the extreme conditions with the greatest potential for toxicity is during the spring runoff when hardness values are lowest, metal concentrations are highest, and sensitive brown trout fry are emerging.

Loading of metals in California Gulch have declined significantly since remediation activities. Concentrations of metals, in particular zinc and cadmium, have declined in the mouth of California Gulch (CG4) since 1997. Stations on the mainstem Arkansas River downstream of California Gulch have also experienced a decline in zinc and cadmium concentration. The sharp decline observed in the late 1990s appears to have slowed or leveled off by 2001. Metal concentrations at stations upstream of California Gulch (EF1, EF3, TC7, and AR1) and tributaries (LF22) did not exhibit any strong or consistent trends of metal concentrations over time.

Decreasing metal concentrations were associated with increasing density and biomass of brown trout in Arkansas River stations below California Gulch. Brown trout density and biomass at impacted sites rapidly responded to improved water quality conditions. Stations that were most impacted by California Gulch experienced the greatest improvement of brown trout density and biomass. Brown trout density at stations upstream of California Gulch (EF1, EF3, TC7, and AR1) and tributaries (LF22) fluctuated but did not exhibit any significant trends over time. Biomass at stations upstream of California Gulch also fluctuated slightly but did not exhibit any significant trends with time. One exception was station EF1. Biomass at EF1 increased 1994-2002 before reaching a plateau.

Benthic macroinvertebrates showed a similar trend, where dramatic improvement in invertebrate metrics was observed at AR3a by as early as 1999. Taxa richness and abundance at sites upstream and downstream of AR3a fluctuated similarly across the ten year sample period, where taxa richness increased over time and abundance metrics were reduced or were more variable during a series of low flow years between 2000-2002. Negative invertebrate responses in 2003 at AR3a and AR1 may have been due to increases in metals observed at these two sites during the spring of that year (see Figure 29).

Intensity of spring runoff appeared to influence metal concentrations in California Gulch and at stations downstream. Metal concentrations in California Gulch and downstream stations were highest during years when spring runoff was highest. A similar relationship was not found at station AR1 which receives metal loading from St Kevins Gulch. The influence of spring runoff on metal concentrations may be different in the California Gulch drainage than St Kevins Gulch. The relationship may also be explained by noting that years with low discharge occurred after remediation and high water years occurred during the time that remediation was taking place. Since remediation, seven of the eight years had below average discharge during spring runoff. In particular, metal concentrations tended to be lowest in 2002 when stream discharge was at record low levels. It is difficult at this time to determine how much of the decline of zinc concentrations in California Gulch is due to low runoff and how much is due to removal of metal sources. Monitoring metal concentrations for several more years, preferably during high flow years, will be necessary to effectively examine the relationship between flow and metal concentrations and loading in California Gulch.

Brown trout recruitment success was inversely related to spring runoff discharge in the South Platte River (Nehring and Anderson 1985) as well as in 11 other Colorado Streams (Nehring and Anderson 1993). In the Arkansas River, brown trout density and growth were negatively related to stream discharge (Nehring 1986, Anderson and Krieger 1994).

Intensity of spring runoff affected brown trout population parameters and recruitment at some stations in the study area. In general, years with low runoff were associated with increased brown trout density. This is consistent with the findings of other investigations in Colorado that brown trout recruitment success was inversely related to spring runoff discharge (Nehring and Anderson 1985, Nehring 1986, Nehring and Anderson 1993). Some of the improvement of the brown trout density and biomass following cleanup may be a result of below average discharge in the last several years. Brown trout density at stations unaffected by metals did not exhibit the same dramatic increases observed at stations downstream from California Gulch. The majority of brown trout density increases must therefore be attributed to reductions of metal concentrations. Improvement observed in the macroinvertebrate community at AR3a after remediation can be partially explained by reduced metal loadings at the site (Clements 2004). However, hepategeniids may still be responding to metals, especially to spikes of metals in some post-remediation years with higher flows (e.g. in 2003). Even in years without such spikes, metals contamination at AR3a and AR5 have shown to have negative impacts to benthic communities. For example a field experiment conducted in spring 2001, where invertebrate communities from upstream sites were exposed to water in AR3a and AR5 *in-situ*, showed negative impacts to the number of stoneflies and mayflies, and the total number of species (Clements 2004). Based on the sum of EPA chronic criteria for zinc, cadmium and copper, water quality at AR3a was ten times higher than the chronic criteria given stream hardness levels. This is consistent with the finding that cumulative criterion unit (similar to "hazard quotients" but using EPA

criteria) with values between 2-10 showed negative impacts to invertebrate communities in metals impacted streams of Colorado (Clements et al. 2000).

The record drought in 2002 had a profound effect on the biota of the Arkansas River. Low runoff in 2002 contributed to low metal concentrations, relative to other years. Densities of brown trout at all stations except EF3 increased significantly over the previous year. For macroinvertebrate communities, heptageniids were reduced at most stations in 2002. The extreme case of 2002 is an illustration of how climate can interact with metals to influence biota. This interaction was not tested statistically in this report, but will be explored in future studies once higher than average flows are captured again at the sites. It is expected that higher flows will also deliver higher metals loads in the future. In general, it is critical to understand how natural environmental gradients can influence biotic metrics and metals-impacted sites in order to detect a "metals" signal (Vieira et al. 2005). This becomes especially important at lower, chronic levels of metals contamination. Our study and those conducted by other researchers in the upper Arkansas demonstrate that long-term datasets are necessary to fully understand how biotic communities respond to metals. Monitoring should capture a wide range of environmental conditions not only throughout the study, but also after remediation activities to determine whether such activities will effectively protect aquatic life.

Brown trout in the Arkansas River downstream from California Gulch responded favorably and rapidly to decreasing concentrations of metals following remediation. Density and biomass increased significantly and quickly as zinc concentrations decreased. As zinc hazard quotients decreased, brown trout density increased at all stations between California Gulch and the confluence with Lake Creek. Brown trout densities increased with decreased HQ values even as HQ values decreased below one. This finding was surprising in that an HQ value equal to one is predicted to be safe for brown trout and adverse effects from zinc are not expected at HQ<1. Higher calcium-magnesium ratios in laboratory water relative to Arkansas River is an unlikely explanation given the general agreement between laboratory and onsite tests conducted using Arkansas River water. It seems more plausible that low stream discharge associated with drought in recent years is interacting with brown trout density in two ways. First, low runoff is related to reduced zinc loading and reduced HQ values and second, the low runoff results in more favorable conditions for brown trout and contributes to increased density.

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Figure 1: Upper Arkansas River Basin

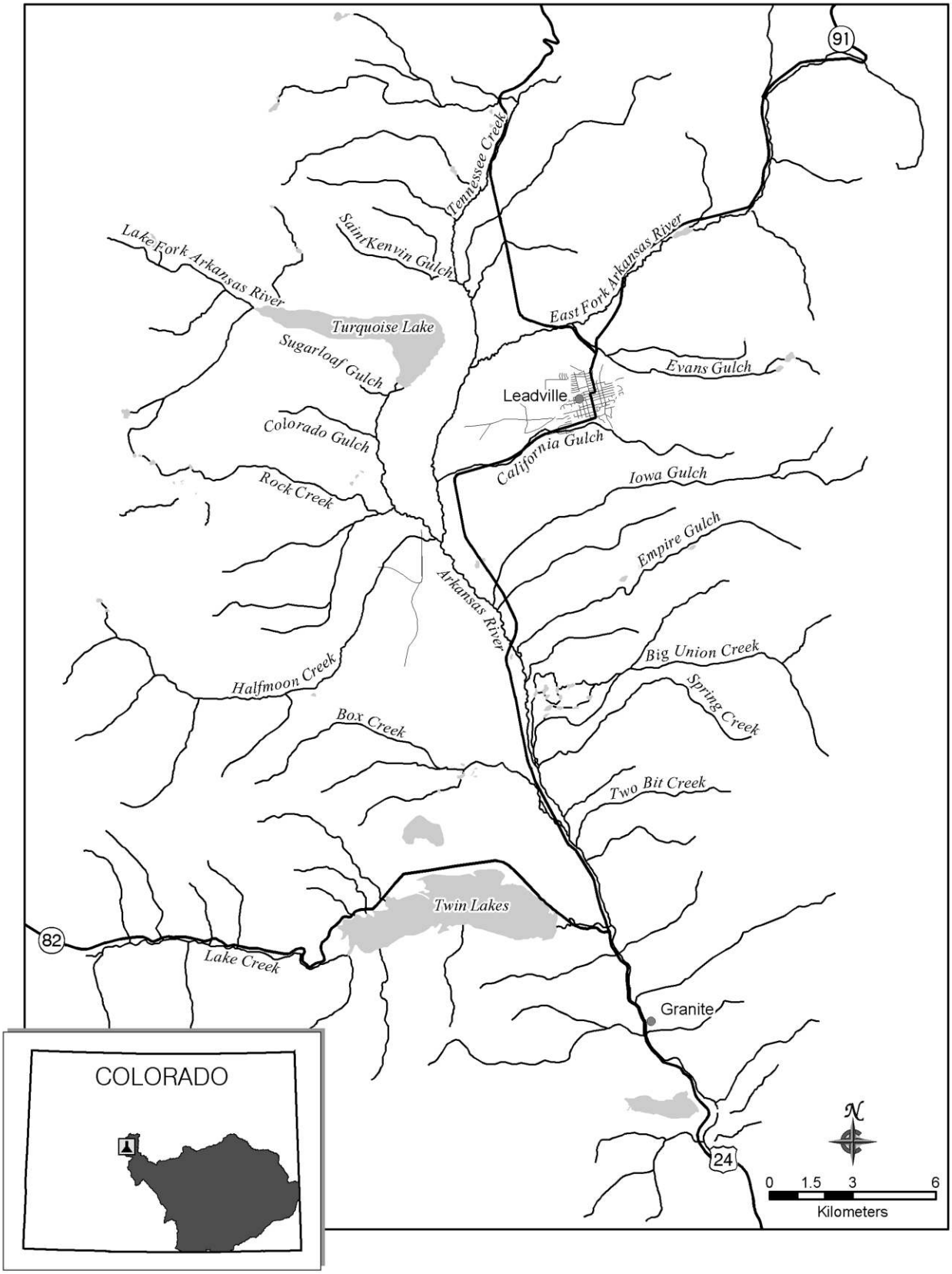
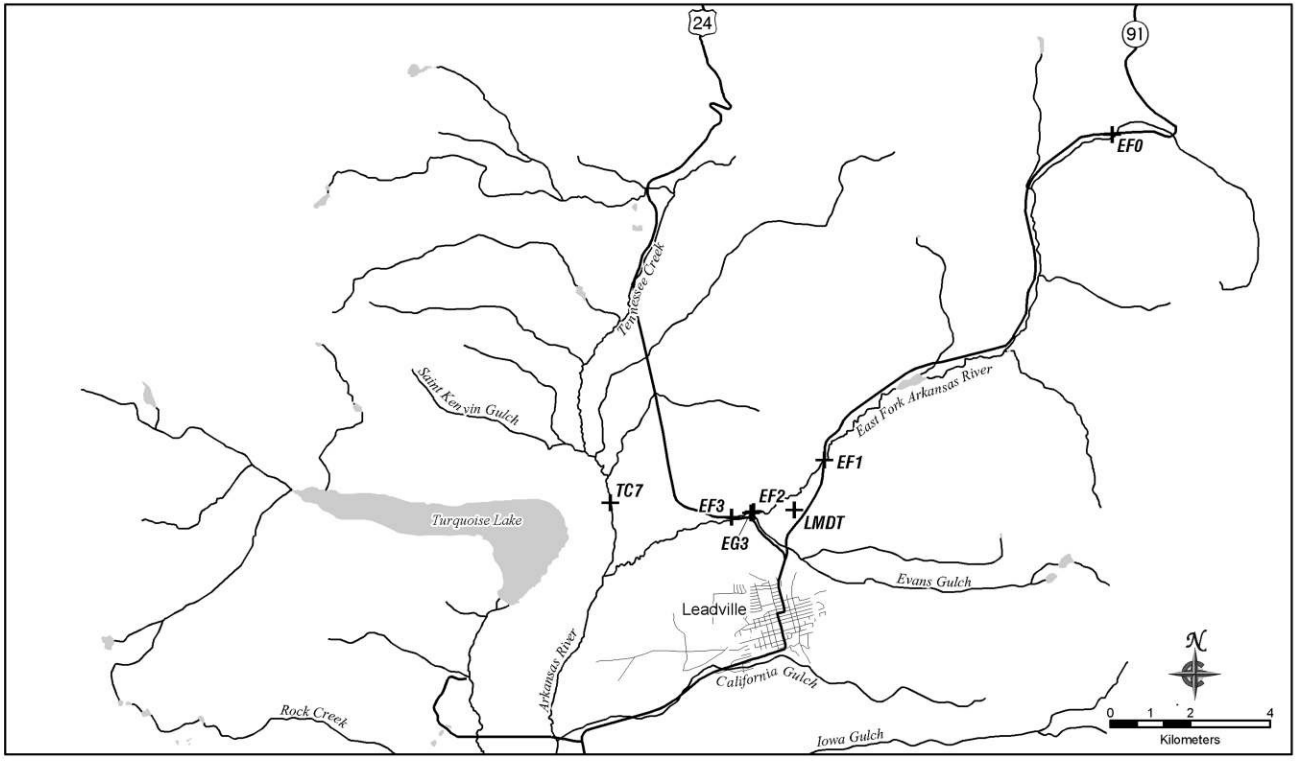
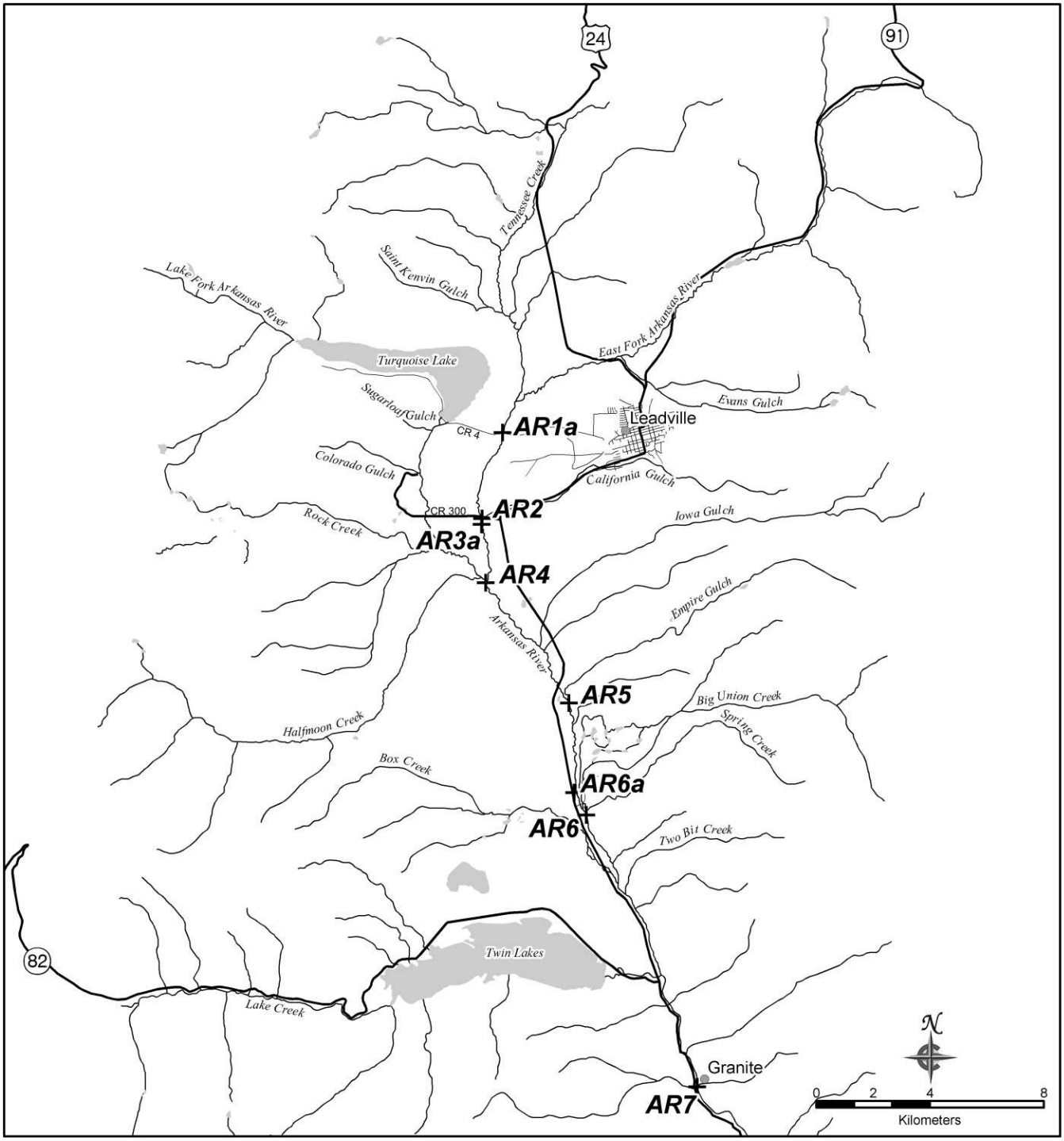


Figure 2A: East Fork Arkansas River, Tennessee Creek and Evans Gulch



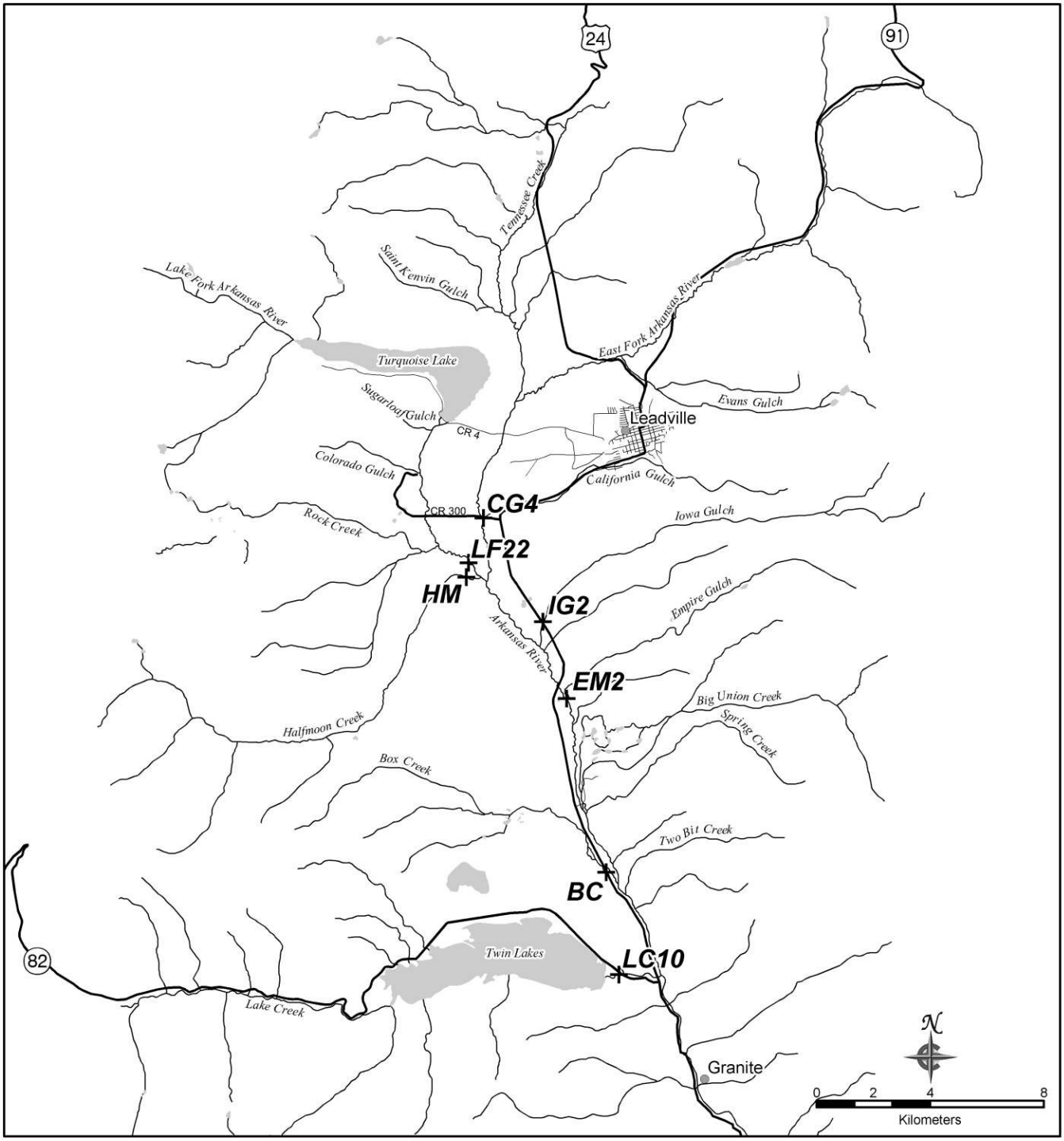
EFO	East Fork Arkansas River below Climax	LMDT	Leadville Mine Drainage Tunnel at BOR Treatment Plant
EF1	East Fork Arkansas River above Colorado Highway 91	TC7	Tennessee Creek below St. Kenvins Gulch at old gage station
EF2	East Fork Arkansas River above confluence of Evans Gulch	EG3	Evans Gulch above confluence with East Fork
EF3	East Fork Arkansas River above USGS gage on U.S. Hwy 24		

Figure 2B: Upper Arkansas River Basin
Mainstem Monitoring Stations



AR1a Arkansas River above C.R. 4, 1/2 mile downstream from USGS gauge
AR2 Arkansas River upstream of California Gulch at C.R. 300
AR3a Arkansas River 1/4 mile below California Gulch on Beck's property
AR4 Arkansas River upstream of C.R. 44 on Dr. Smith's ranch
AR5 Arkansas River downstream of U.S. Hwy 24 below Empire Gulch
AR6a Arkansas River 1/2 mile upstream of C.R. 55 above Big Union Gulch
AR6 Arkansas River downstream of C.R. 55 at Kobe
AR7 Arkansas River at USGS gauge Granite, CO
AR8 Arkansas River at Buena Vista (off map)

Figure 2C: Tributaries to the Upper Arkansas River



CG4	California Gulch above confluence of Arkansas River	EM2	Empire Gulch above confluence of Arkansas River
LF22	Lake Fork below C.R. 11/Forest Route 160	BC	Box Creek above U.S. Hwy 24 bridge
HM	Halfmoon Creek below Forest Route 160	LC10	Lake Creek below Twin Lake Reservoirs
IG2	Iowa Gulch above U.S. Hwy 24		

Figure 3. Mean hardness during spring runoff in the East Fork and mainstem of Arkansas River and major tributaries 1994-2005.

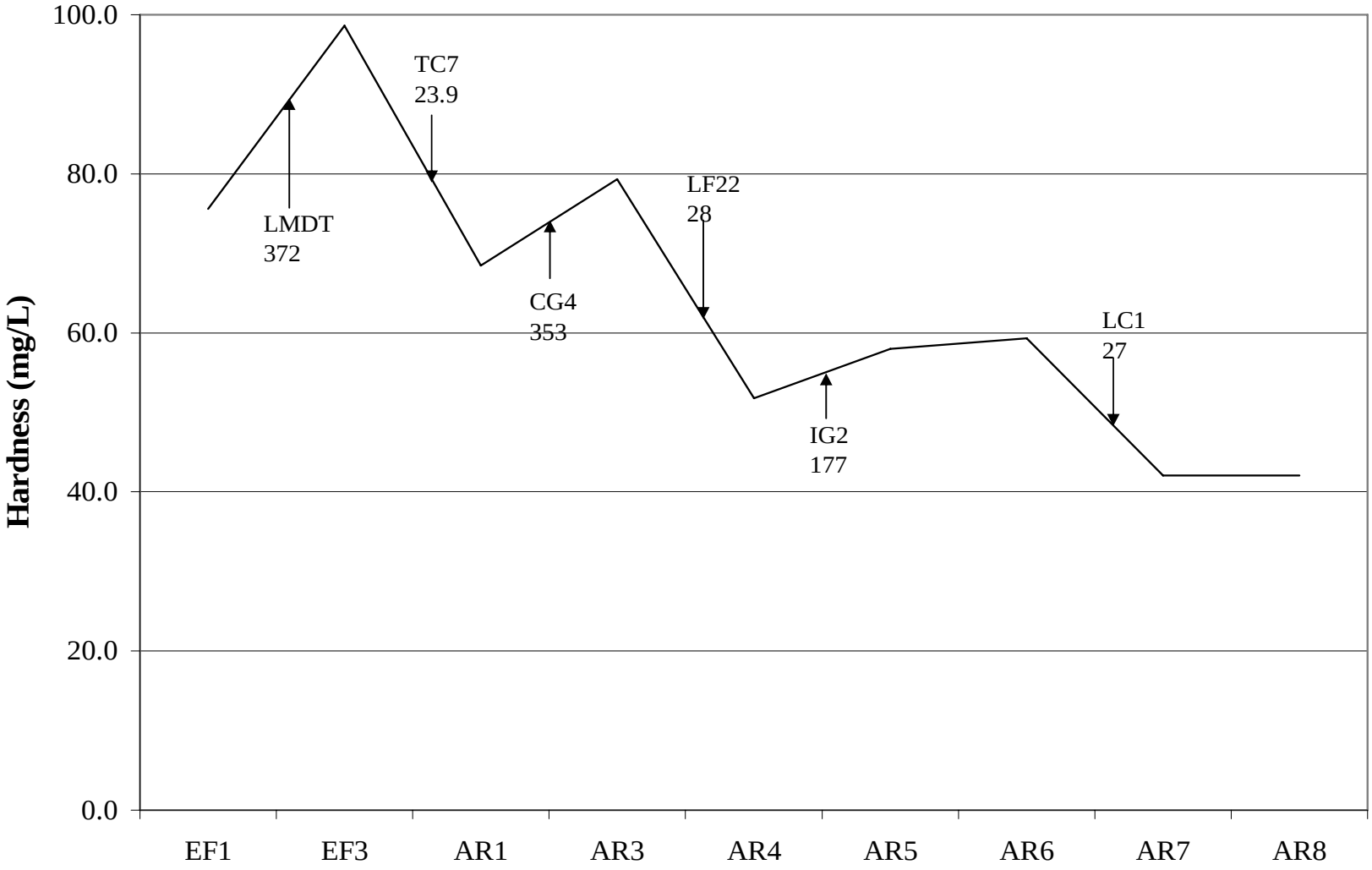


Figure 4. Mean monthly discharge (cfs) and water hardness (mg/L) in the Arkansas River at Leadville (AR1) 1994-2005.

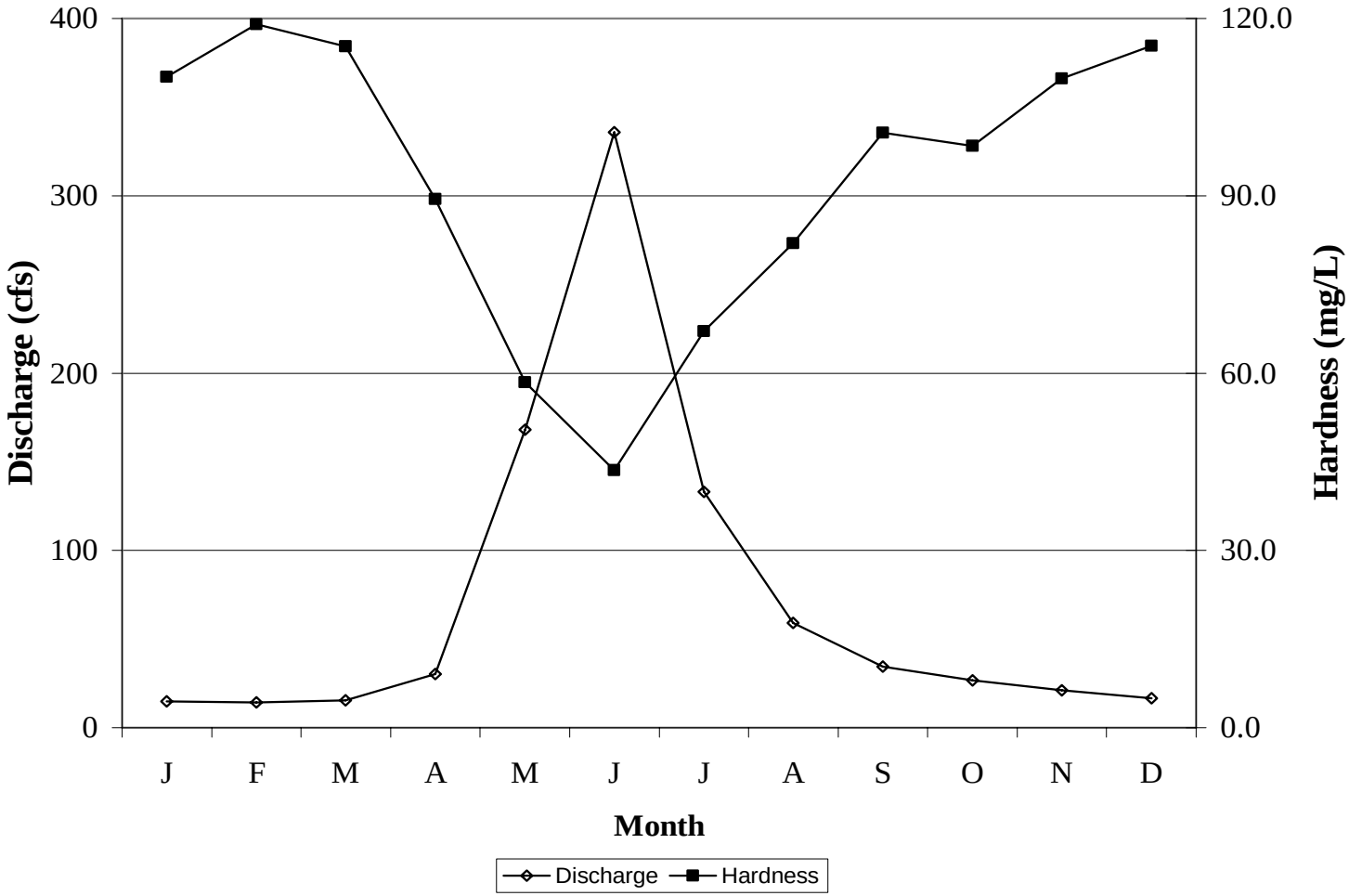


Figure 5. Water hardness (mg/L) as a function of stream discharge (cfs) in the Arkansas River at Leadville (AR1) 1994-2005.

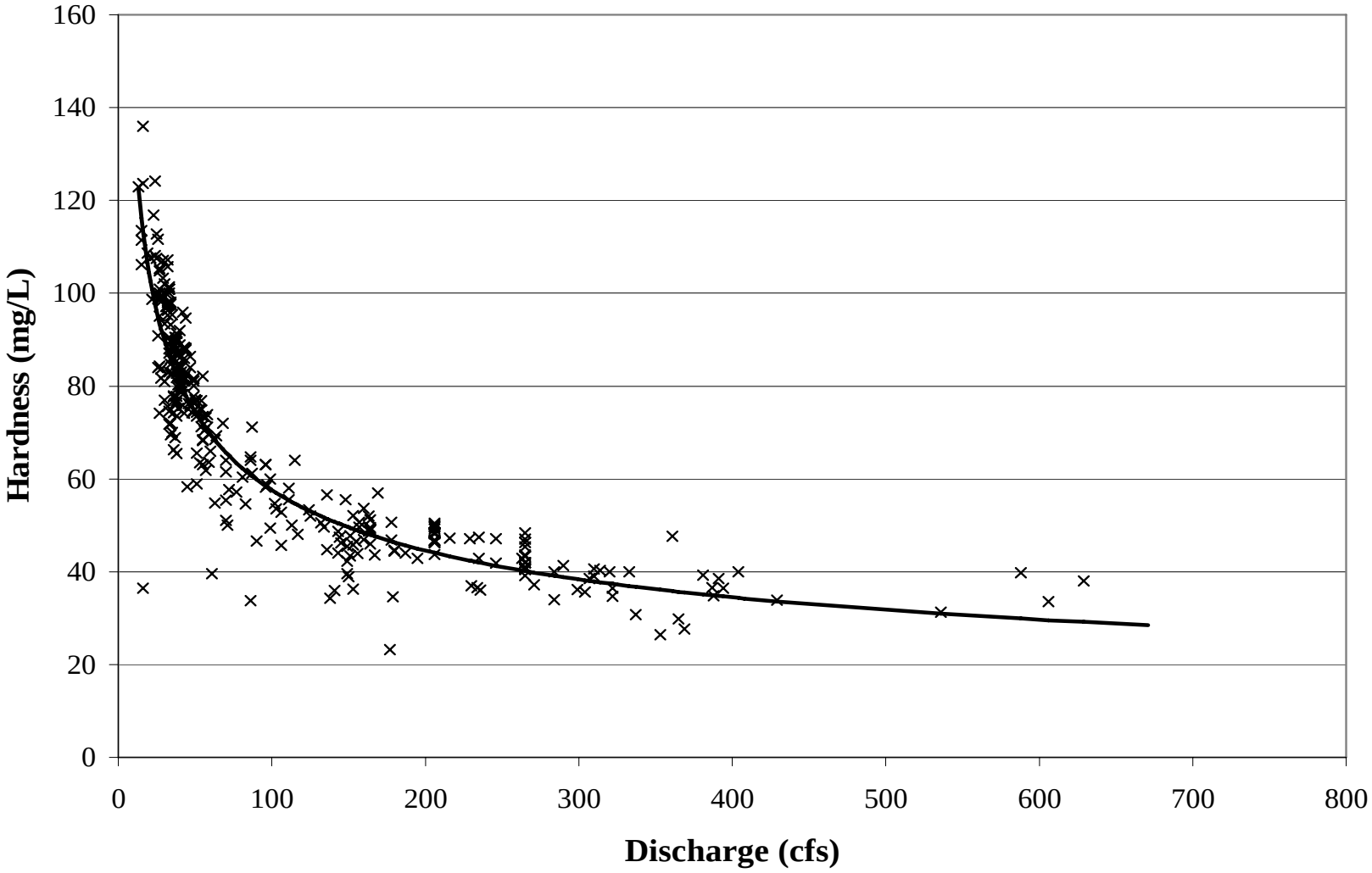


Figure 6. Water hardness (mg/L) as a function of stream discharge (cfs) in the Arkansas River at Granite (AR7) 1994-2005.

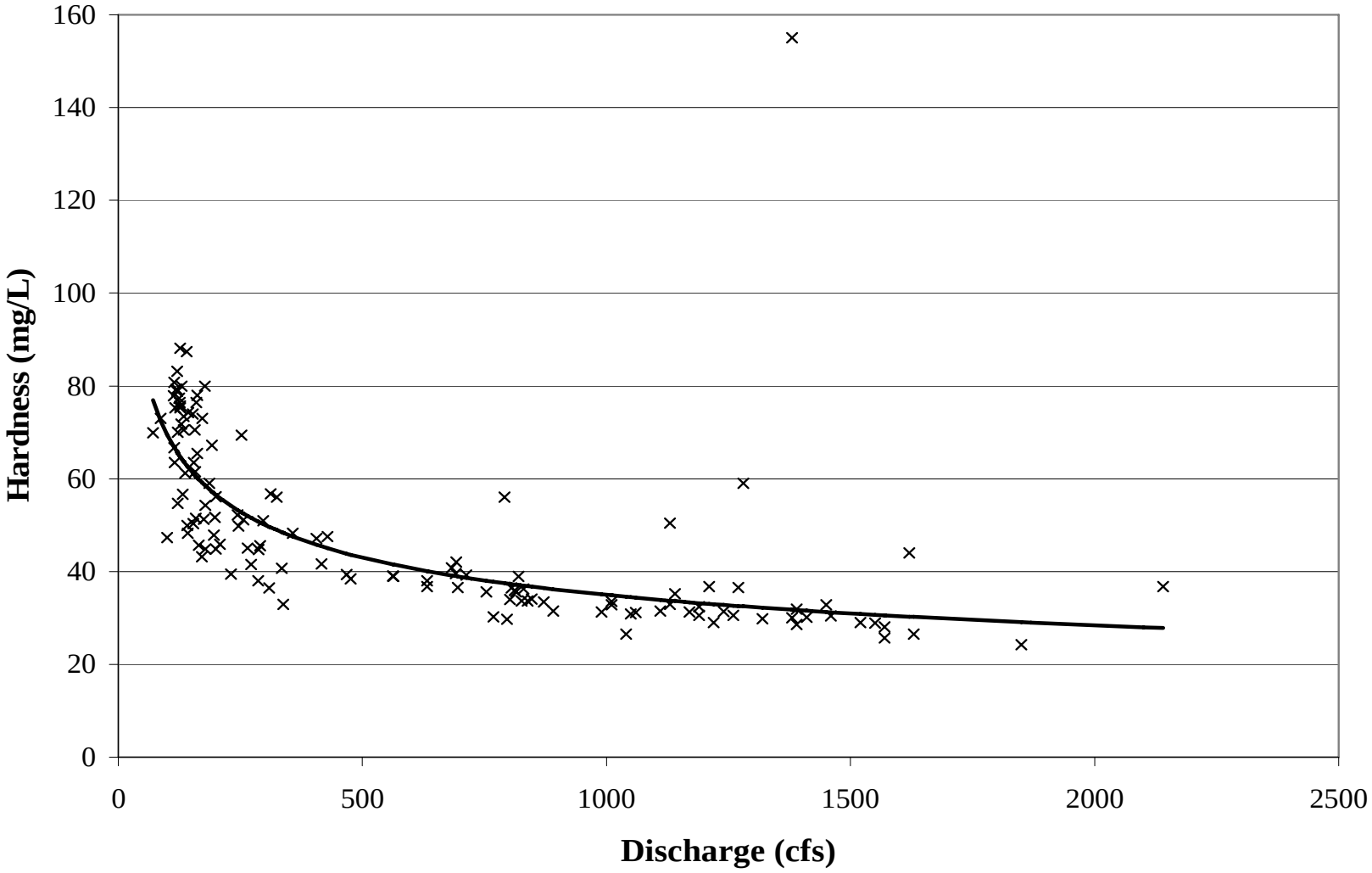
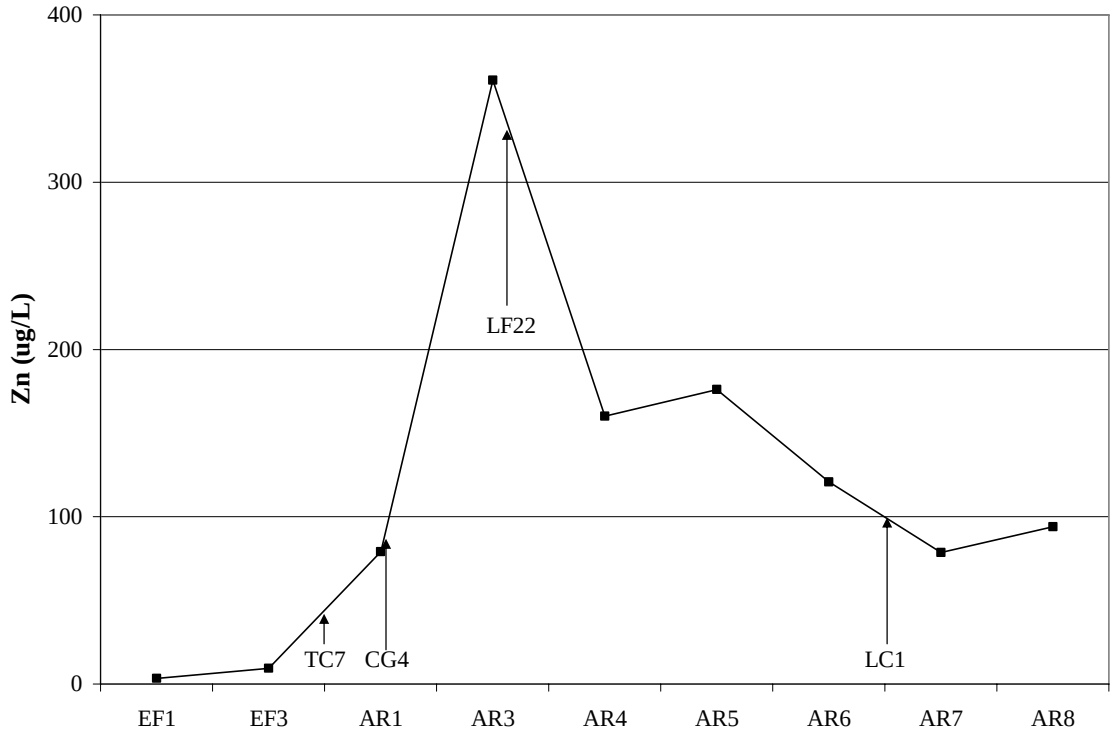


Figure 7. Mean dissolved zinc and cadmium concentrations in the Arkansas River 1994-2005.



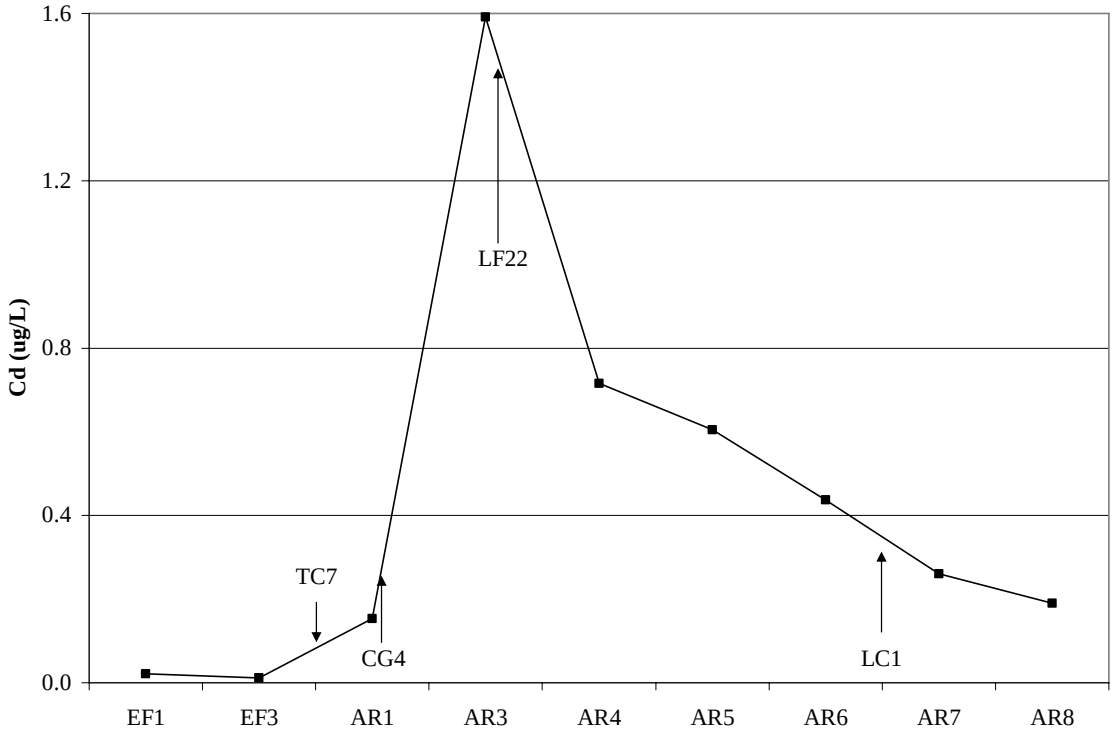


Figure 8. Box and whisker plot of dissolved zinc concentrations (µg/L) in the Arkansas River at Leadville (AR1) during spring runoff 1994-2005.

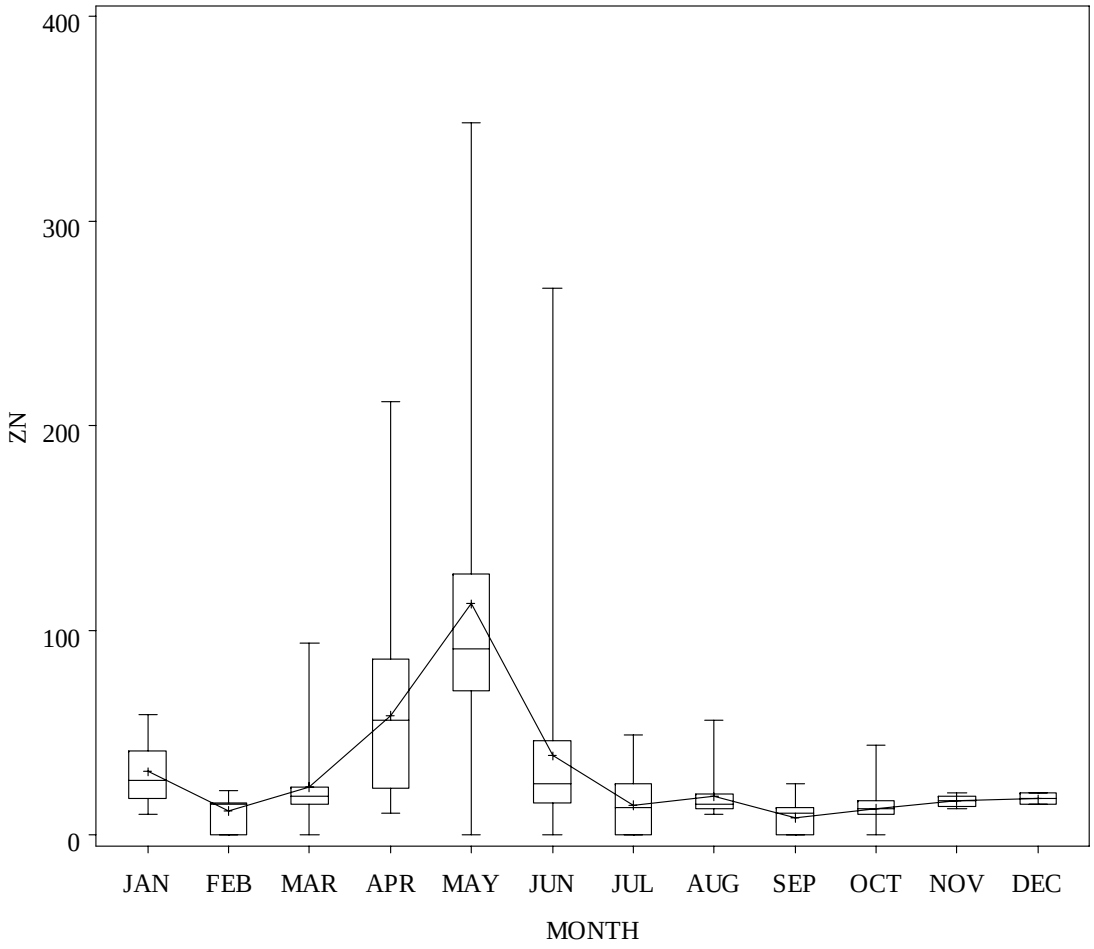


Figure 9. Box and whisker plot of dissolved zinc concentrations (µg/L) at the mouth of California Gulch (CG4) during spring runoff 1994-2005.

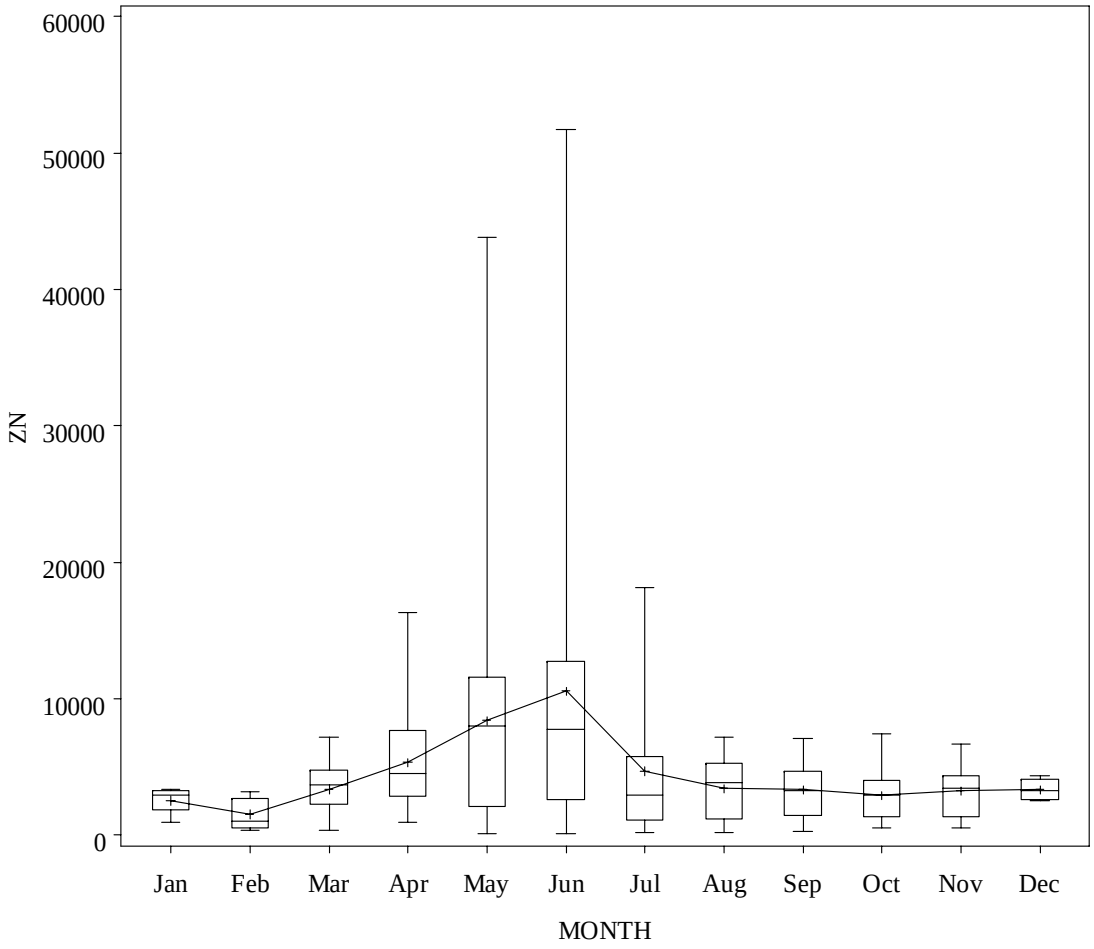


Figure 10. Box and whisker plot of dissolved zinc concentrations (µg/L) in the Arkansas River below California Gulch (AR3a) during spring runoff 1994-2005.

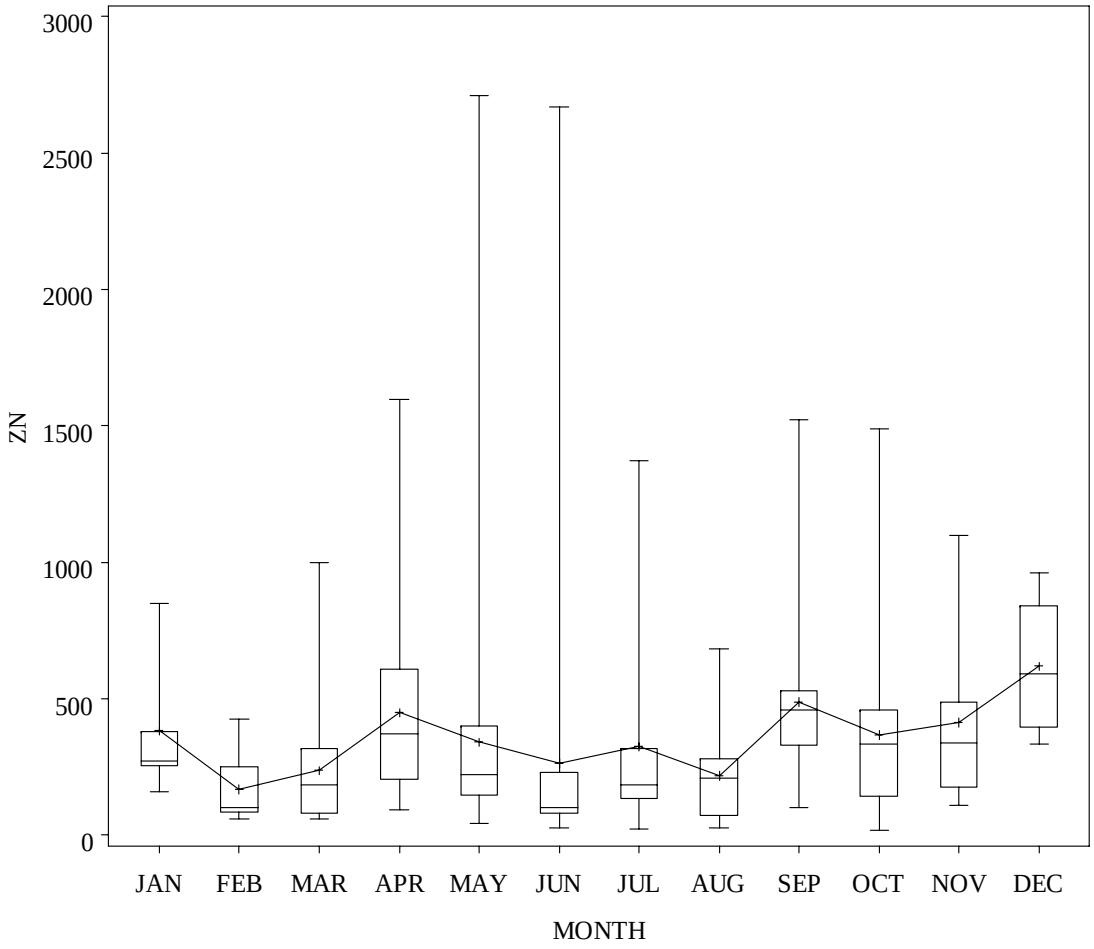


Figure 11. Box and whisker plot of dissolved zinc concentrations (µg/L) in the Arkansas River between the Lake Fork and Lake Creek (AR4, AR5, AR6) during spring runoff 1994-2005.

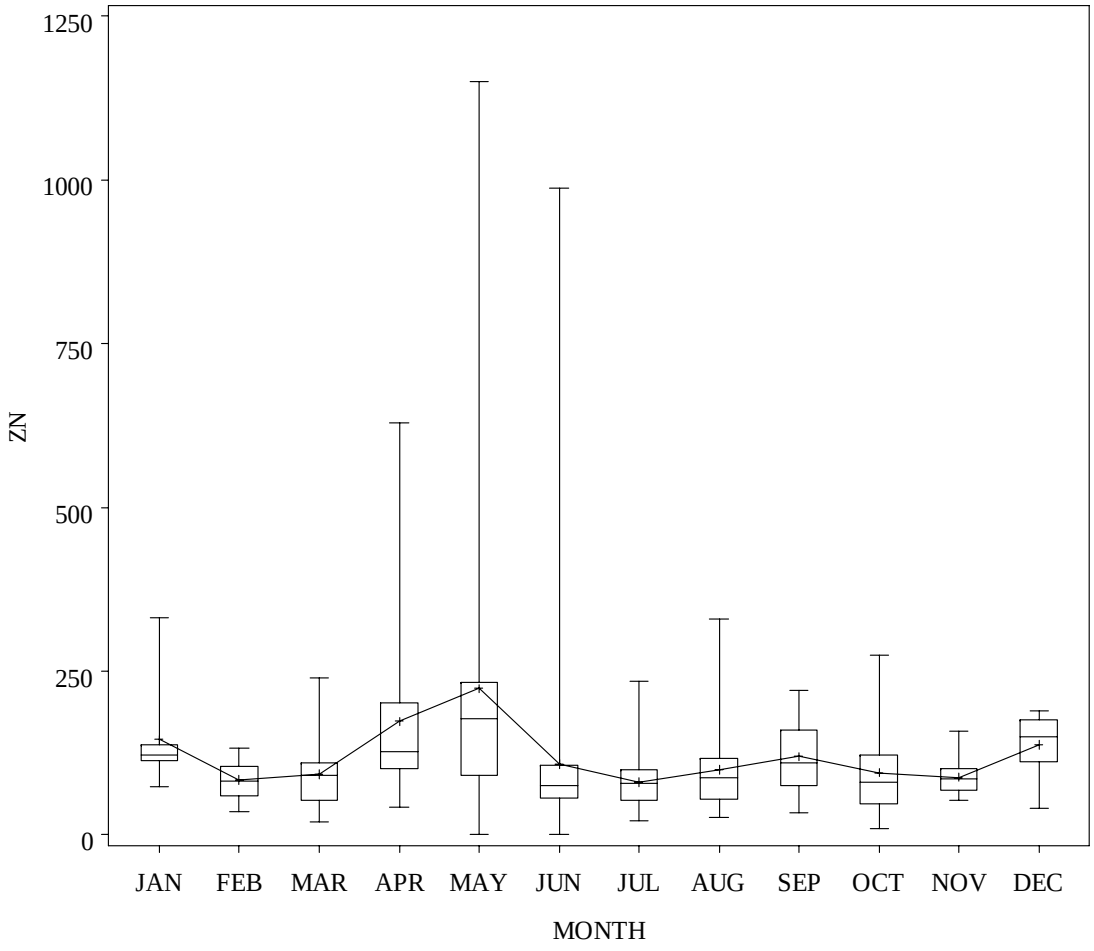


Figure 12. Box and whisker plot of dissolved zinc concentrations (µg/L) in the Arkansas River at Granite (AR7) during spring runoff 1994-2005.

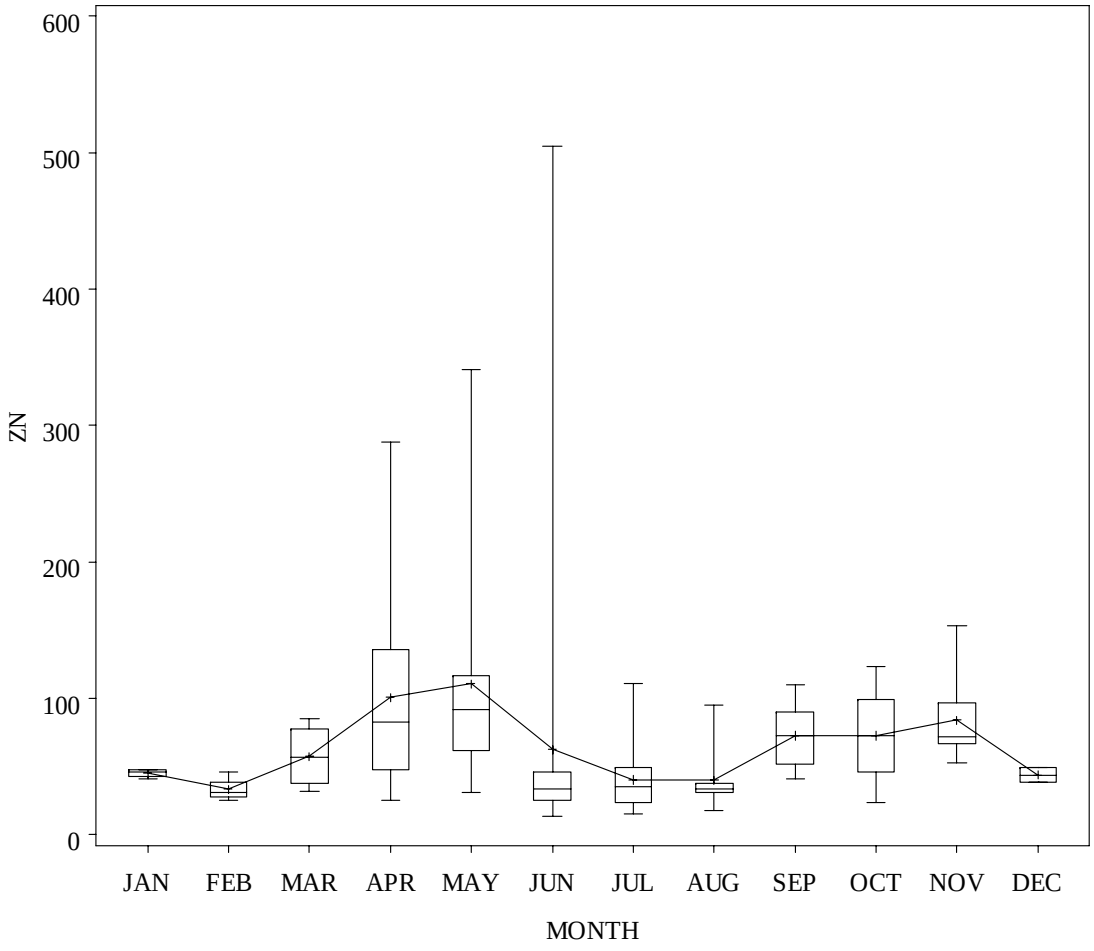


Figure 13. Discharge (cfs) in the Arkansas River at Leadville (AR1) 1994-2005.

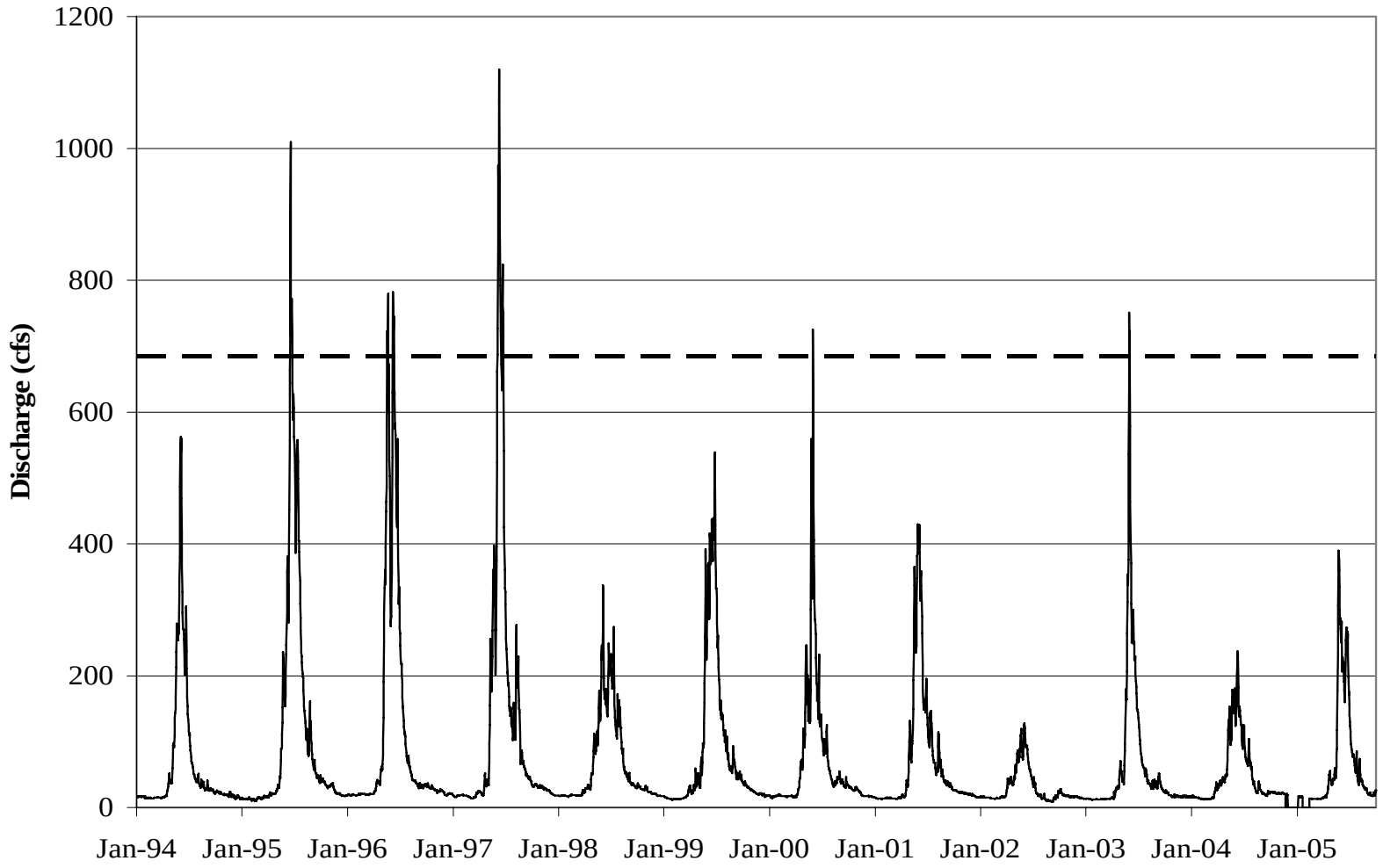


Figure 14. Mean discharge (cfs) in the Arkansas River at Leadville (AR1) during spring runoff (April-July) 1994-2005.

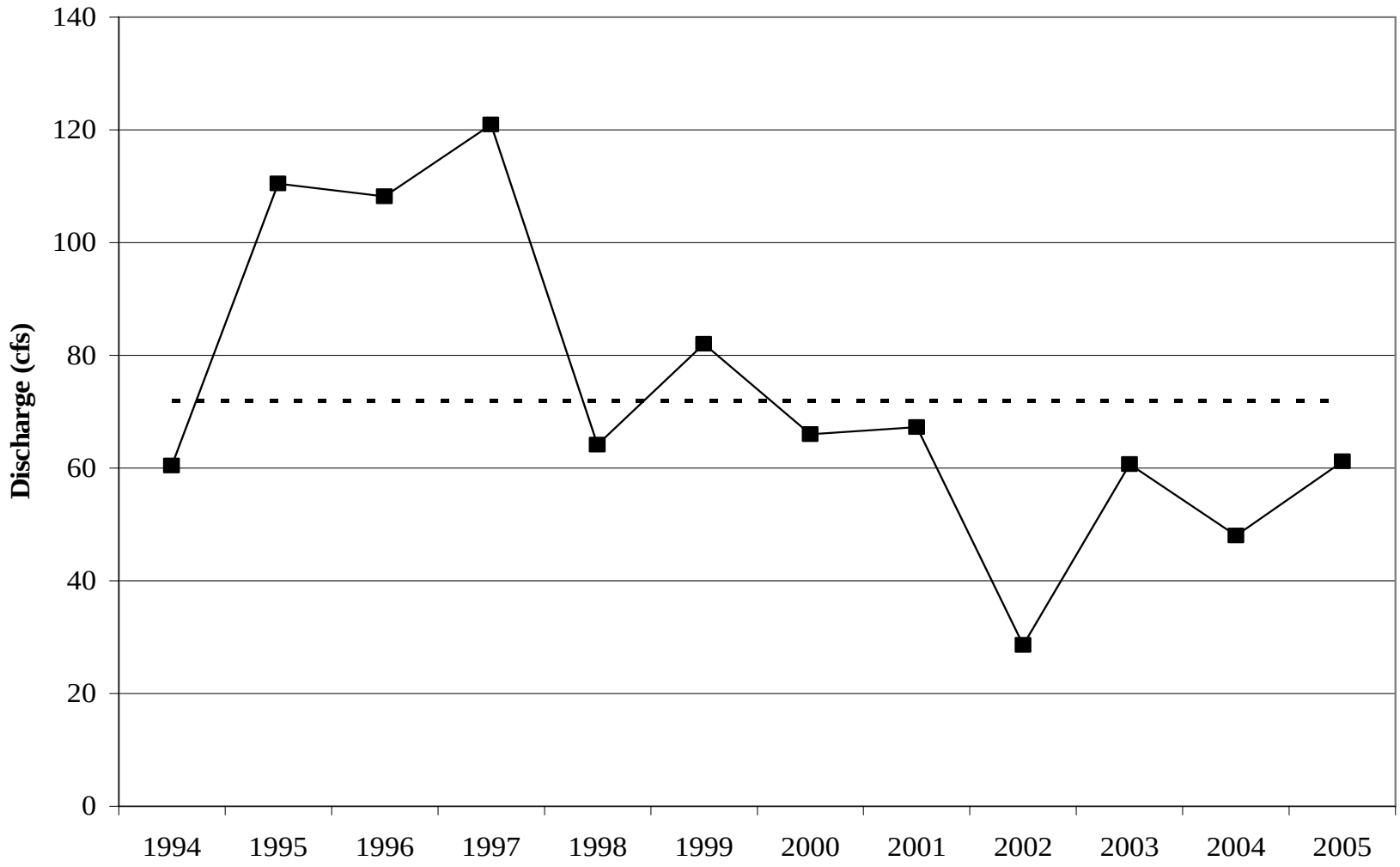


Figure 15. Discharge (cfs) in the Arkansas River at Granite (AR7) 1994-2005.

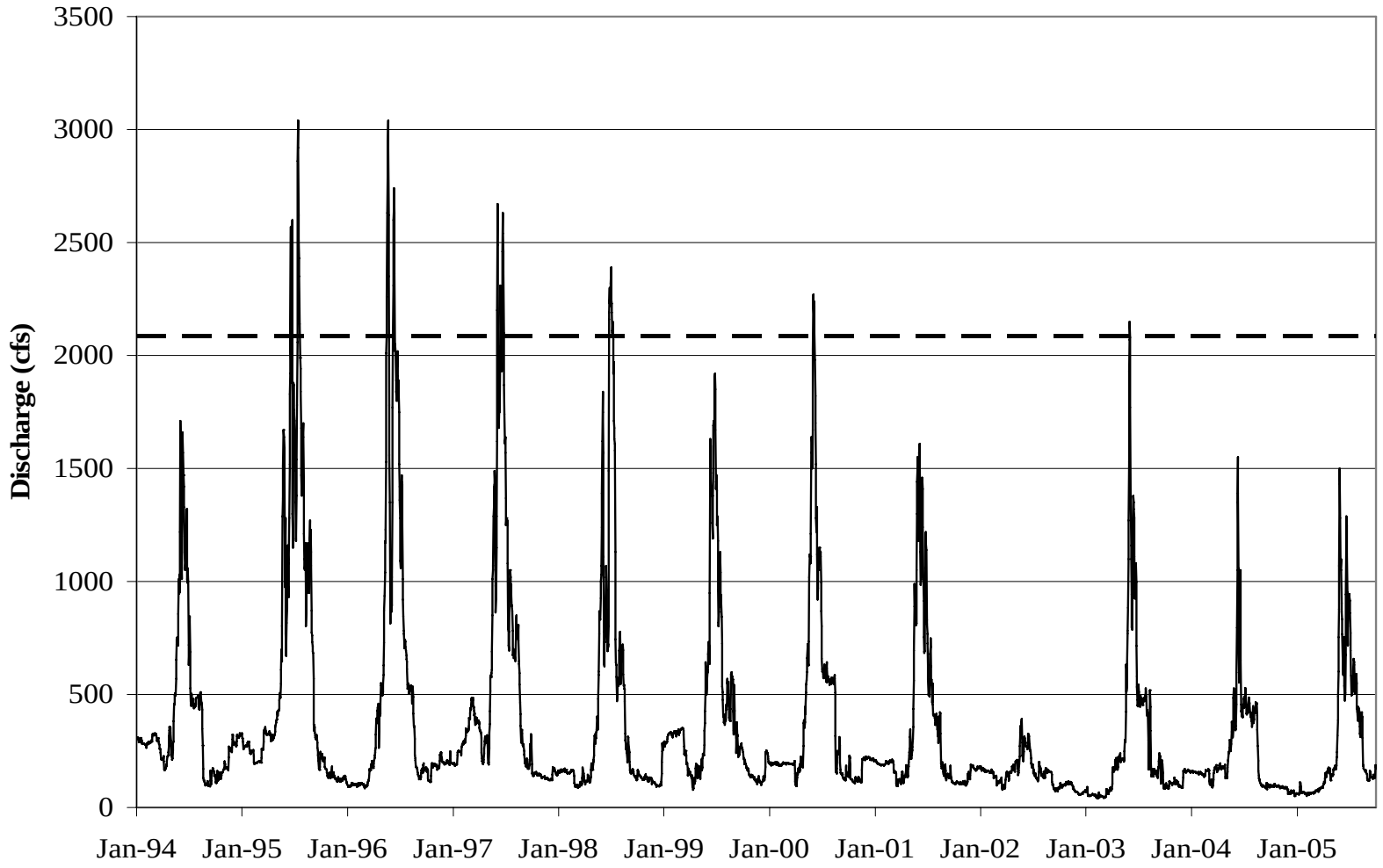


Figure 16. Measured dissolved zinc concentrations as a function of stream discharge at Arkansas River near Leadville (AR1) 1994-2005.

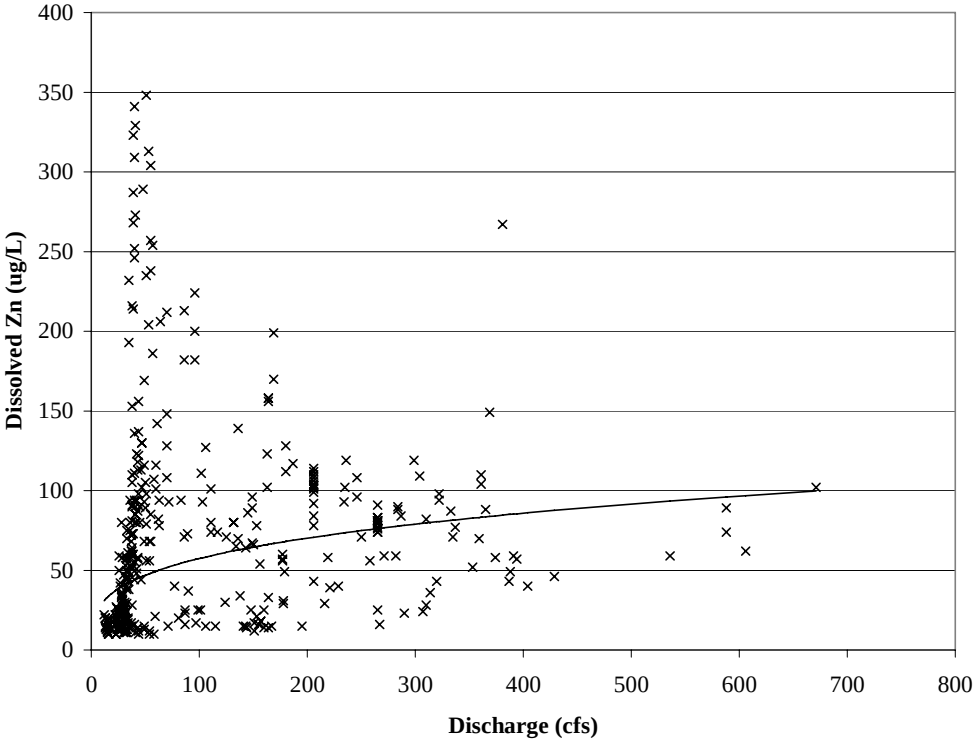


Figure 17a. Mean dissolved zinc concentrations in relation to mean stream discharge at Arkansas River near Leadville (AR1) during spring runoff 1994-2005. Not significant, p>0.85.

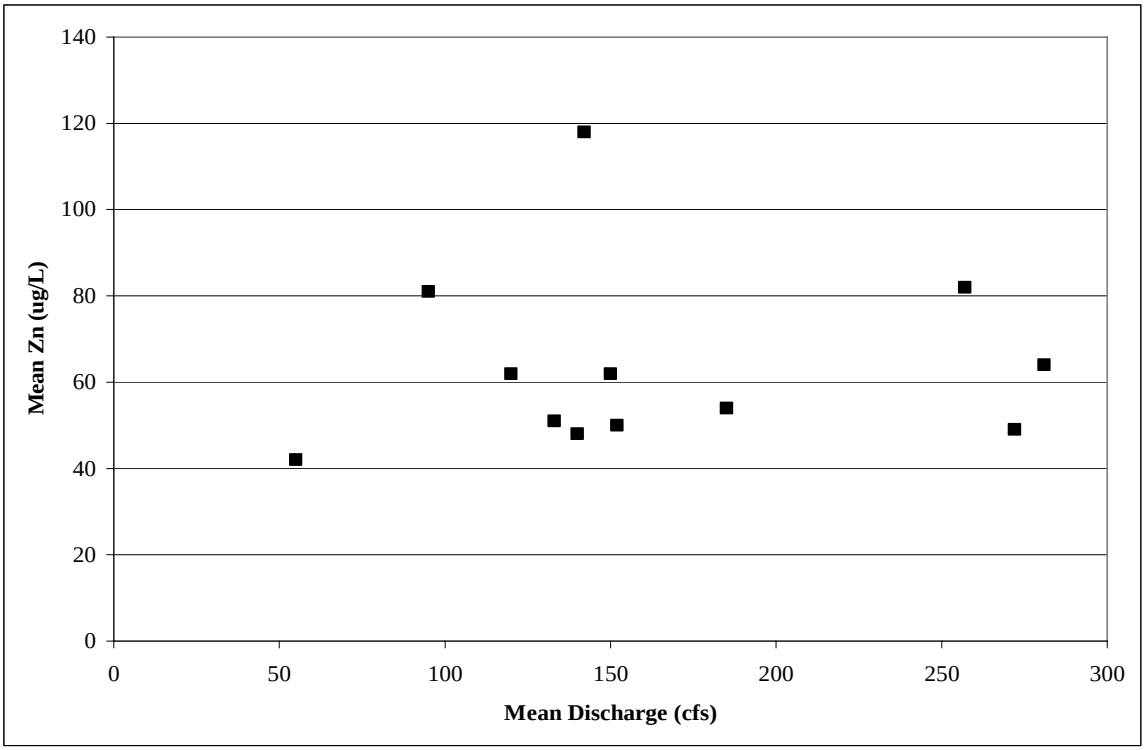


Figure 17b. Mean dissolved zinc concentrations in relation to peak stream discharge at Arkansas River near Leadville (AR1) during spring runoff 1994-2005. Not significant, p>0.27

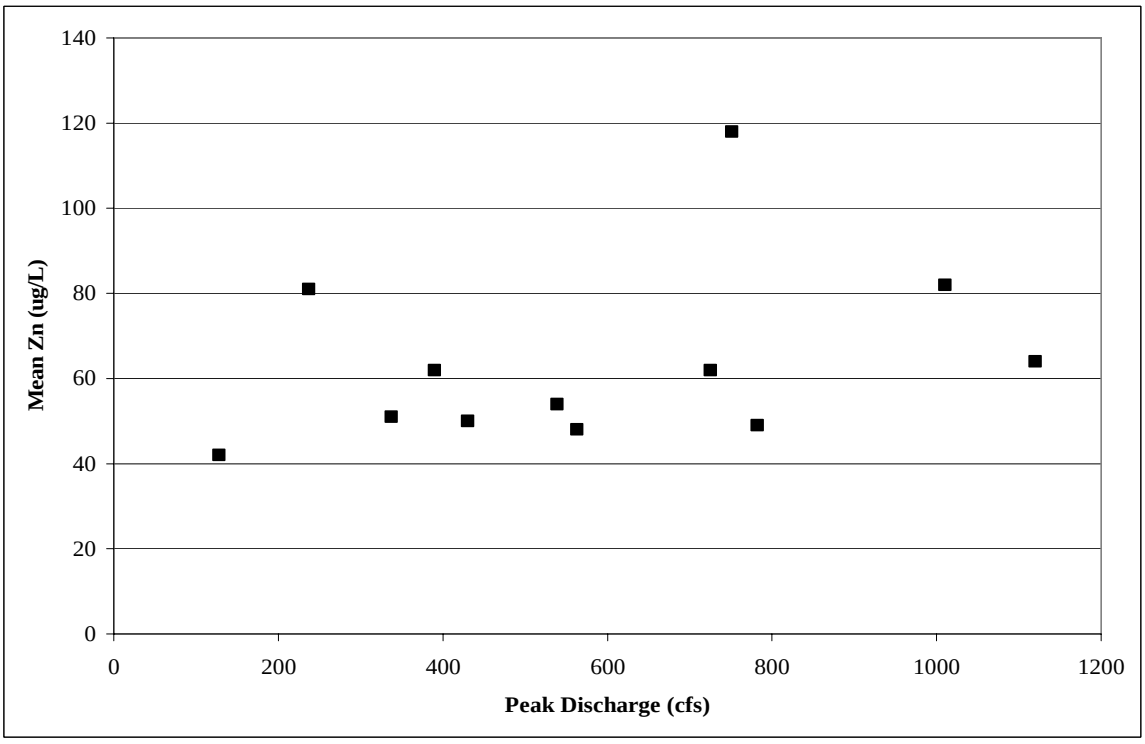


Figure 18. Measured dissolved zinc concentrations (ug/L) in relation to discharge (cfs) at the mouth of California Gulch (CG4) 1994-2005. Quadratic regression, $p<0.0001$; $r^2=0.70$.

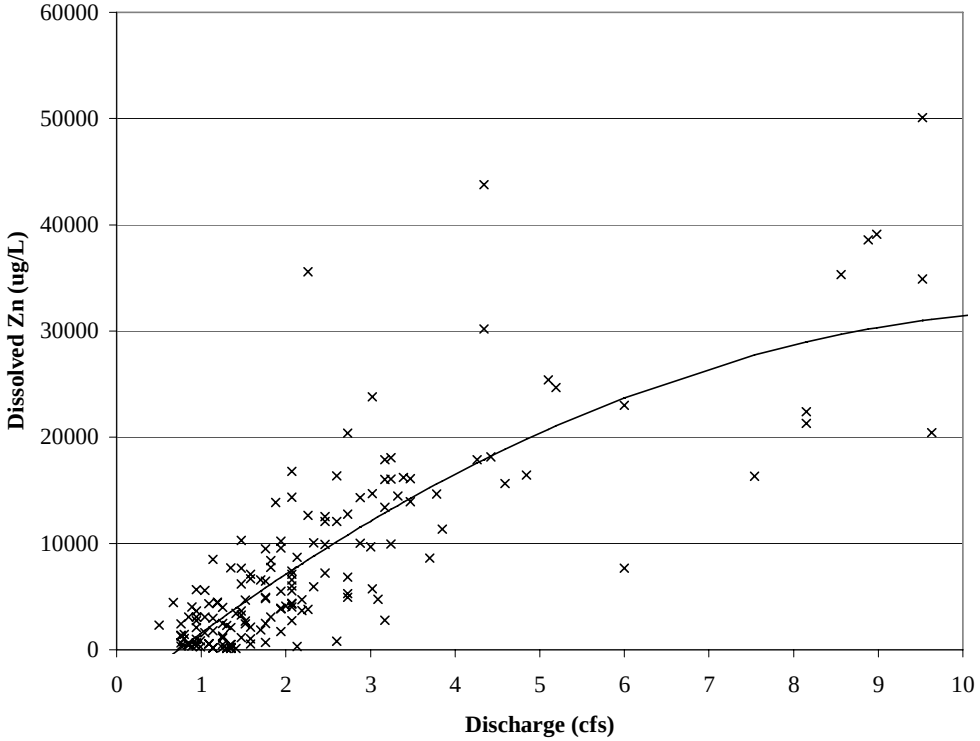


Figure 19. Measured dissolved zinc concentrations in the Arkansas River below California Gulch (AR3a) as a function of stream discharge at Arkansas River near Leadville (AR1) 1994-2005. ($p<0.001$; $r^2 = 0.06$)

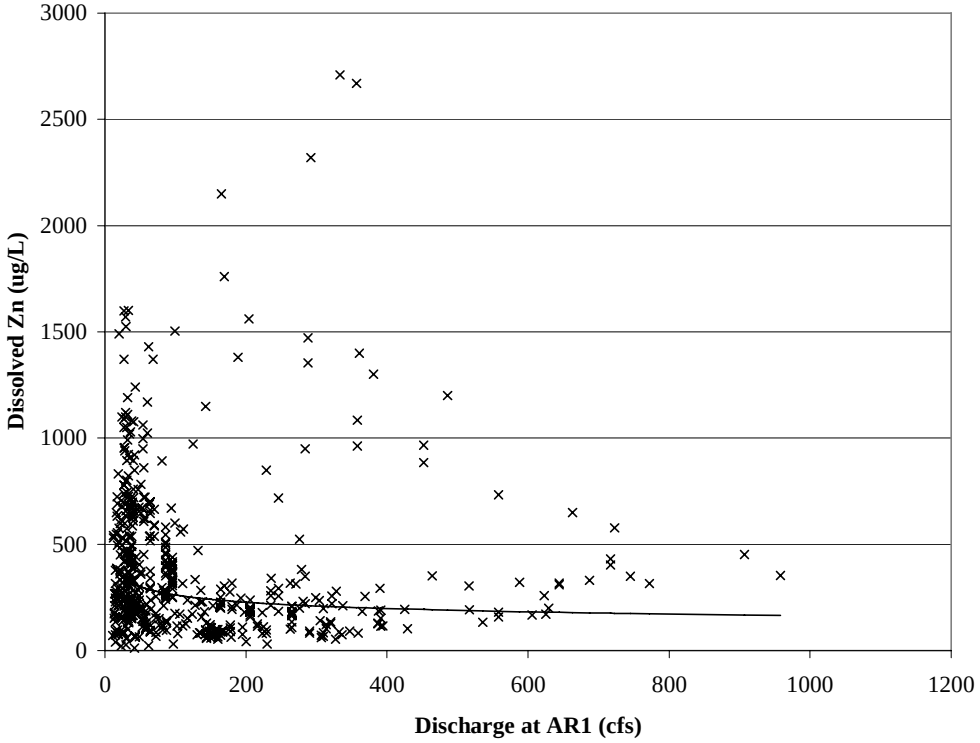


Figure 20a. Mean dissolved zinc concentrations in the Arkansas River below California Gulch (AR3a) in relation to mean stream discharge at Arkansas River near Leadville (AR1) during spring runoff 1994-2005. Linear regression, $p<0.001$; $r^2=0.70$.

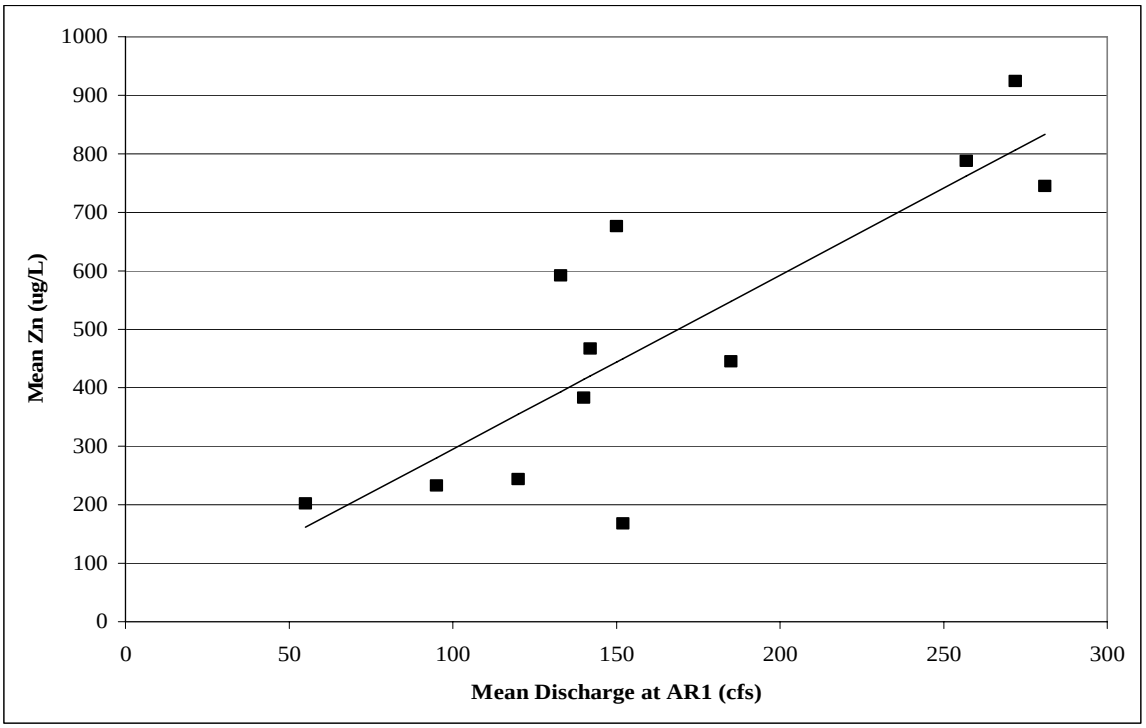


Figure 20b. Mean dissolved zinc concentrations in the Arkansas River below California Gulch (AR3a) in relation to peak stream discharge at Arkansas River near Leadville (AR1) during spring runoff 1994-2005. Linear regression, $p<0.003$; $r^2=0.62$.

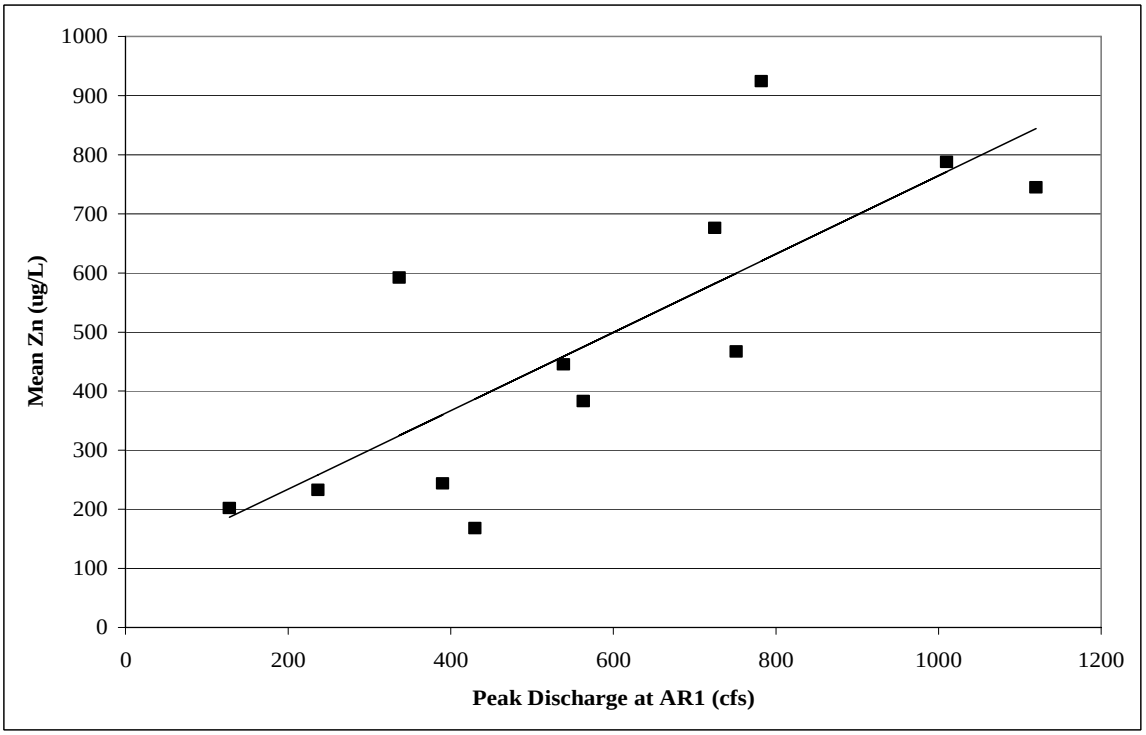


Figure 21. Measured dissolved zinc concentrations as a function of stream discharge in the Arkansas River at Granite (AR7) 1994-2005. Not significant, p>0.08.

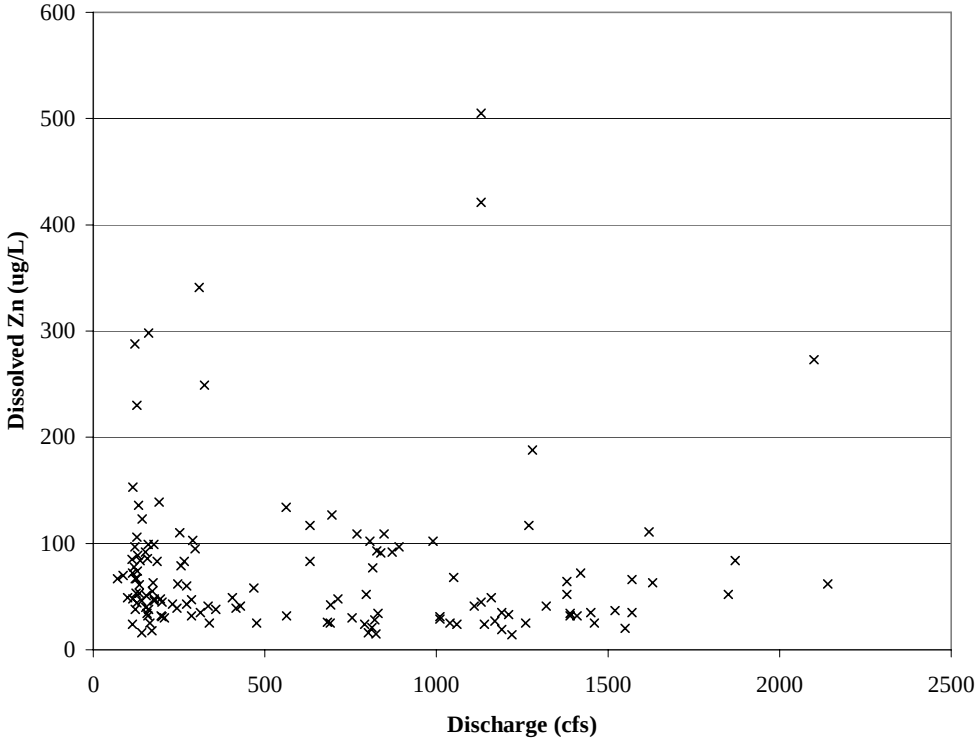


Figure 22a. Mean dissolved zinc concentrations in the Arkansas River at Granite (AR7) in to relation to mean stream discharge during spring runoff 1994-2005. Linear regression $p<0.016$; $r^2=0.59$.

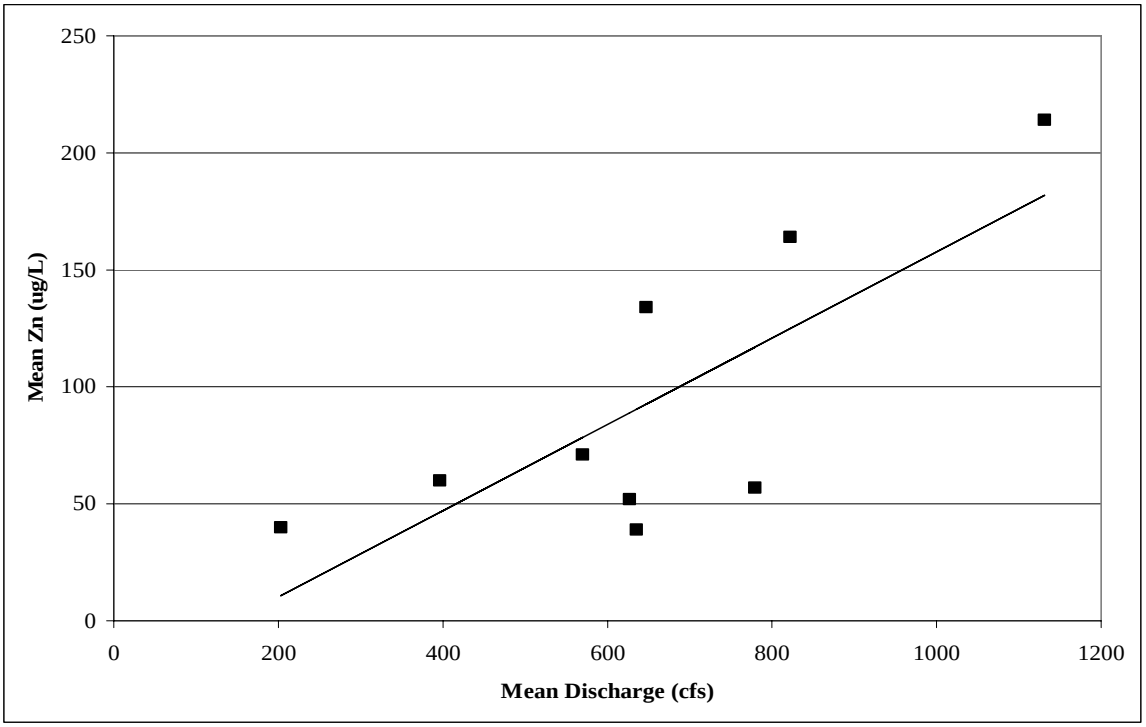


Figure 22b. Mean dissolved zinc concentrations in the Arkansas River at Granite (AR7) in to relation to peak stream discharge during spring runoff 1994-2005. Linear regression $p<0.026$; $r^2=0.53$.

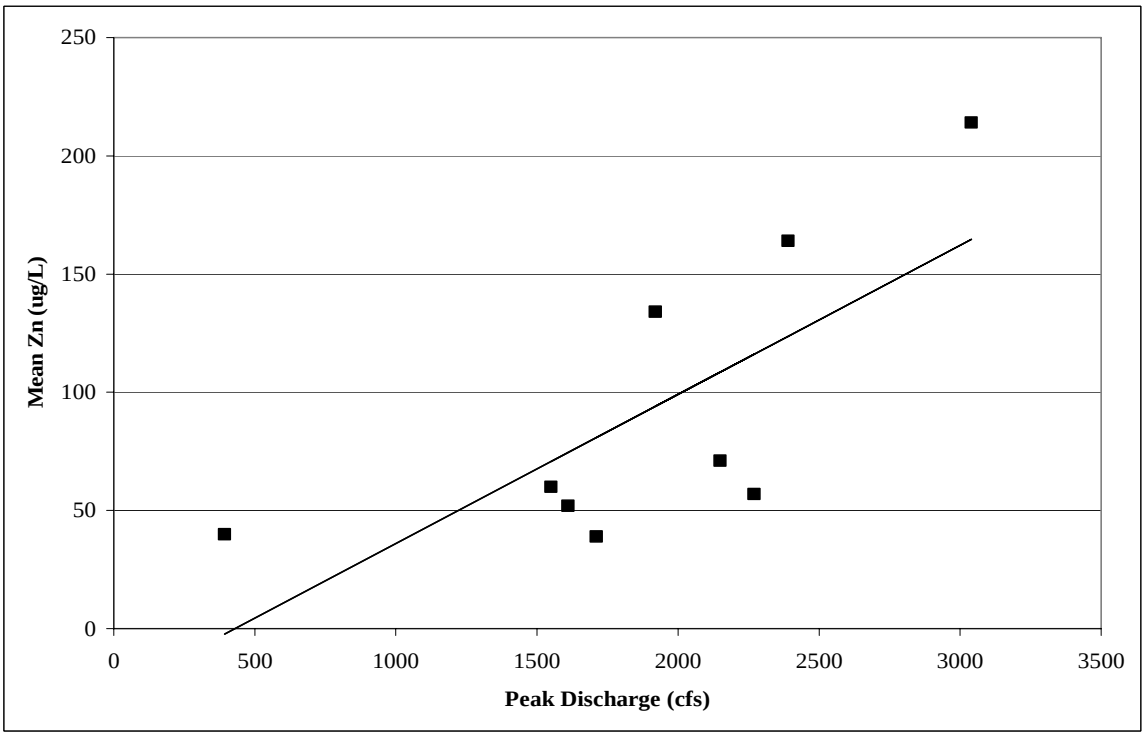


Figure 23. Box and whisker plot of dissolved zinc concentrations (µg/L) at station CG4 during spring runoff 1994-2005.

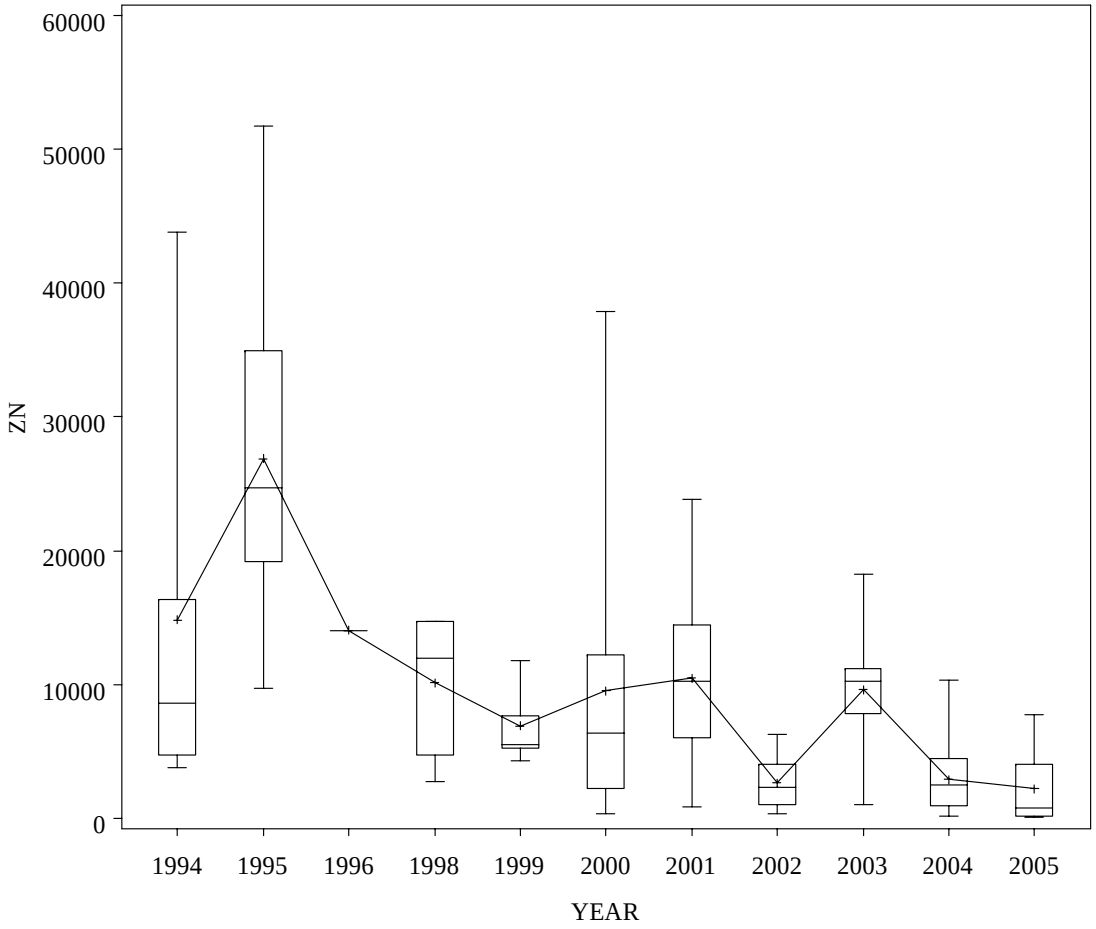


Figure 24. Box and whisker plot of dissolved zinc concentrations (µg/L) at station AR3a during spring runoff 1994-2005.

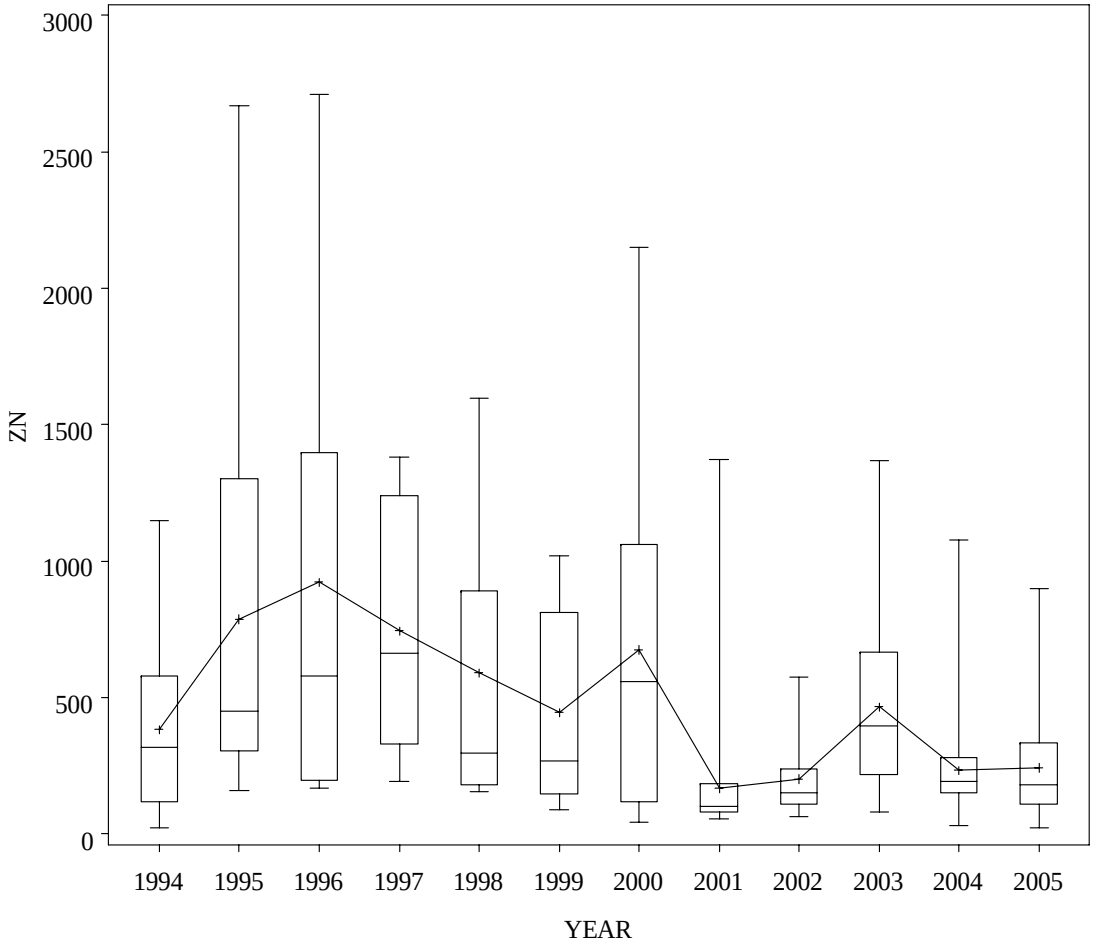


Figure 25. Box and whisker plot of dissolved zinc concentrations (µg/L) at station AR4 during spring runoff 1994-2005.

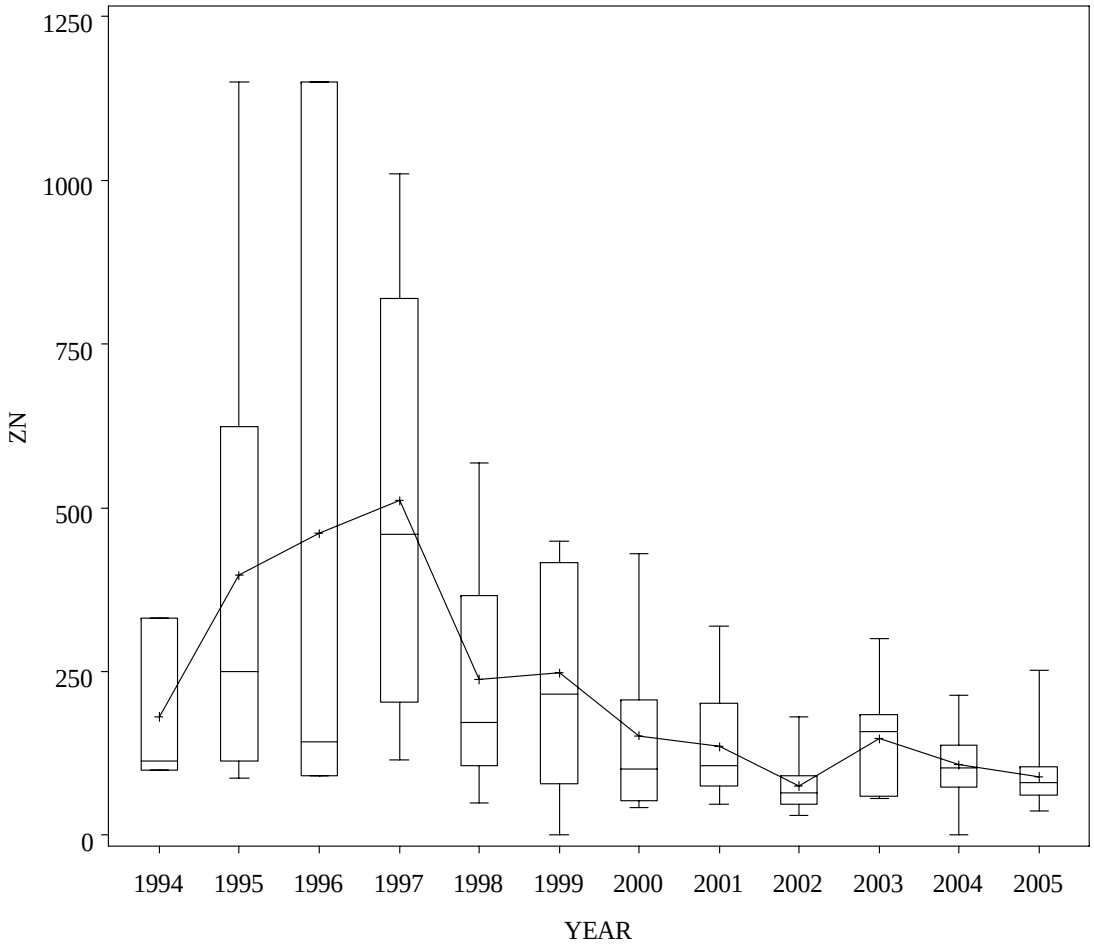


Figure 26. Box and whisker plot of dissolved zinc concentrations (µg/L) at station AR5 during spring runoff 1994-2005.

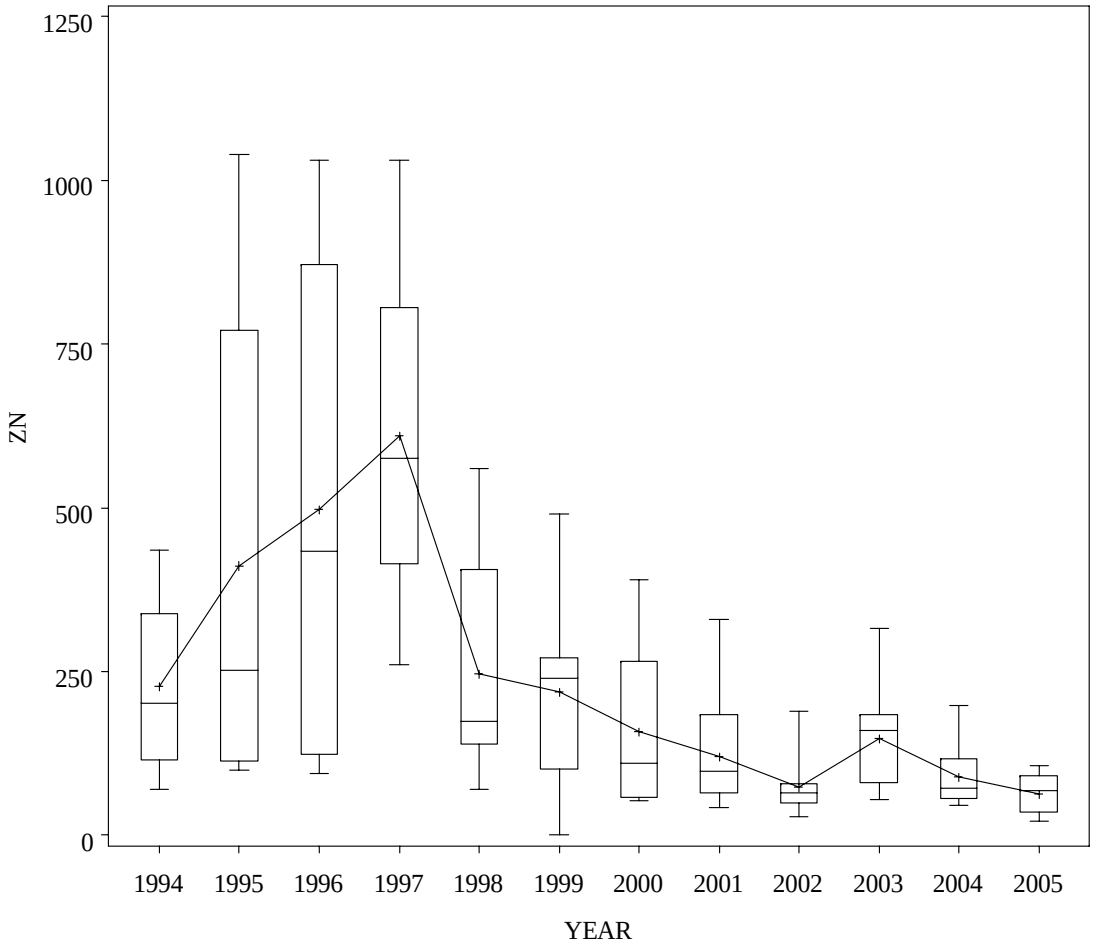


Figure 27. Box and whisker plot of dissolved zinc concentrations (µg/L) at station AR6 during spring runoff 1998-2005.

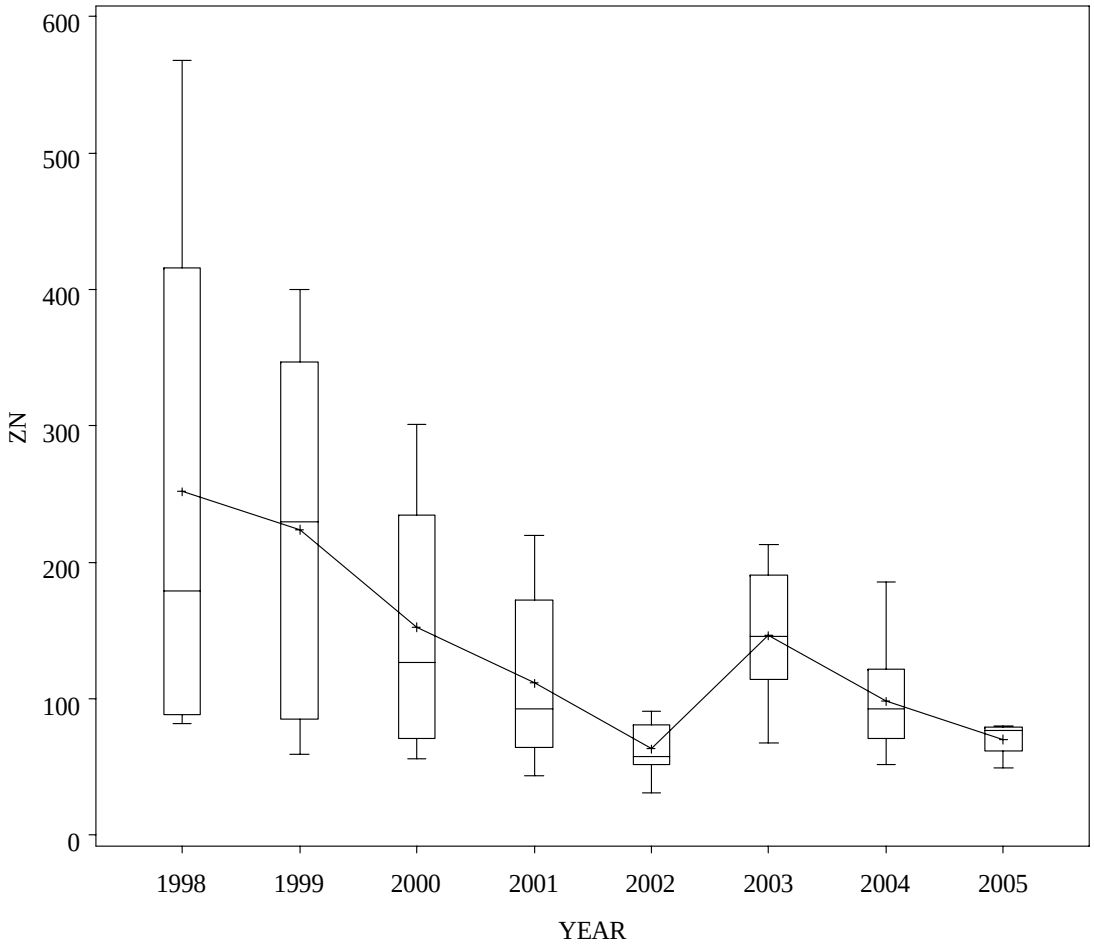


Figure 28. Box and whisker plot of dissolved zinc concentrations (µg/L) at station AR7 during spring runoff 1998-2005.

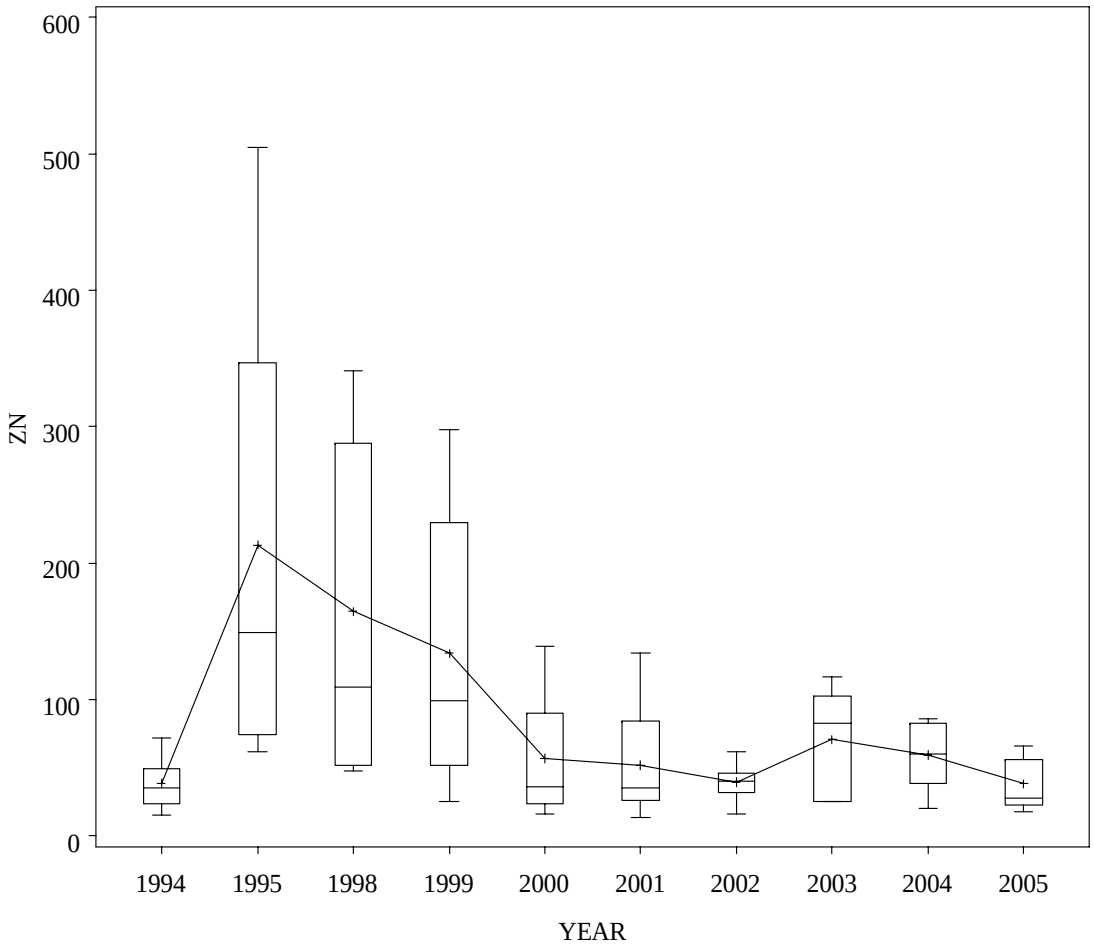


Figure 29. Mean dissolved zinc concentrations (µg/L) in the Arkansas River during spring runoff 1994-2005.

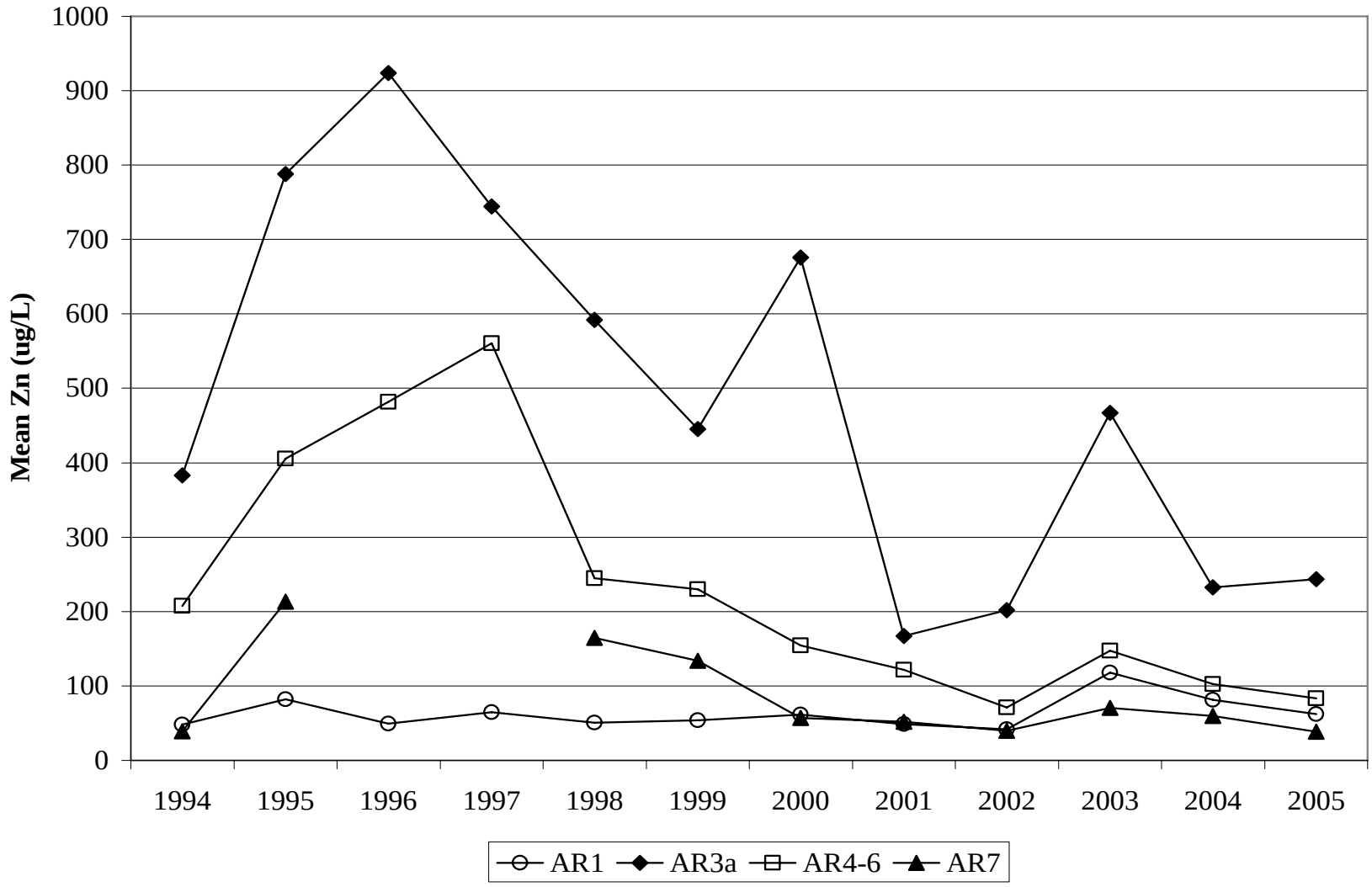
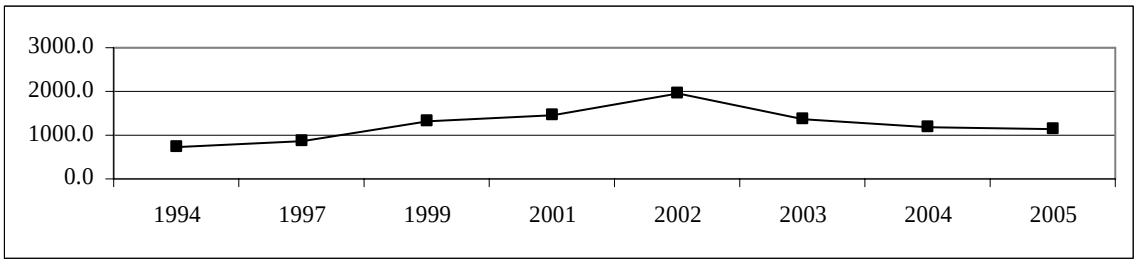
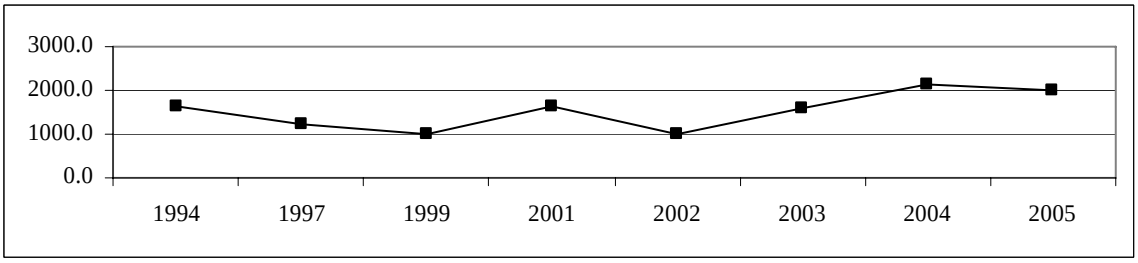


Figure 30. Brown trout population estimates (>10 cm/ha) at Arkansas River stations unimpacted by California Gulch discharges.

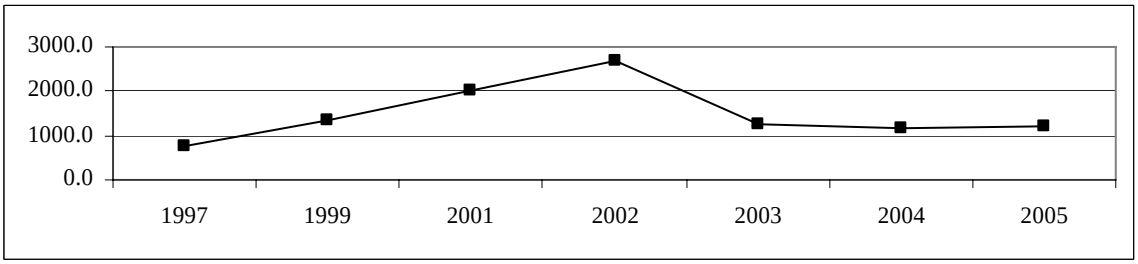
EF1



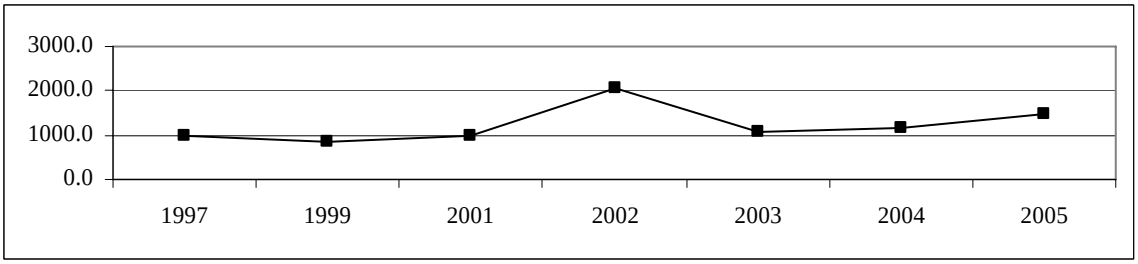
EF3



TC7



AR1



LF22

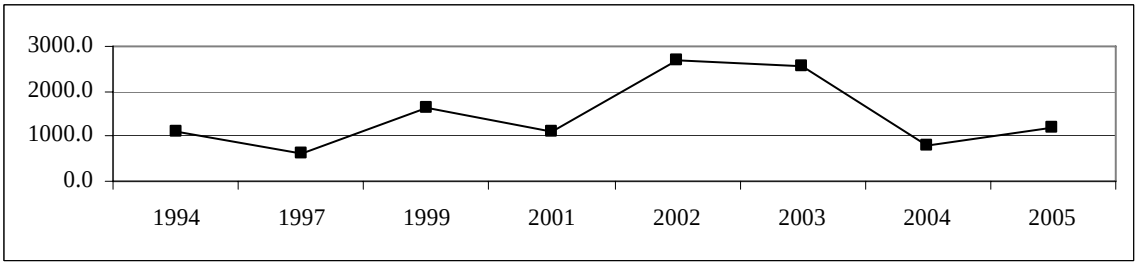
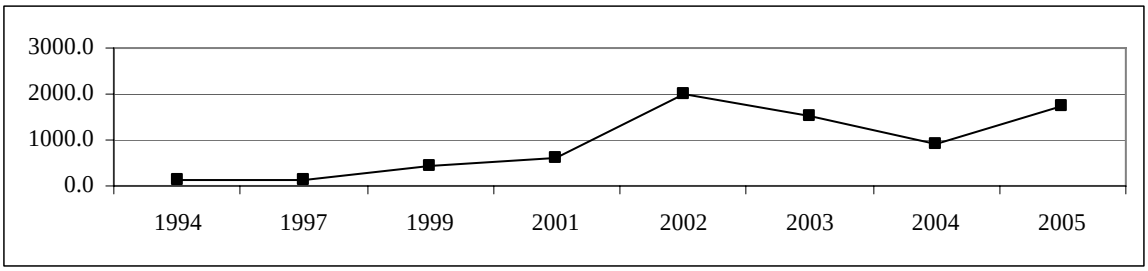
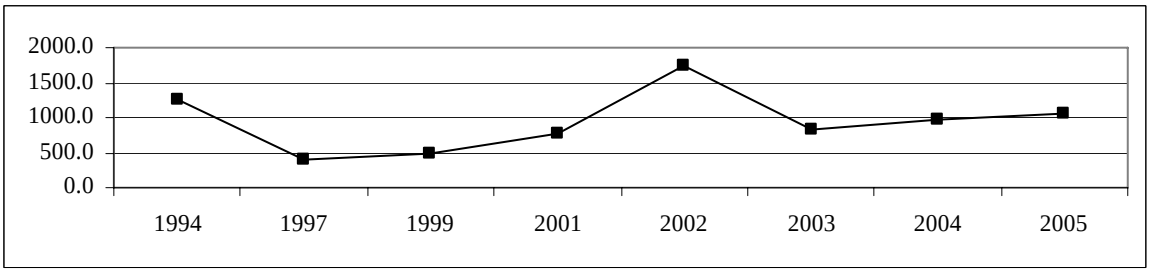


Figure 31. Brown trout population estimates (>10 cm/ha) at Arkansas River stations downstream from California Gulch discharges.

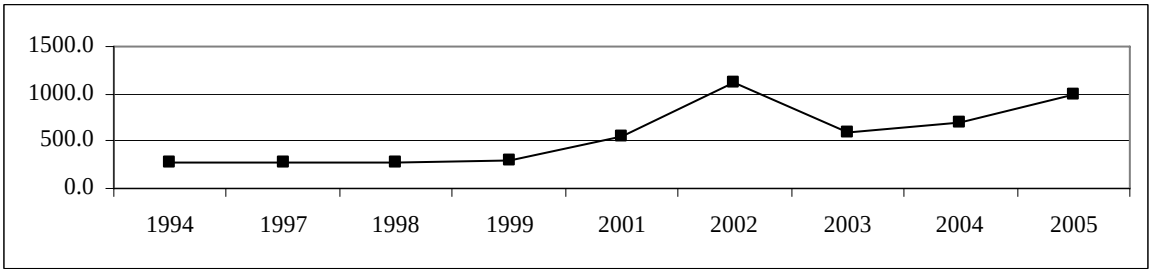
AR3a



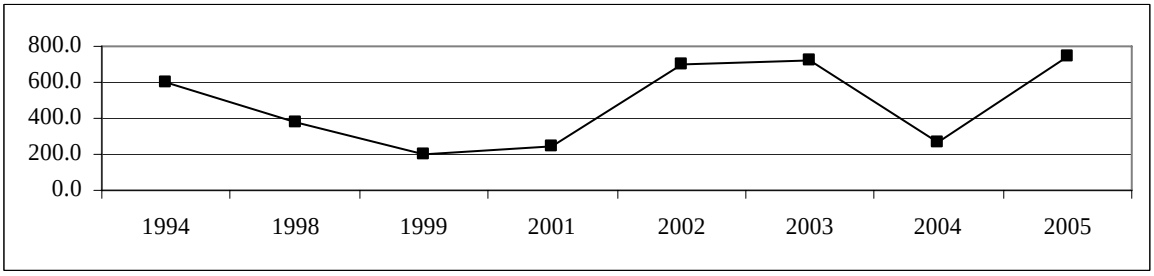
AR4



AR5



AR6



AR7

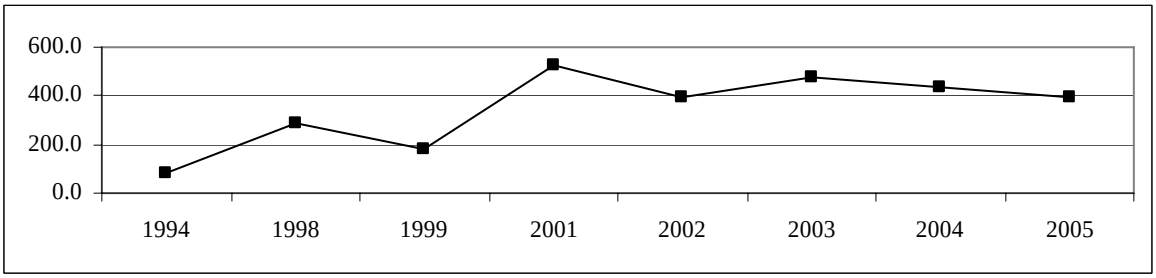


Figure 32. Length-frequency distributions of brown trout collected at station EF1 during fall sampling 1994-2005.

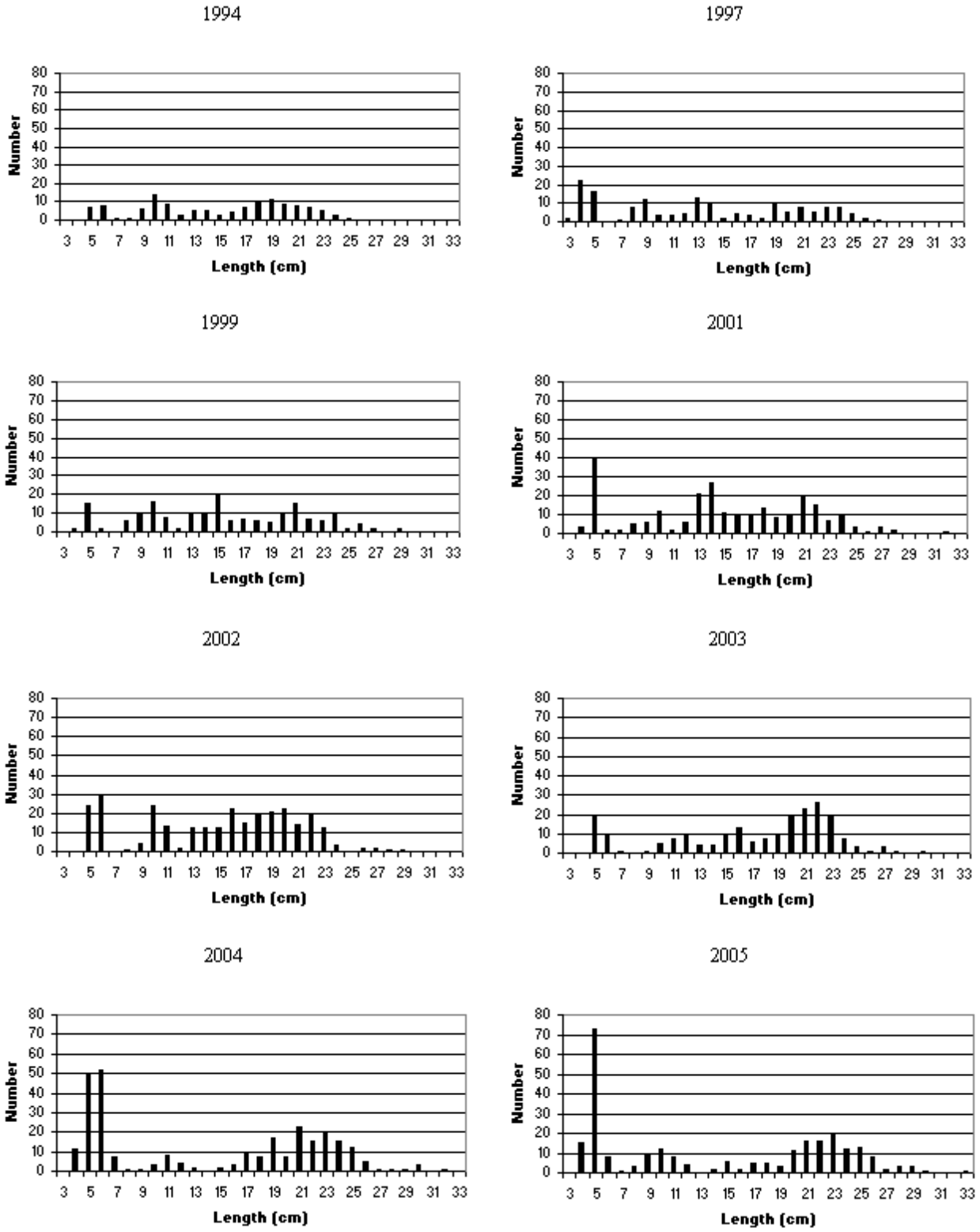


Figure 33. Length-frequency distributions of brown trout collected at station EF3 during fall sampling 1994-2005.

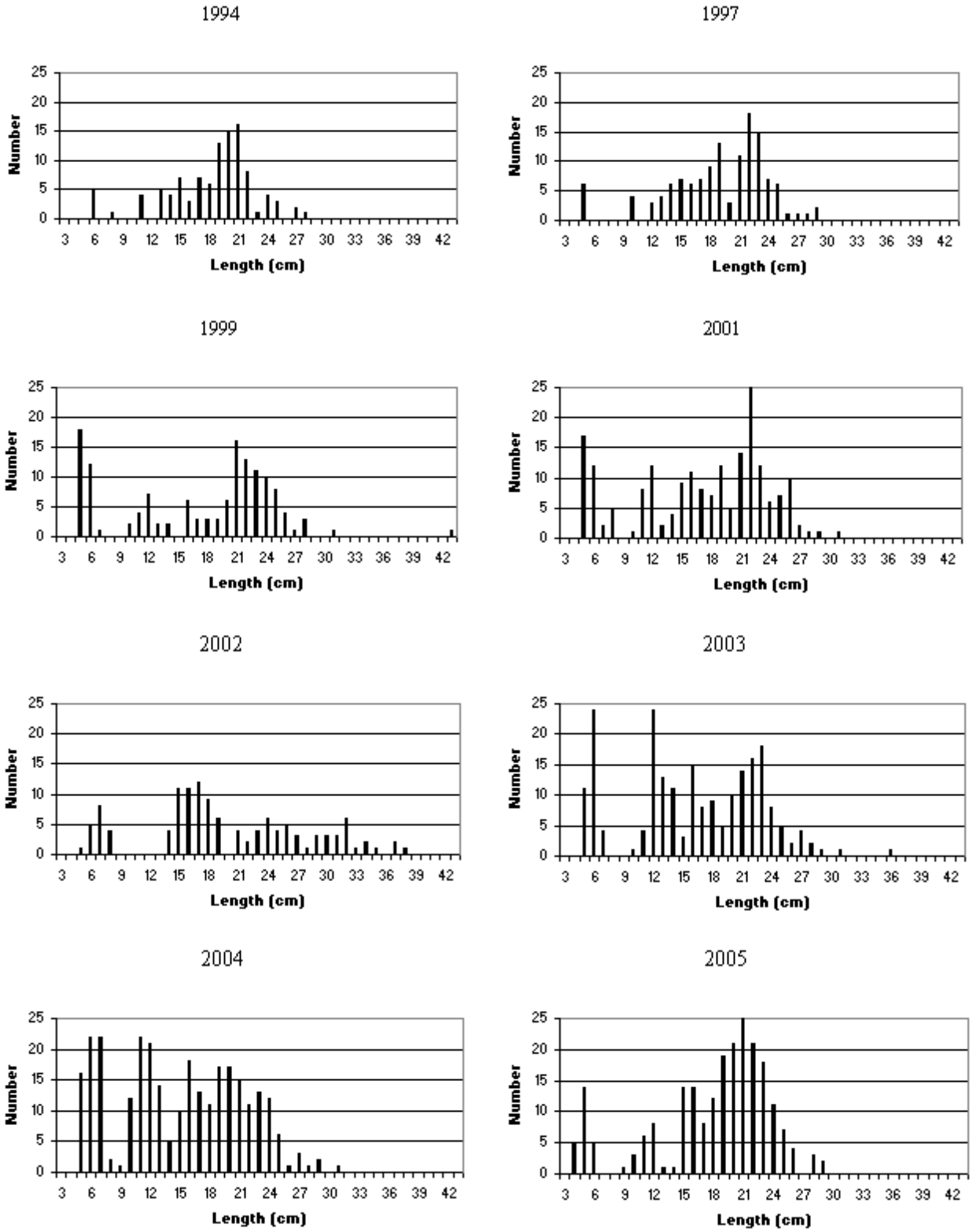


Figure 34. Length-frequency distributions of brown trout collected at station TC7 during fall sampling 1997-2005.

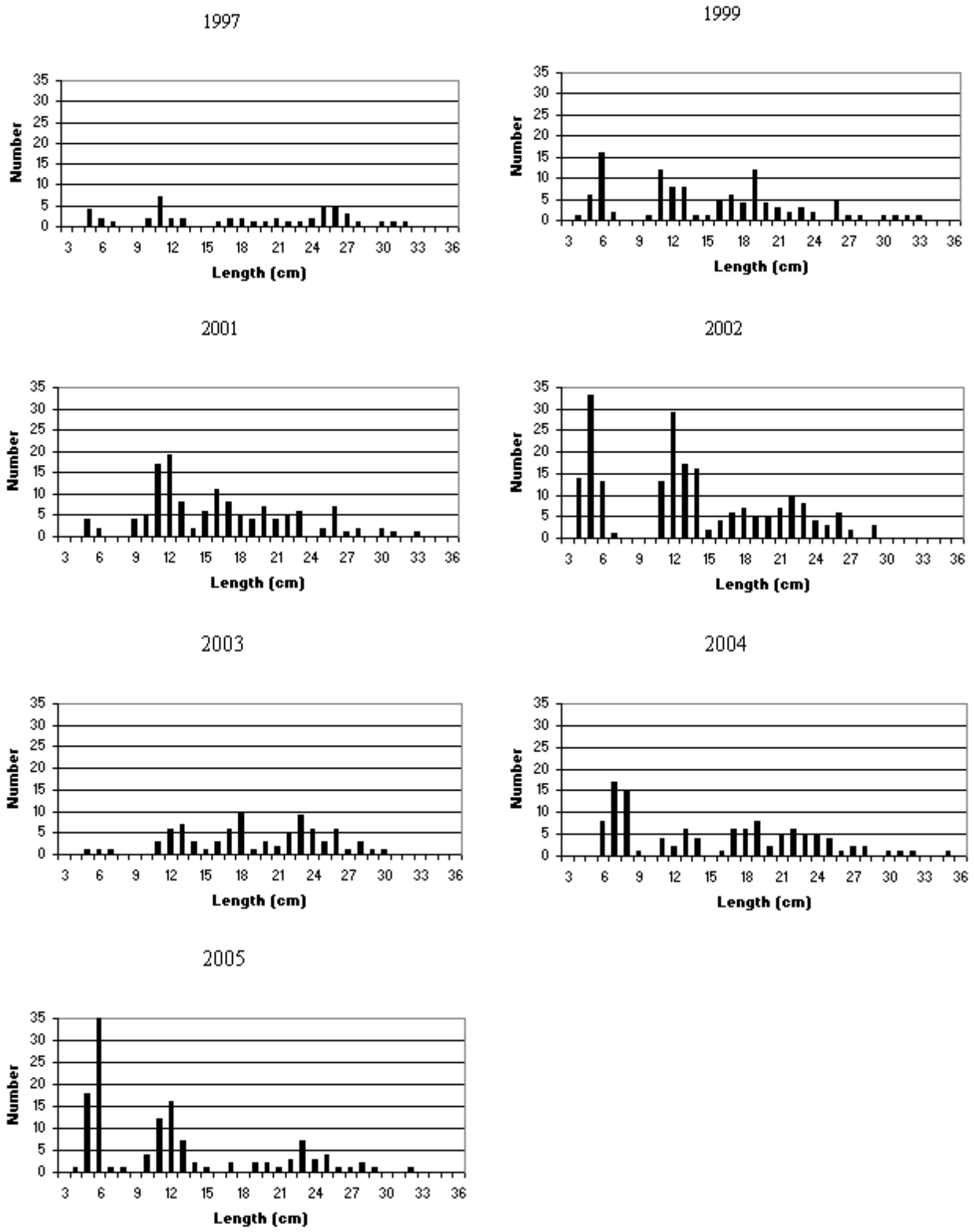


Figure 35. Length-frequency distributions of brown trout collected at station AR1 during fall sampling 1997-2005.

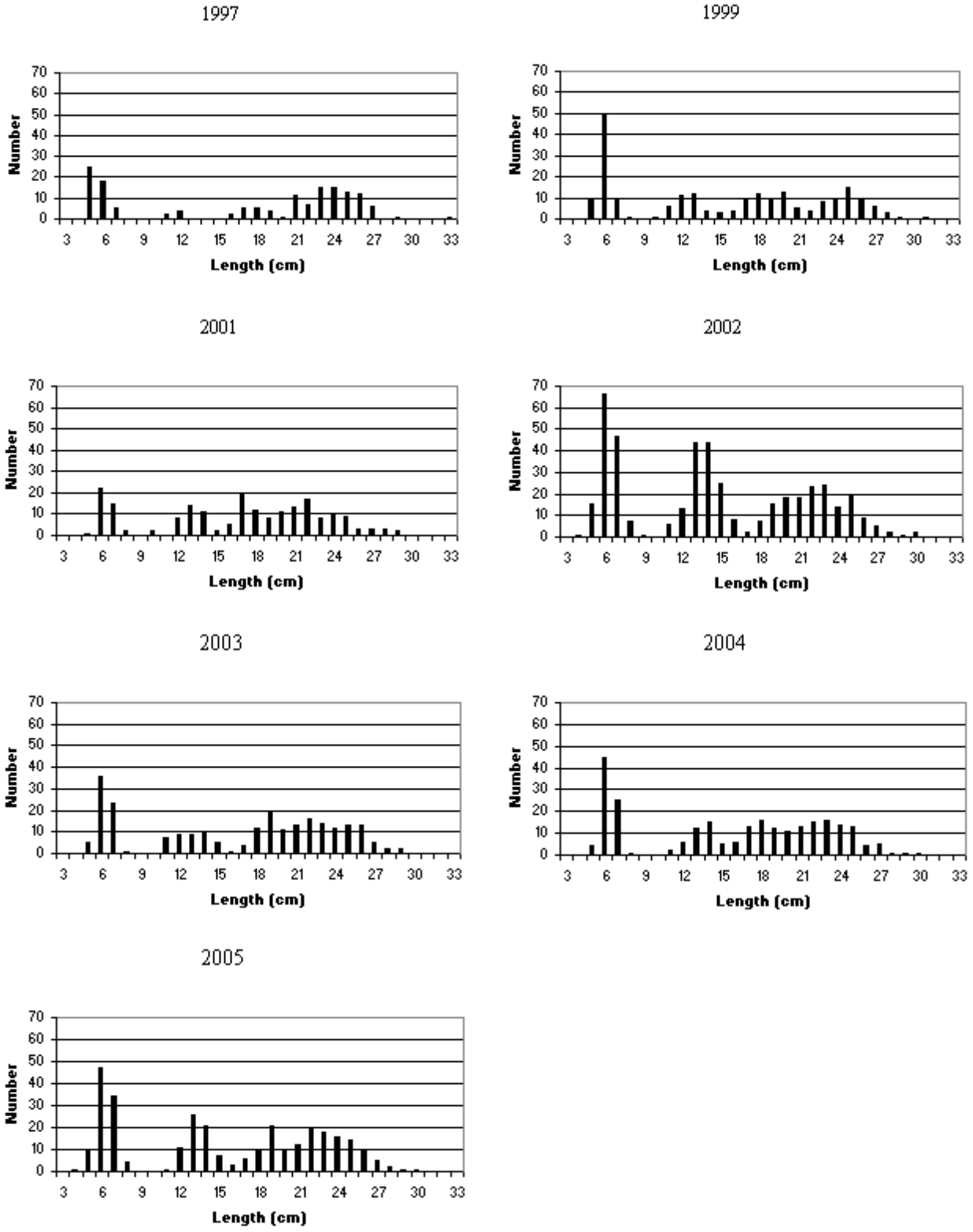


Figure 36. Length-frequency distributions of brown trout collected at station AR3a during fall sampling 1994-2005.

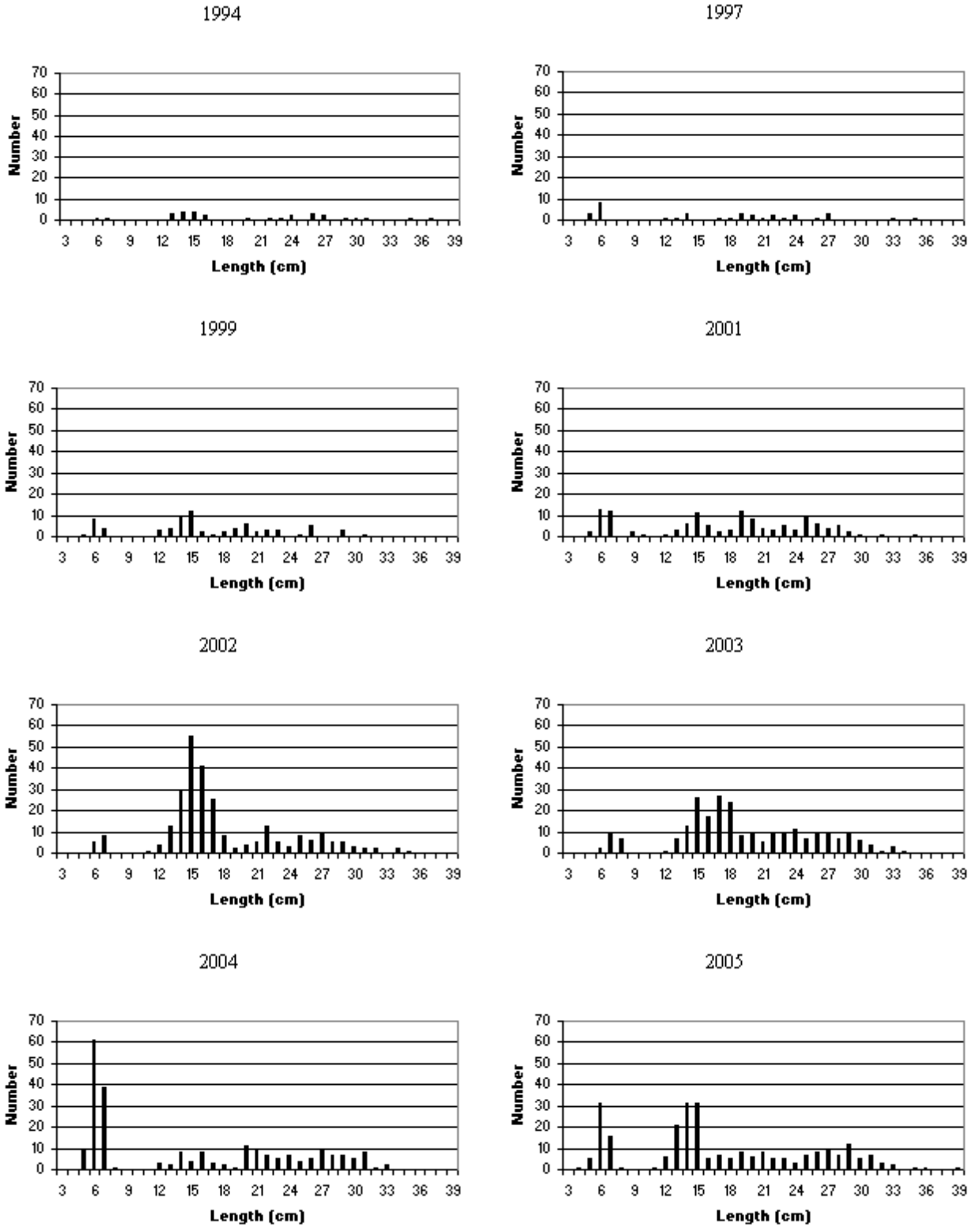


Figure 37. Length-frequency distributions of brown trout collected at station LF22 during fall sampling 1994-2005.

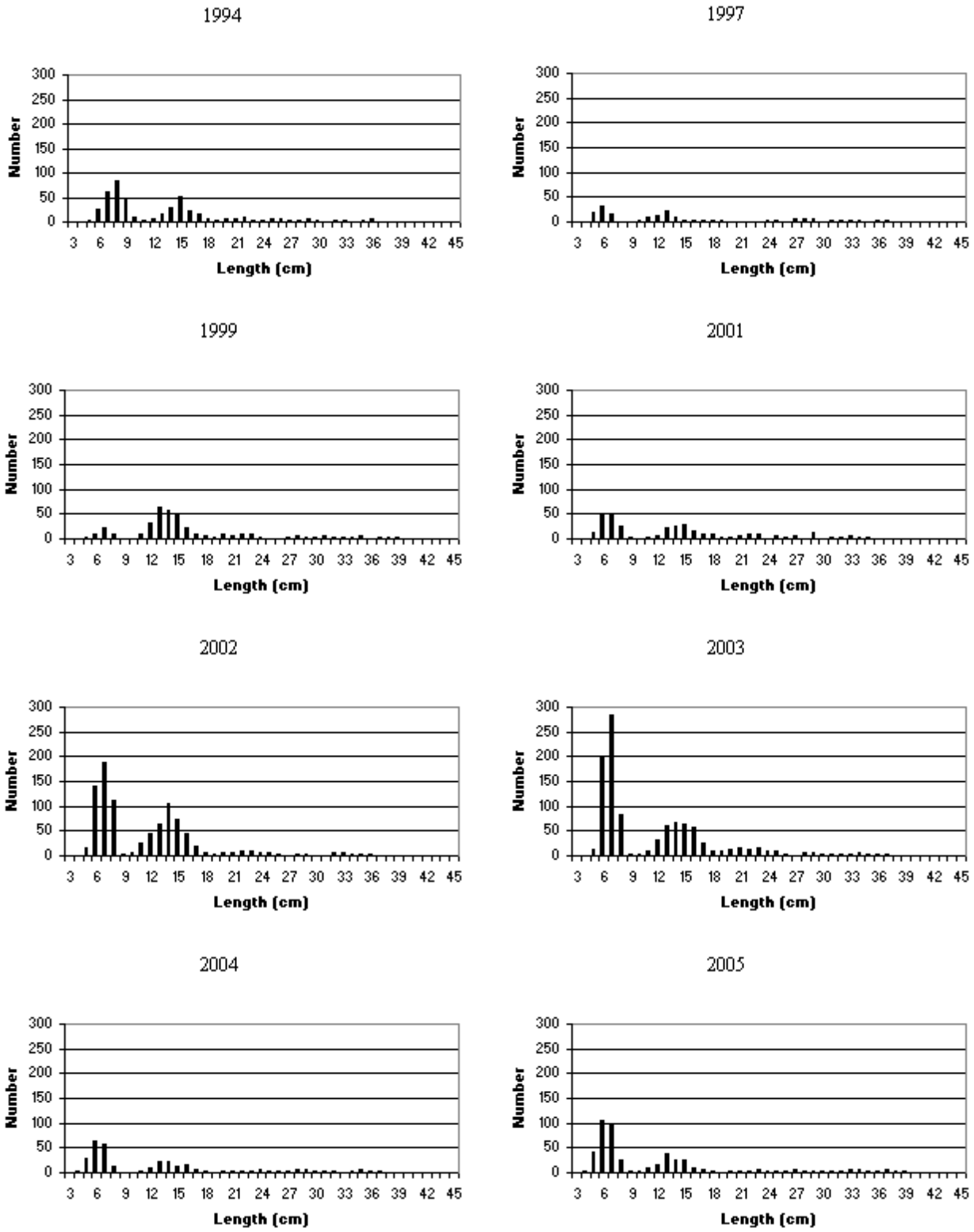


Figure 38. Length-frequency distributions of brown trout collected at station AR4 during fall sampling 1994-2005.

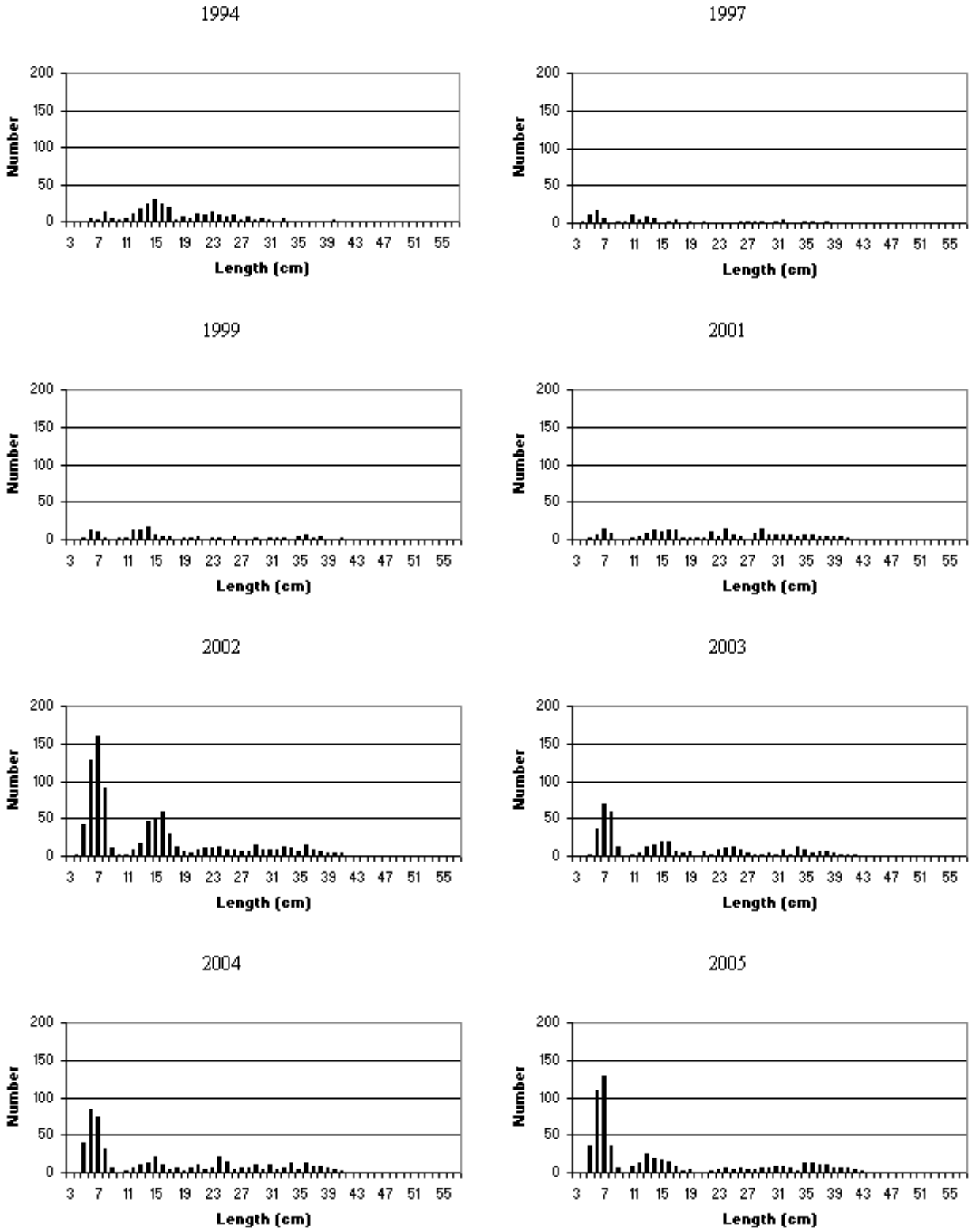


Figure 39. Length-frequency distributions of brown trout collected at station AR5 during fall sampling 1994-2005.

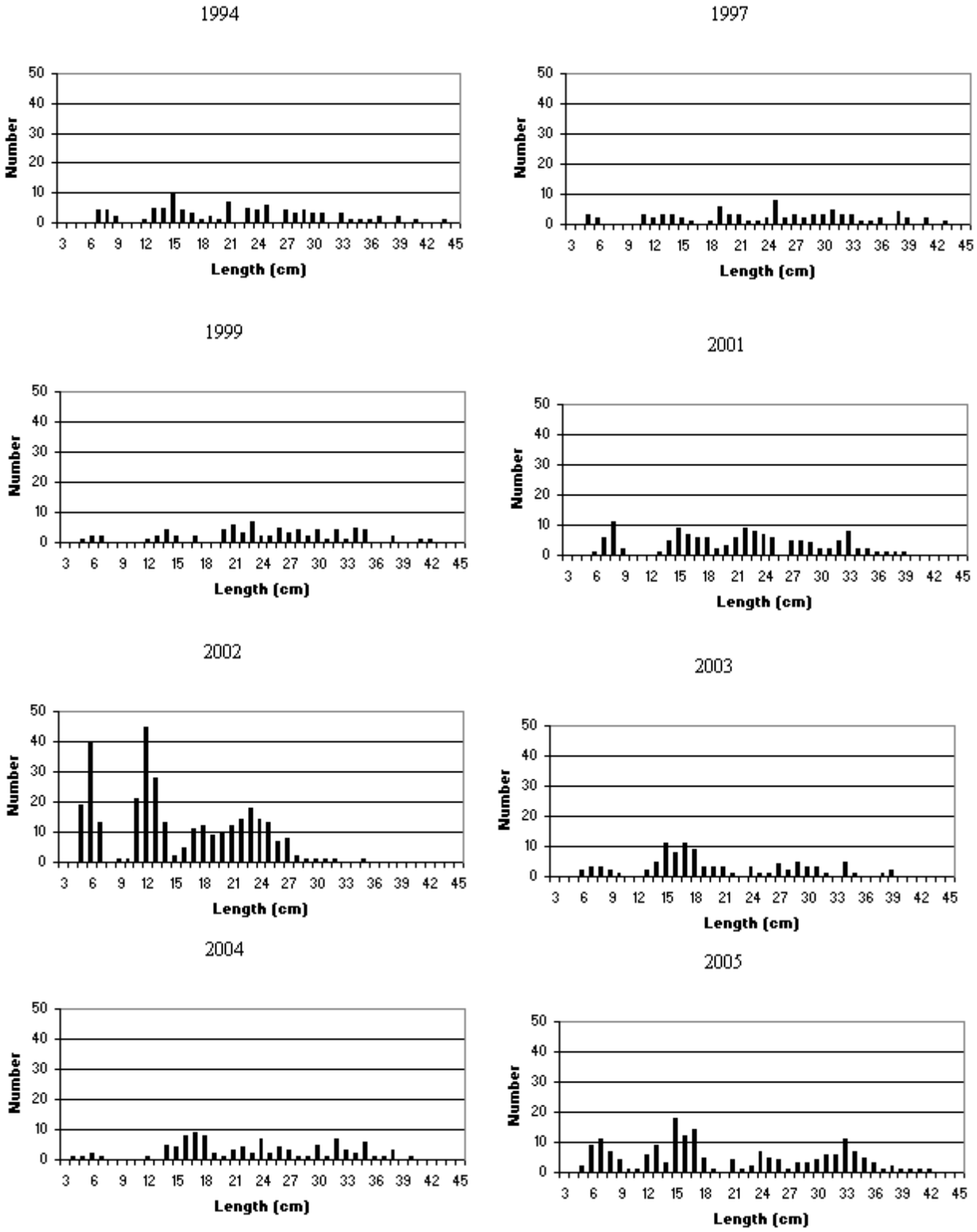


Figure 40. Length-frequency distributions of brown trout collected at station AR6 during fall sampling 1994-2005.

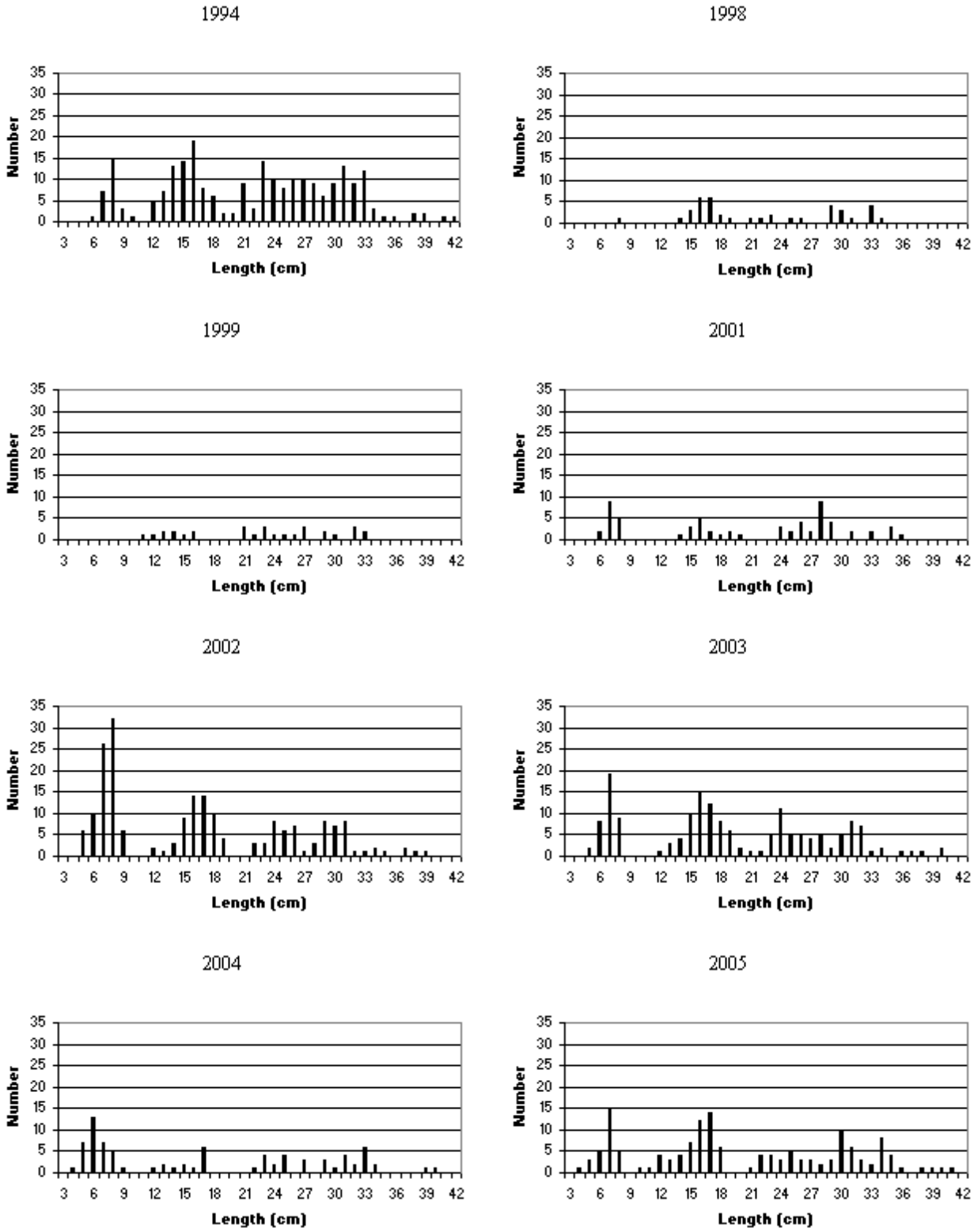


Figure 41. Length-frequency distributions of brown trout collected at station AR6a during fall sampling 1999-2005.

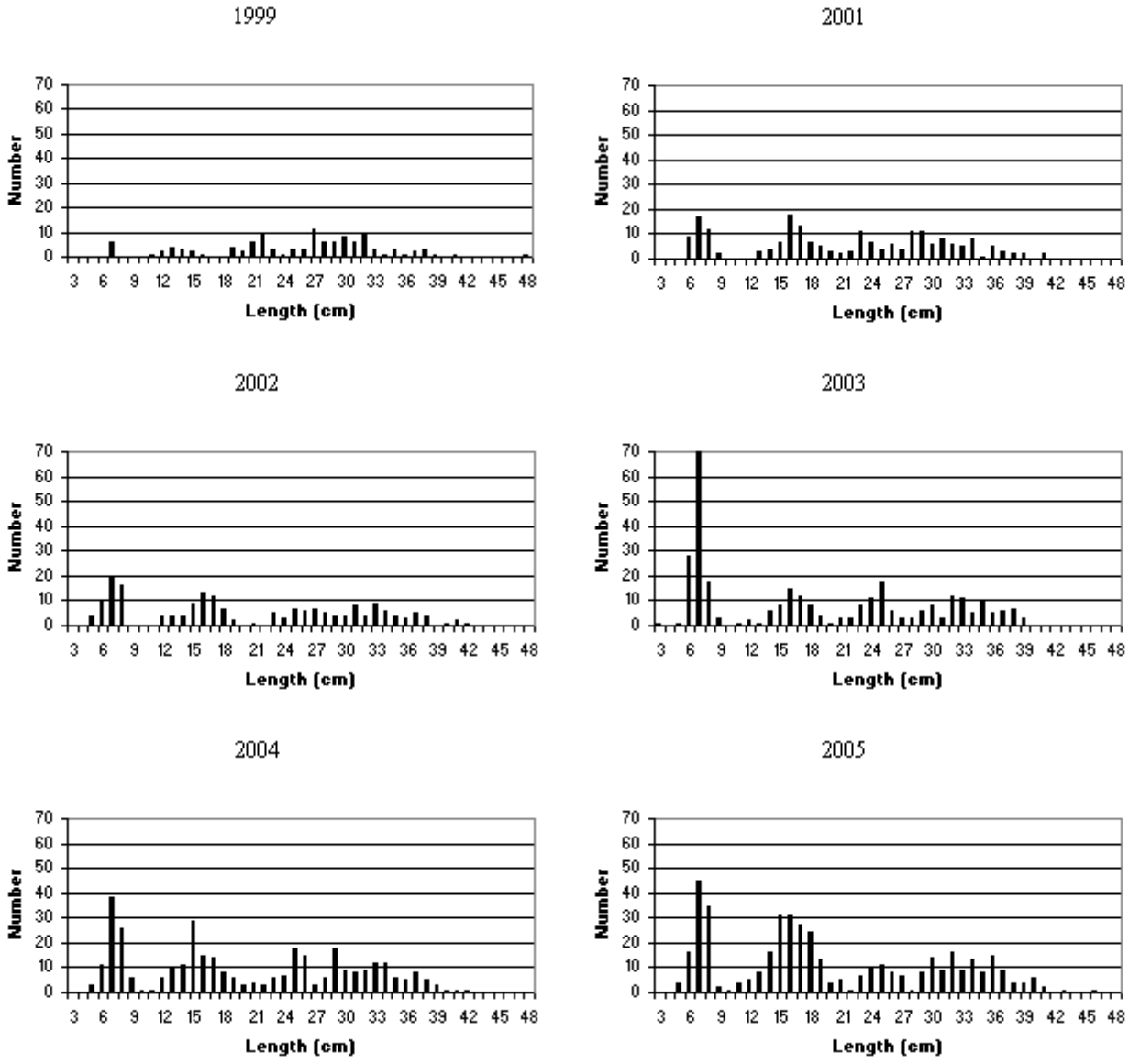


Figure 42. Length-frequency distributions of brown trout collected at station AR7 during fall sampling 1994-2005.

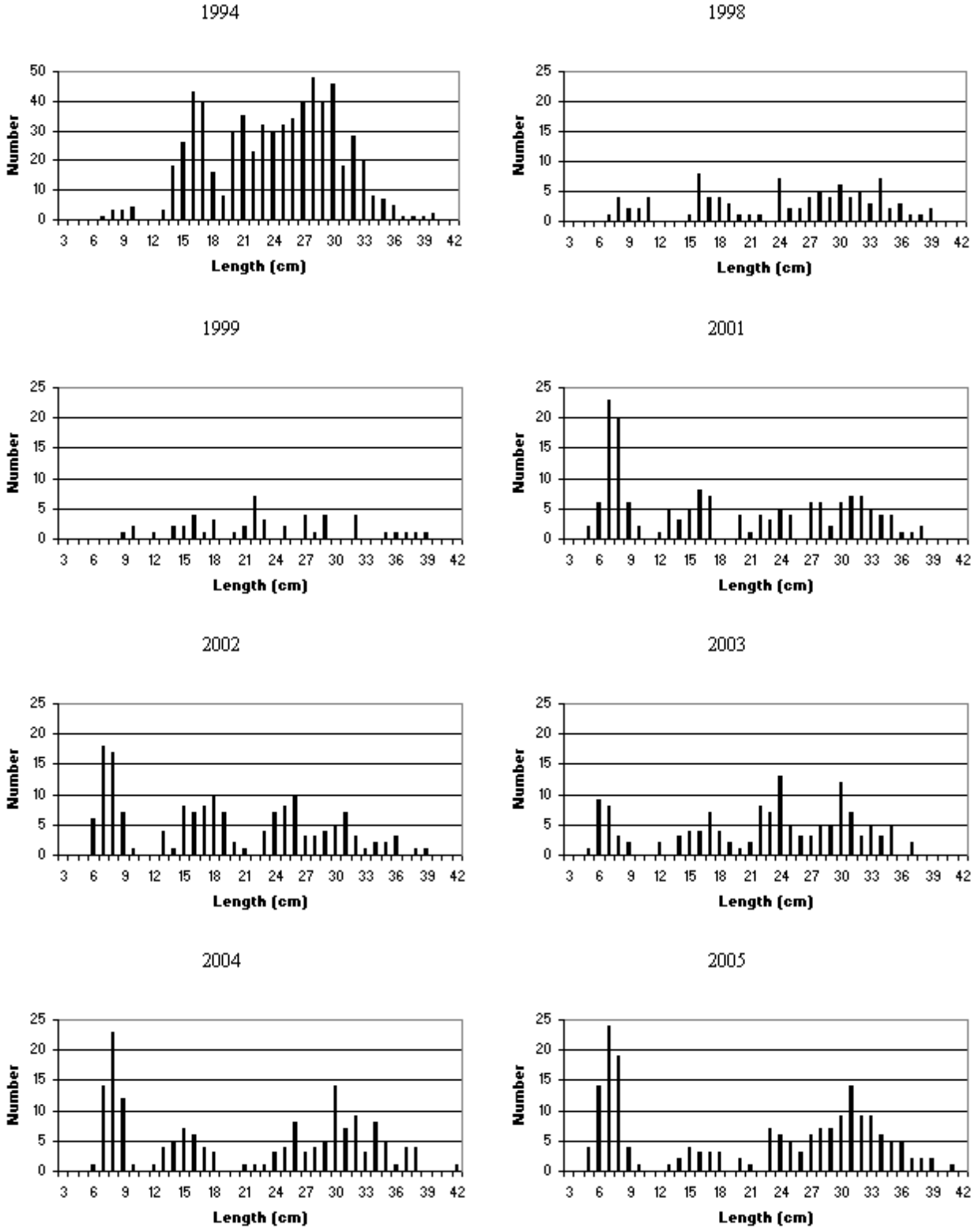


Figure 43a. Brown trout densities (#>10cm/ha) in relation to mean spring discharge in the East Fork of the Arkansas River at Highway 24 (EF3). Not significant p>0.46.

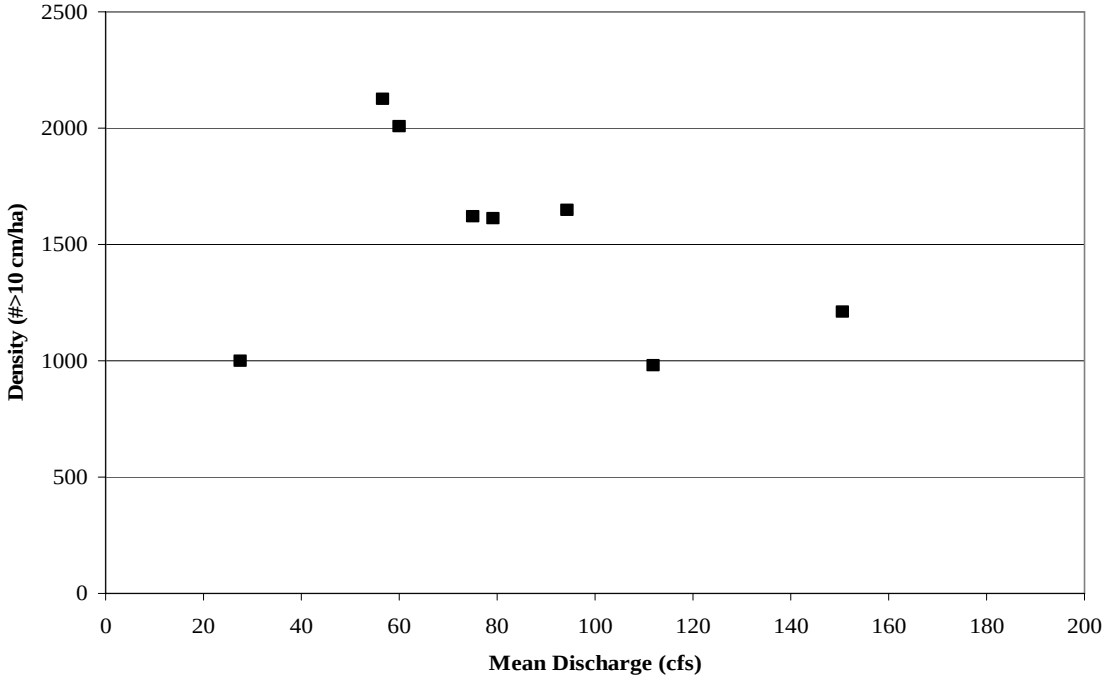


Figure 43b. Brown trout densities (#>10cm/ha) in relation to peak discharge in the East Fork of the Arkansas River at Highway 24 (EF3). Not significant p>0.50.

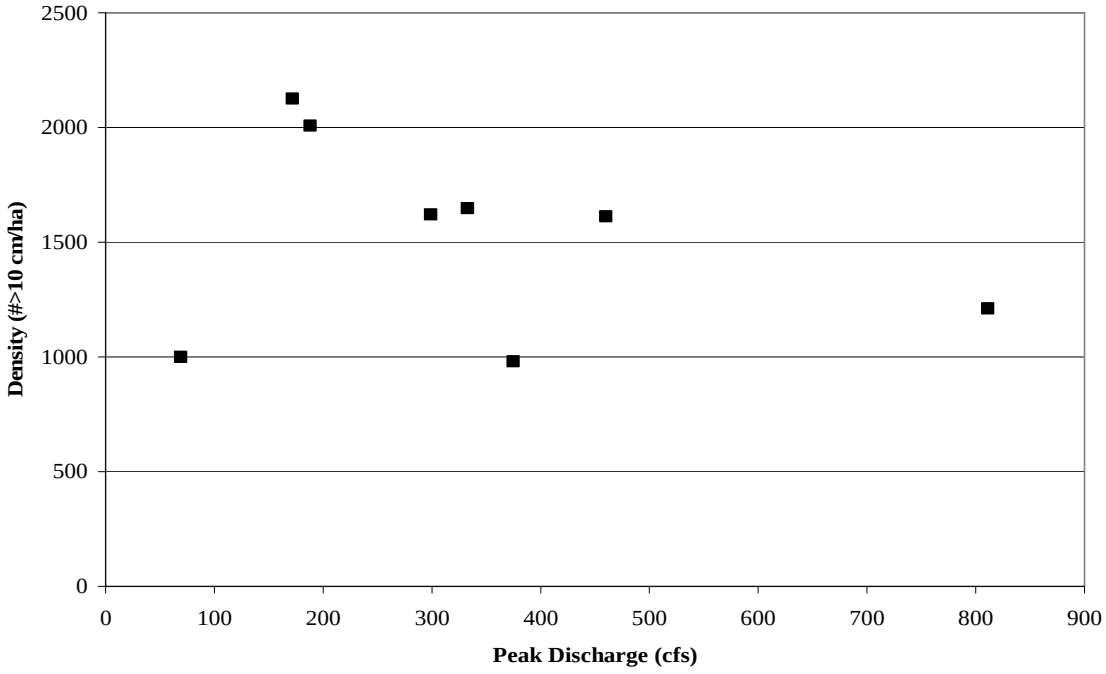


Figure 44a. Brown trout densities (#>10cm/ha) in relation to mean spring discharge in Tennessee Creek (TC7); $p<0.039$; $r^2=0.60$.

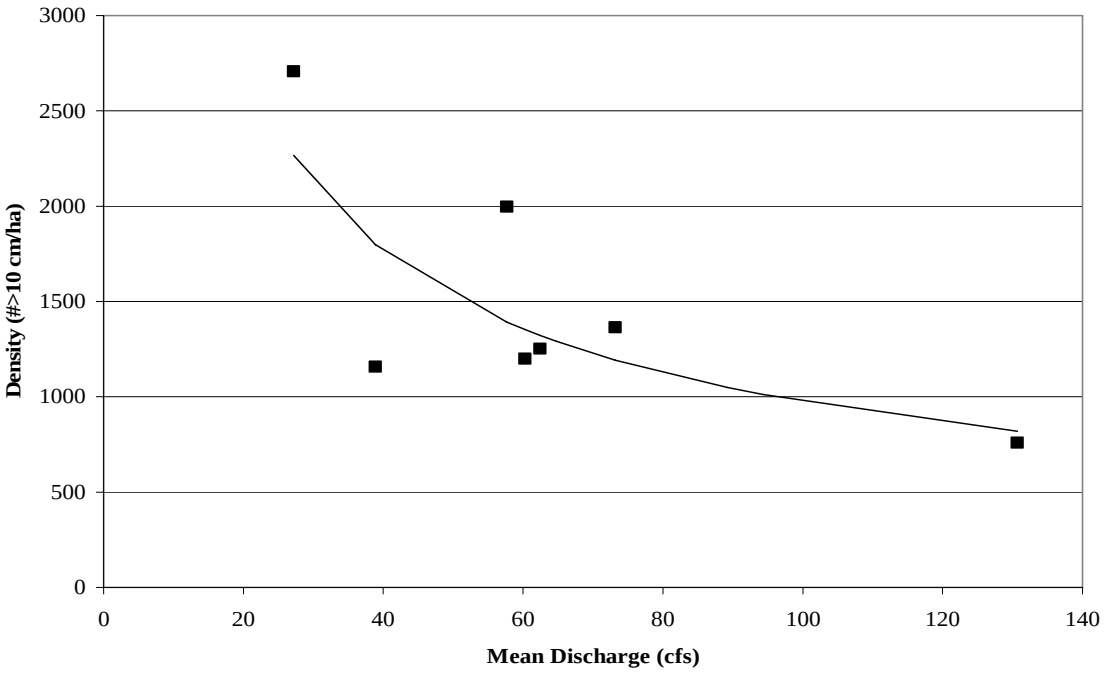


Figure 44b. Brown trout densities (#>10cm/ha) in relation to peak discharge in Tennessee Creek (TC7). Not significant $p>0.09$.

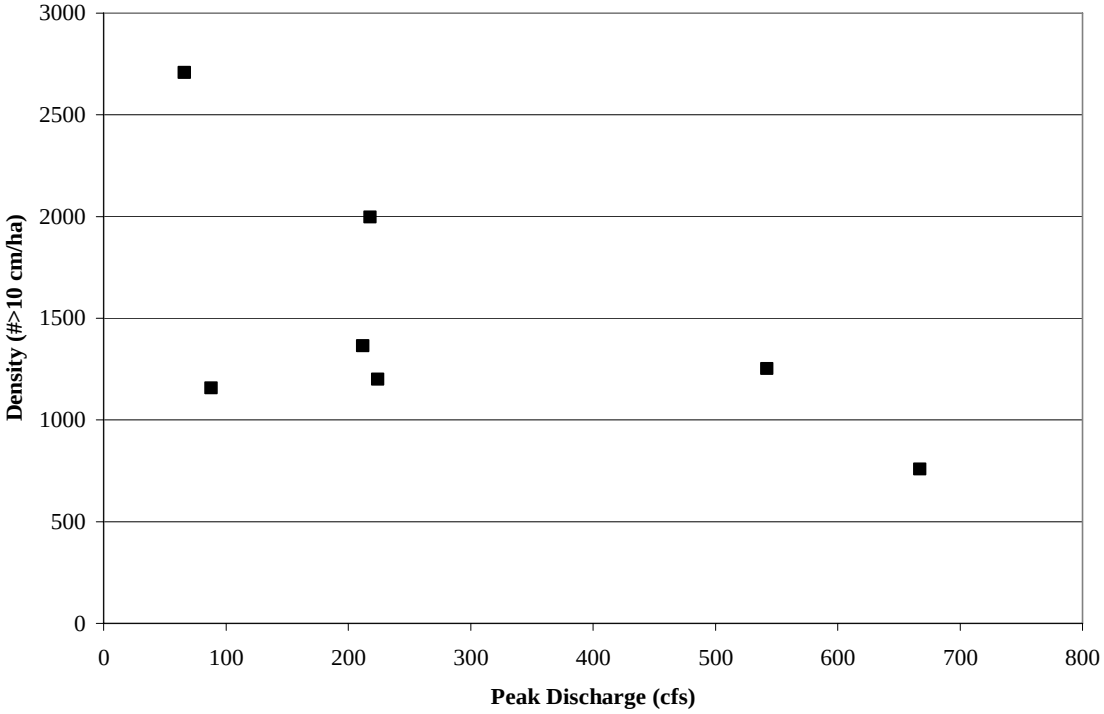


Figure 45a. Brown trout densities (#>10cm/ha) in relation to mean spring discharge in the Arkansas River near Leadville (AR1); $p<0.018$; $r^2=0.70$.

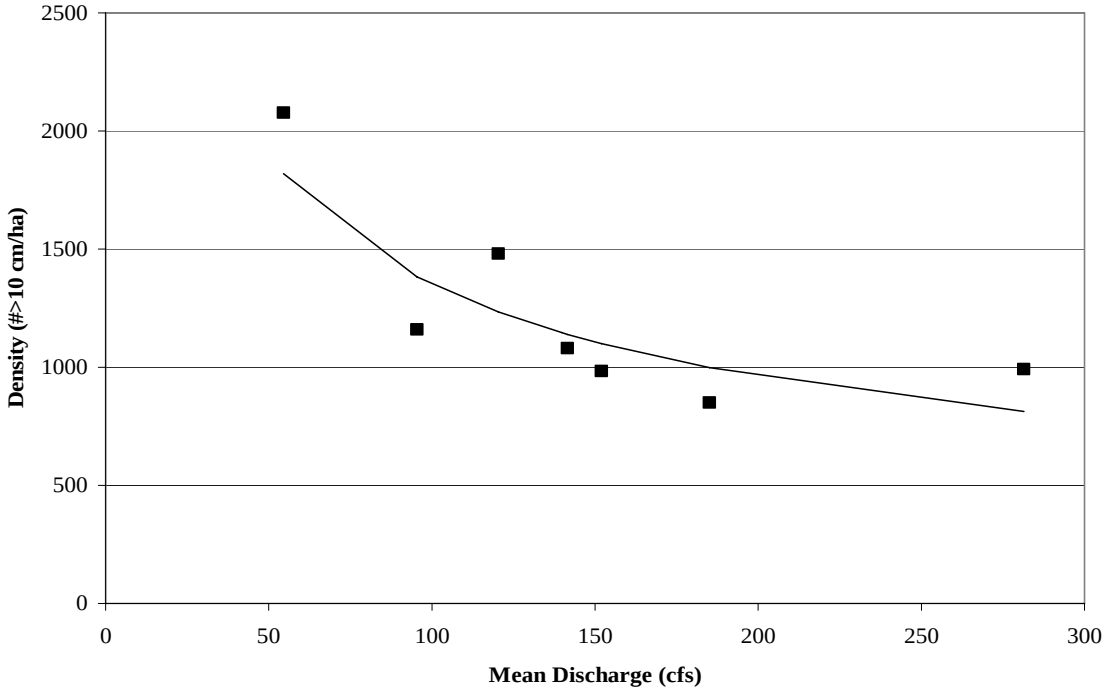


Figure 45b. Brown trout densities (#>10cm/ha) in relation to peak discharge in the Arkansas River near Leadville (AR1). Not significant $p>0.05$.

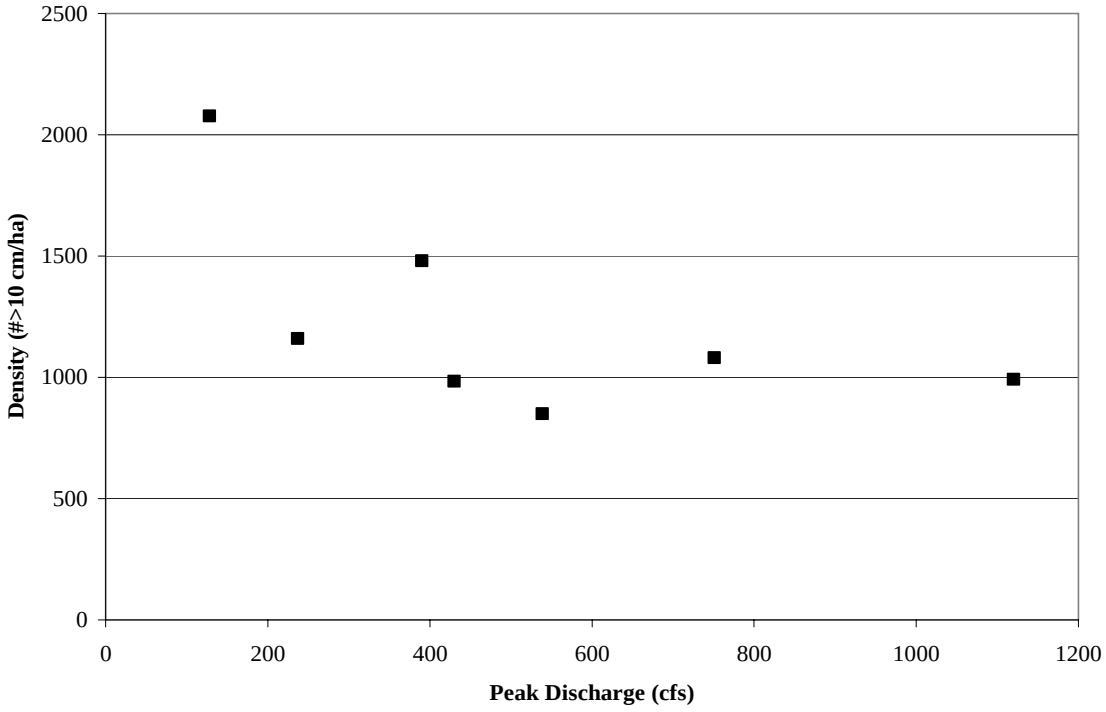


Figure 46a. Brown trout densities (#>10cm/ha) in the Arkansas River below California Gulch (AR3a) in relation to mean discharge in the Arkansas River near Leadville (AR1); p=0.047, r^2=0.51.

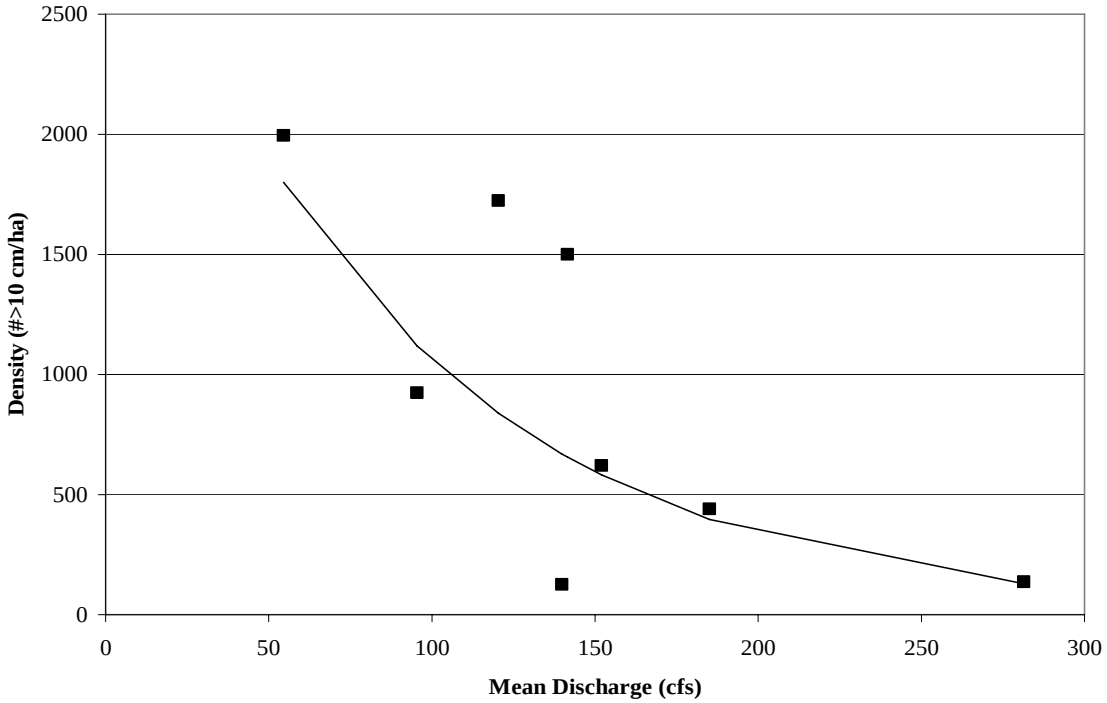


Figure 46b. Brown trout densities (#>10cm/ha) in the Arkansas River below California Gulch (AR3a) in relation to peak discharge in the Arkansas River near Leadville (AR1). Not significant p>0.095.

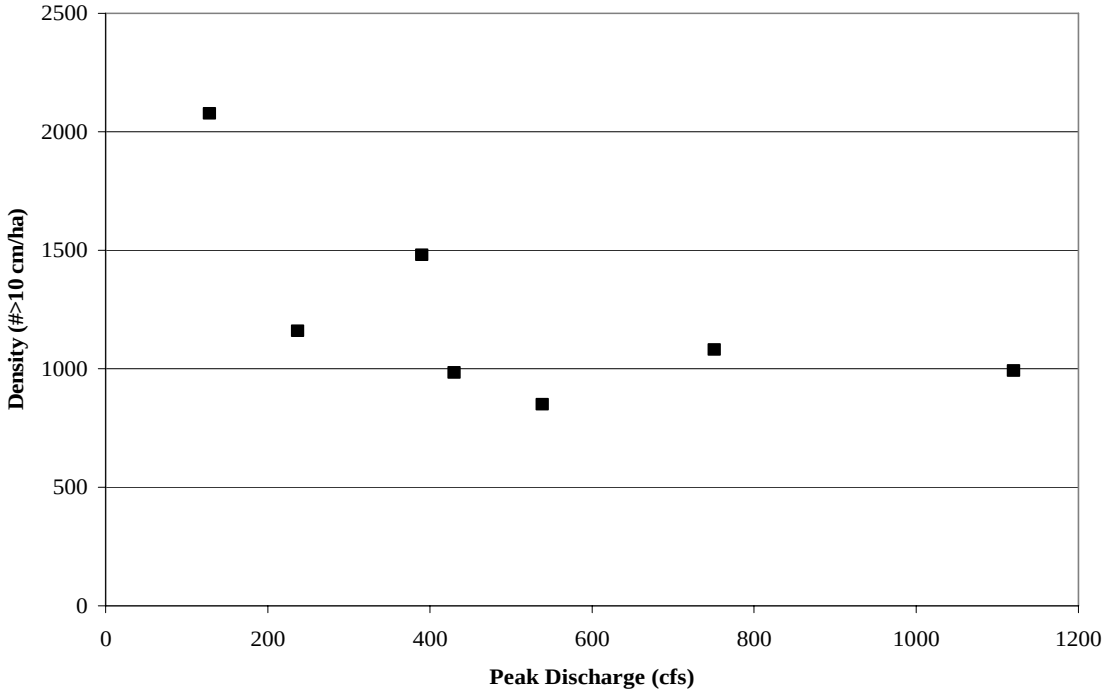


Figure 47a. Brown trout densities (#>10cm/ha) in the Arkansas River at Granite (AR7) in relation to mean discharge. Not significant p>0.45.

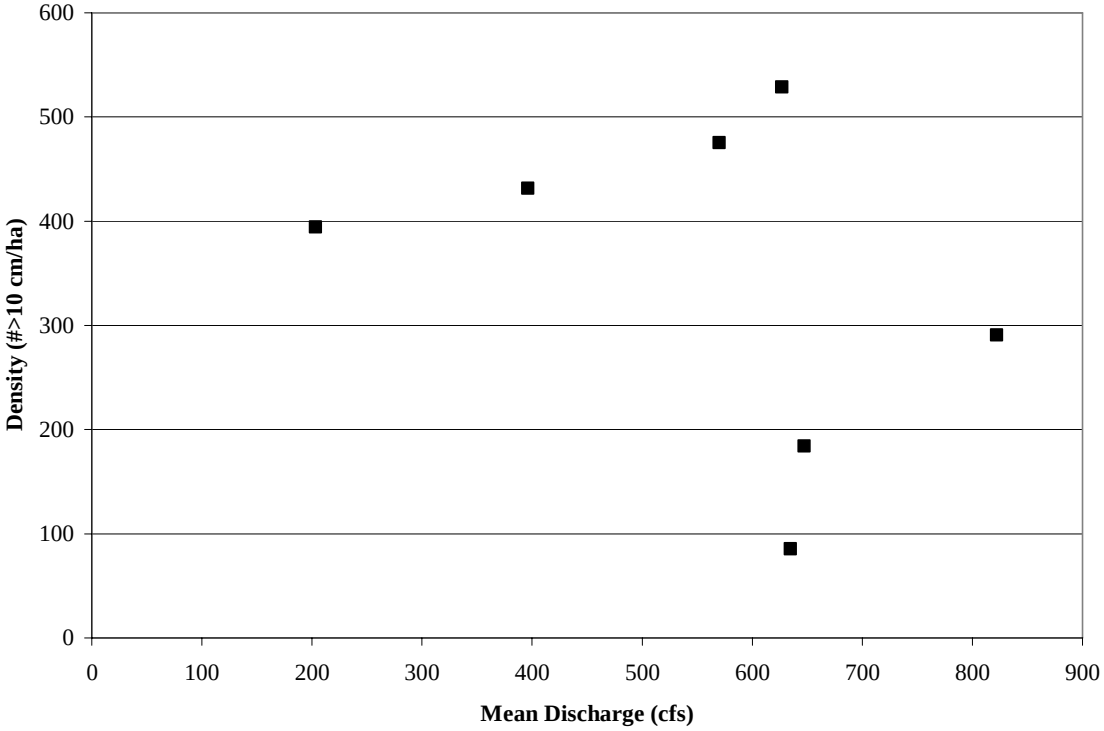


Figure 47b. Brown trout densities (#>10cm/ha) in the Arkansas River at Granite (AR7) in relation to peak discharge. Not significant p>0.69.

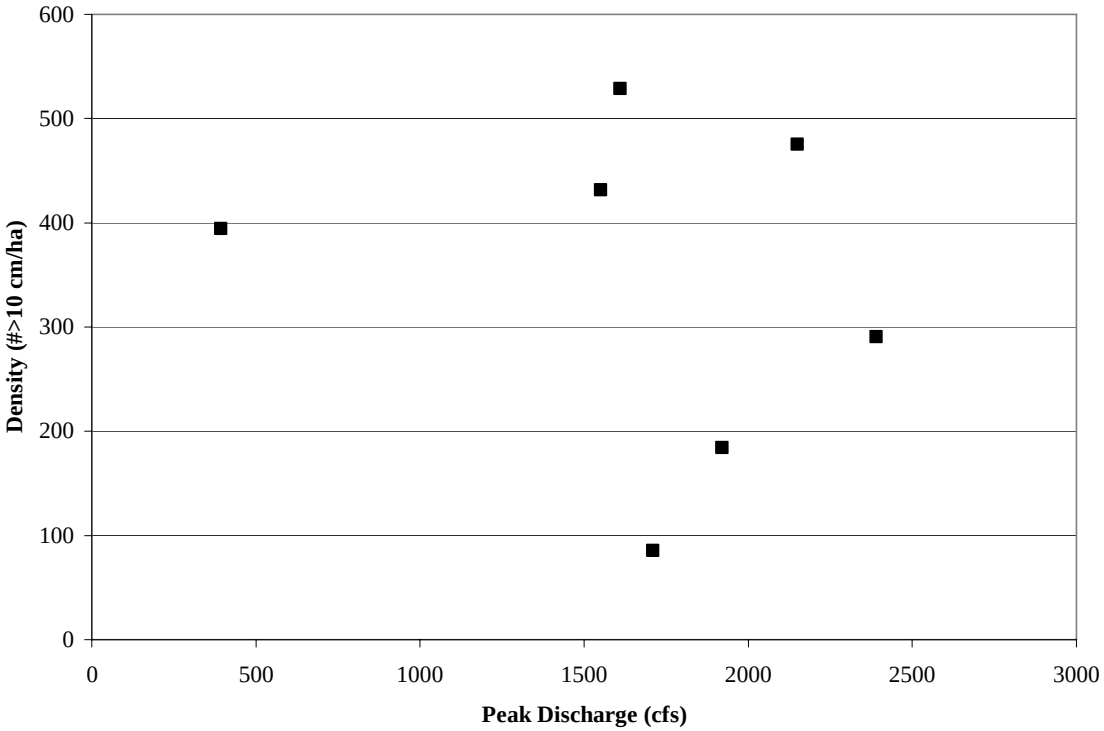


Figure 48a. Brown trout densities (#/ha) in relation to mean chronic Hazard Quotients at the Arkansas River below California Gulch (AR3a).

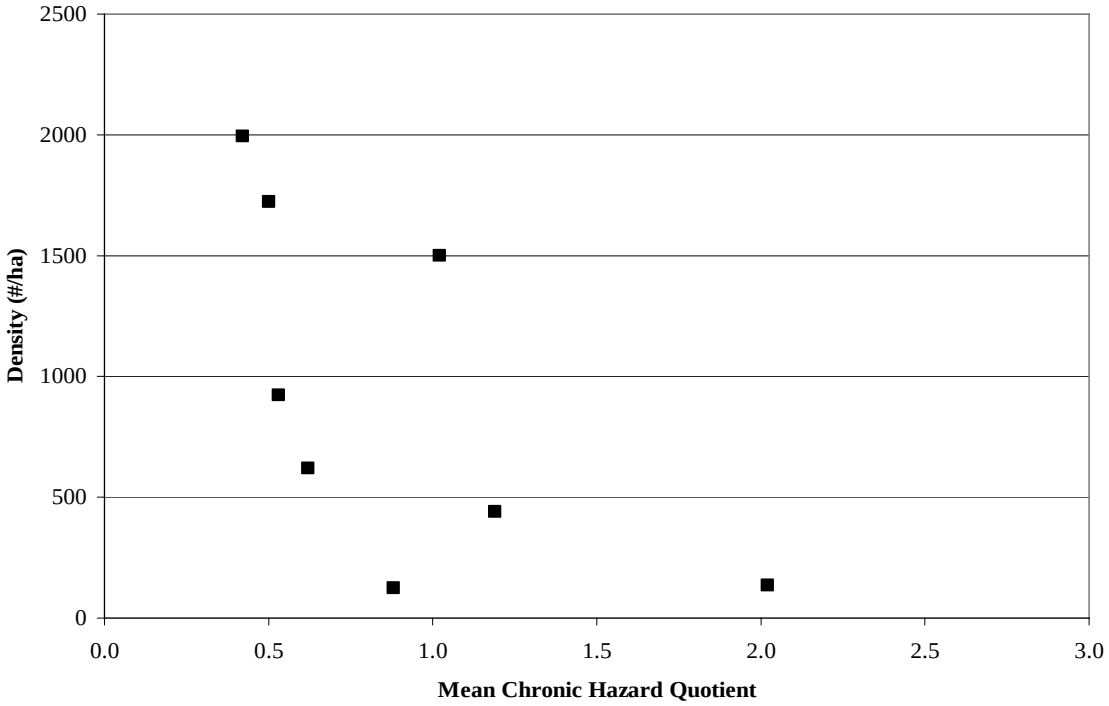


Figure 48b. Brown trout densities (#/ha) in relation to maximum acute Hazard Quotients at the Arkansas River below California Gulch (AR3a).

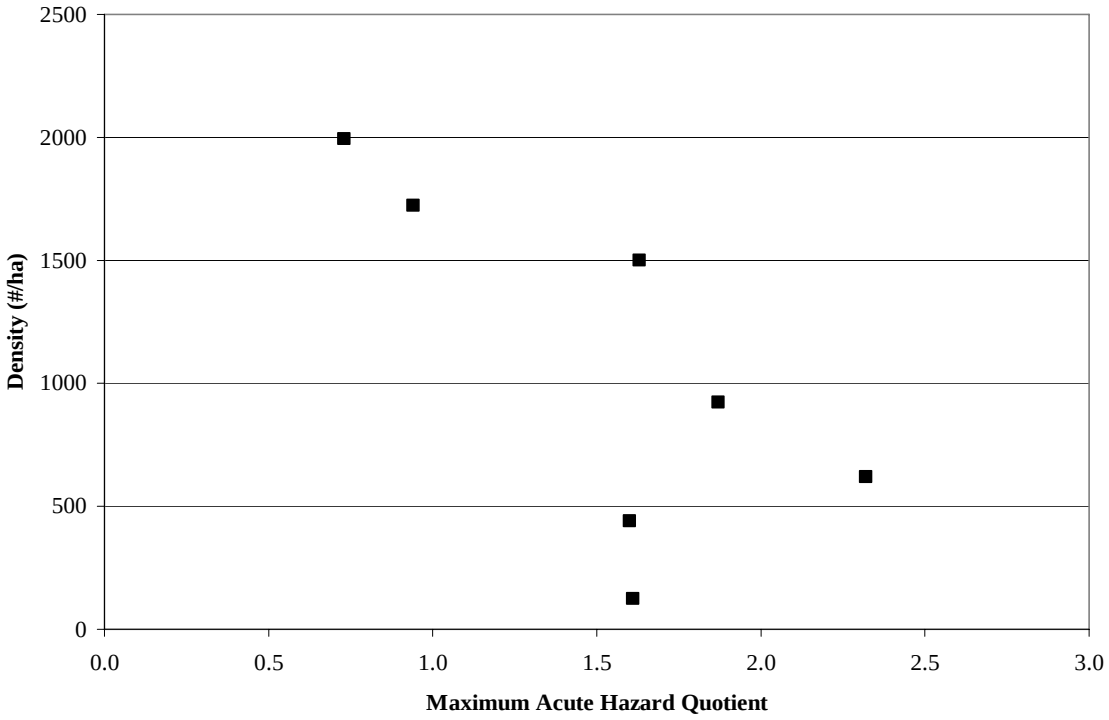


Figure 49a. Brown trout densities (#/ha) in relation to mean chronic Hazard Quotients at the Arkansas River between the Lake Fork and Lake Creek.

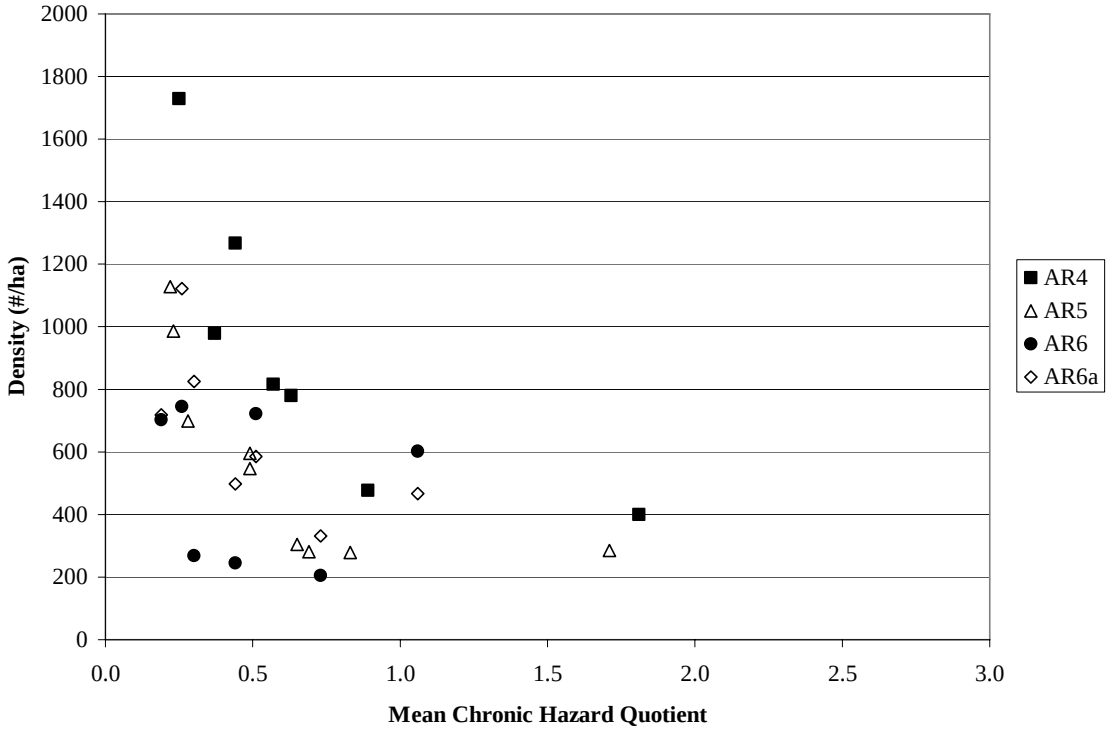


Figure 49b. Brown trout densities (#/ha) in relation to maximum acute Hazard Quotients at the Arkansas River between the Lake Fork and Lake Creek.

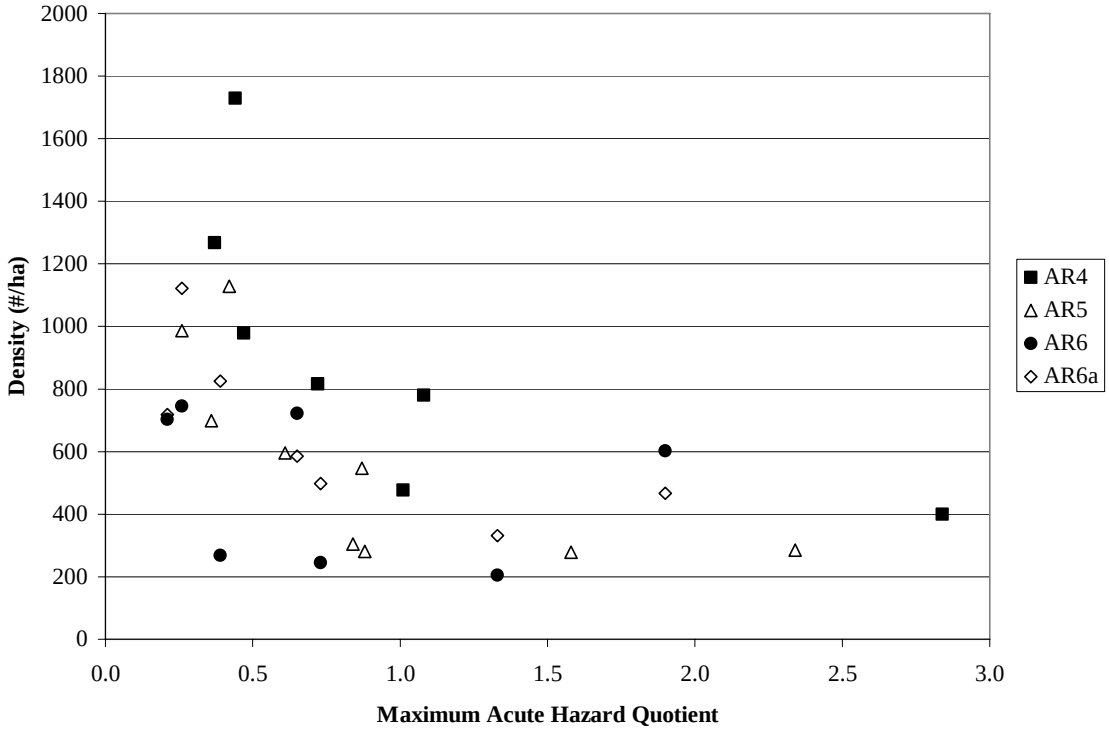


Figure 50a. Brown trout densities (#/ha) in relation to mean chronic Hazard Quotients at the Arkansas River below Lake Creek (AR7).

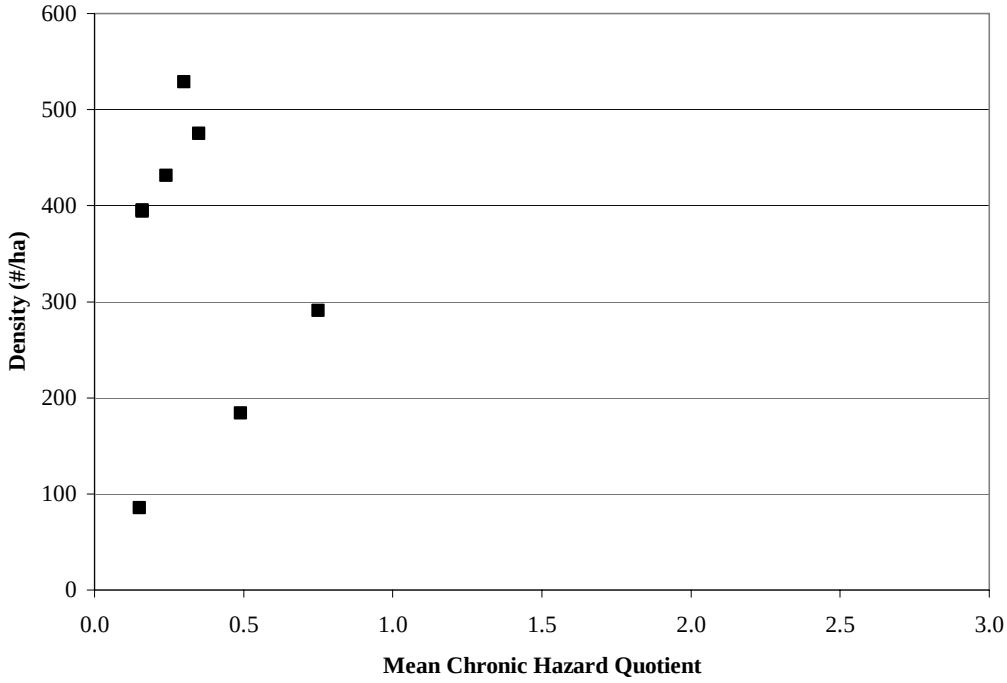


Figure 50b. Brown trout densities (#/ha) in relation to maximum acute Hazard Quotients at the Arkansas River below Lake Creek (AR7).

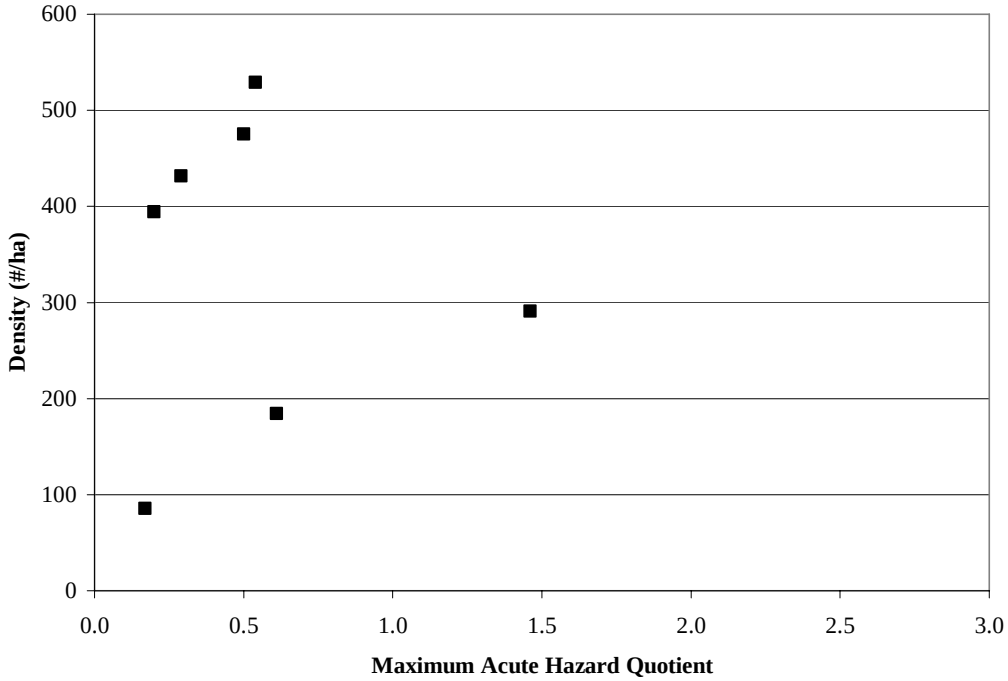


Figure 51. Number of heptagenidae, total abundance, and number of taxa of macroinvertebrates collected at station EF3 1993-2005.

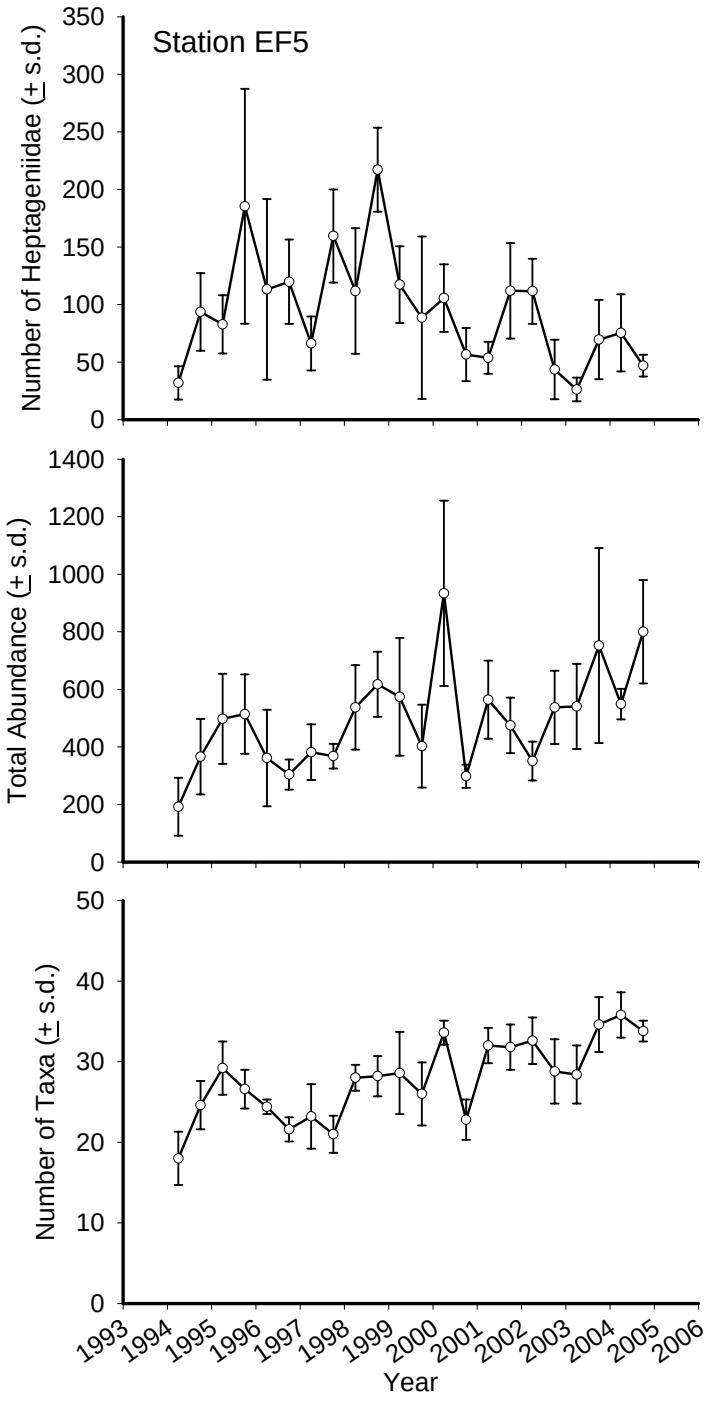


Figure 52. Number of heptagenidae, total abundance, and number of taxa of macroinvertebrates collected at station AR1 1993-2005.

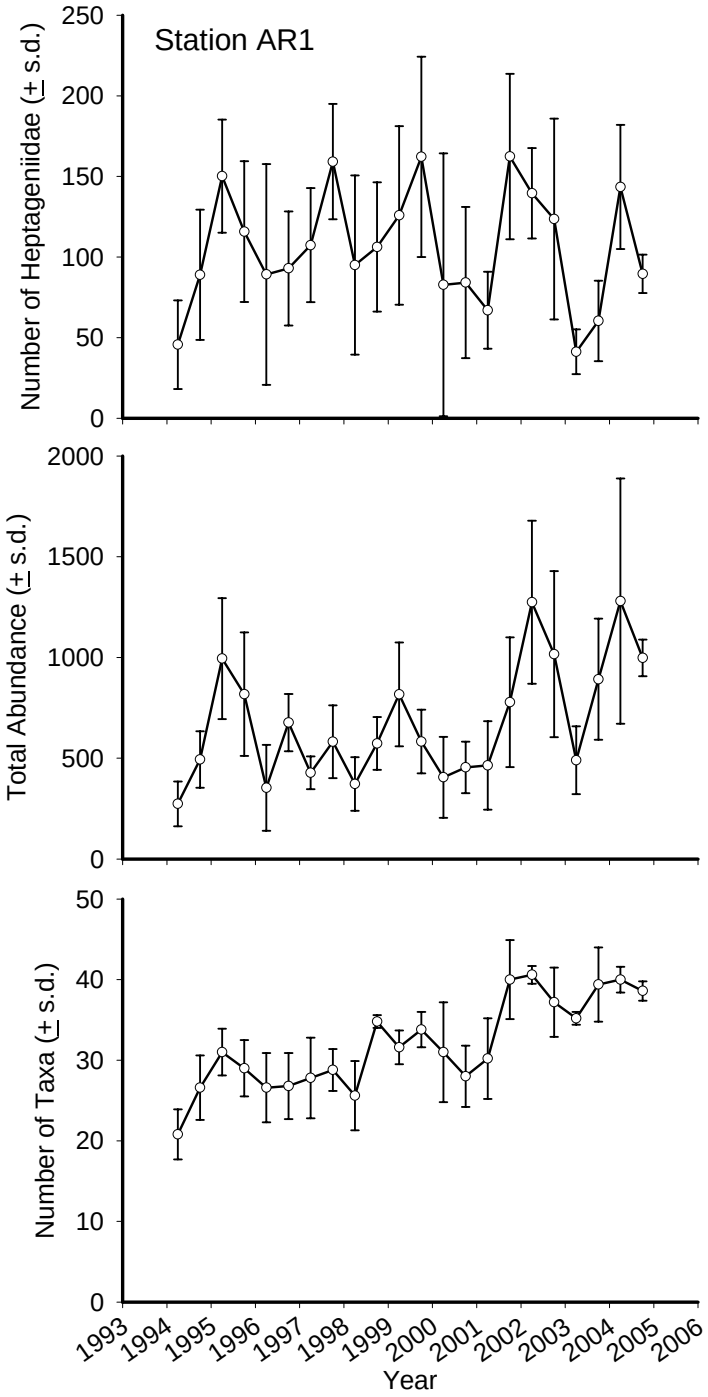


Figure 53. Number of heptagenidae, total abundance, and number of taxa of macroinvertebrates collected at station AR3a 1993-2005.

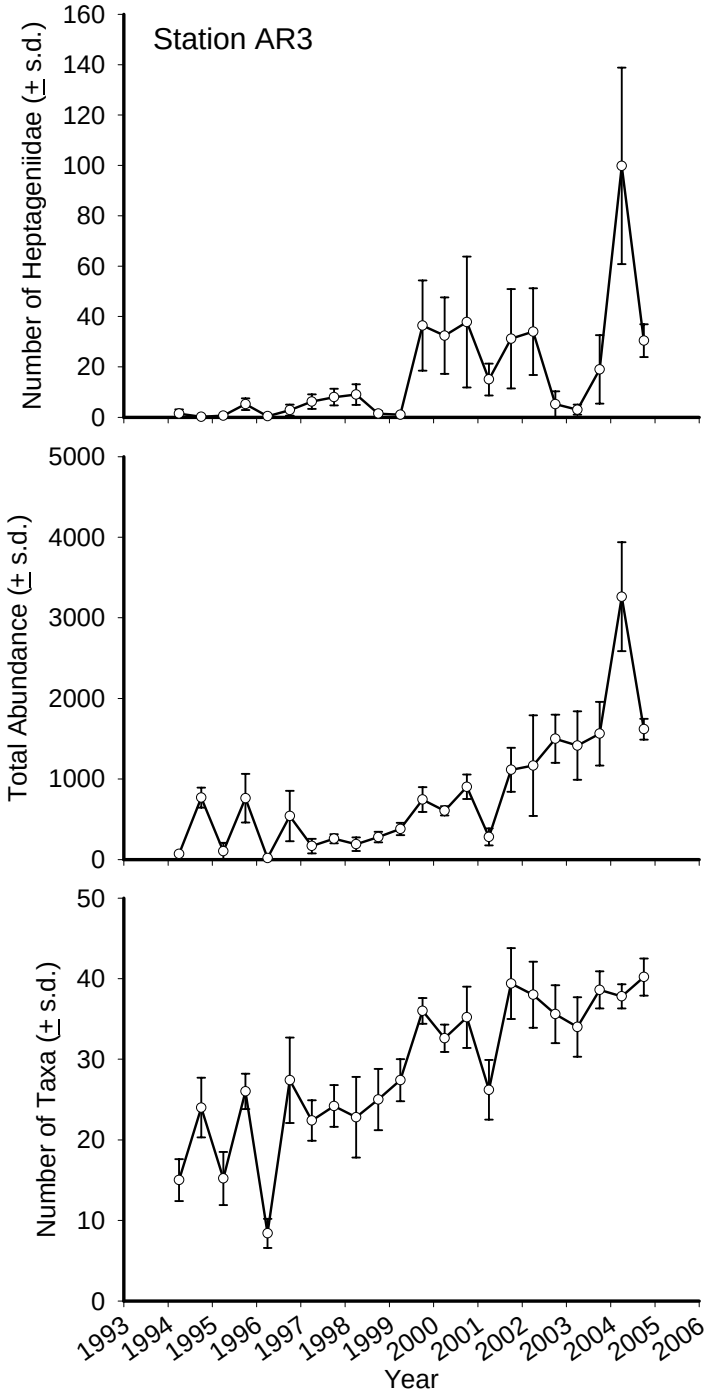


Figure 54. Number of heptagenidae, total abundance, and number of taxa of macroinvertebrates collected at station AR5 1993-2005.

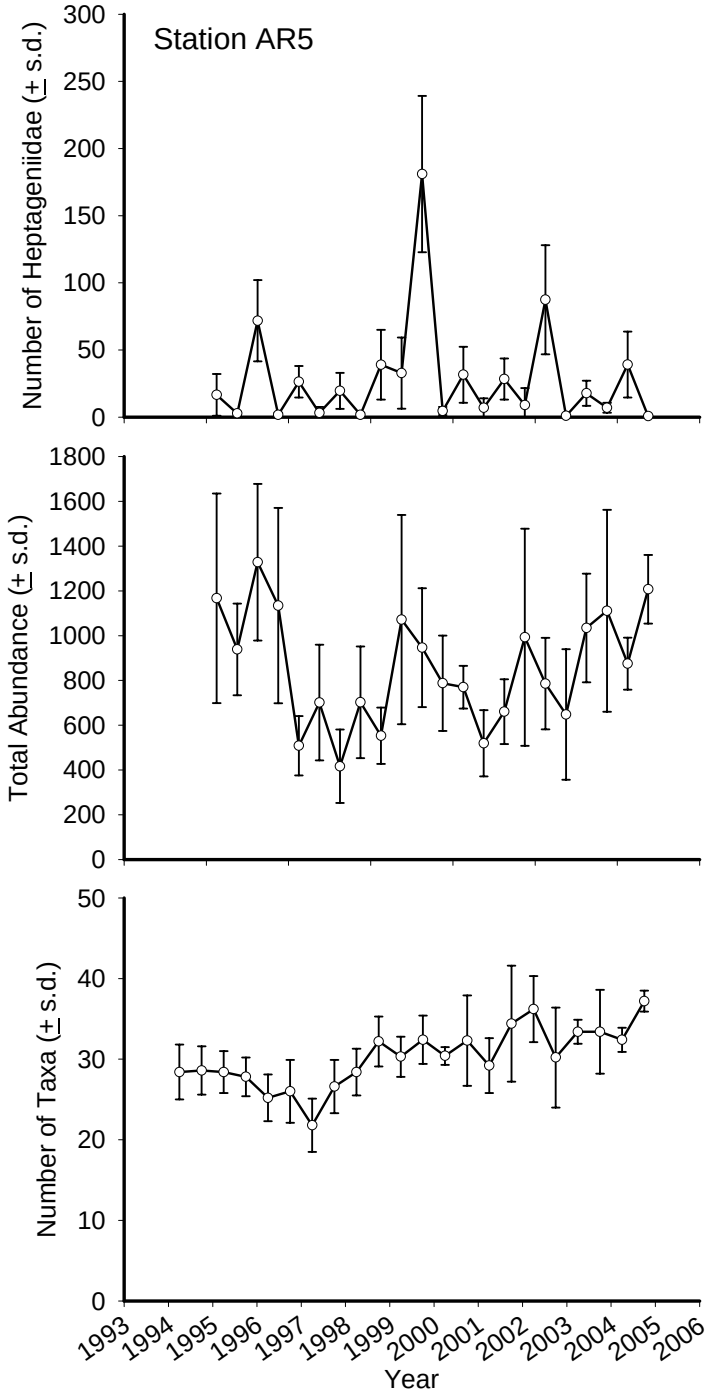


Figure 55. Number of heptagenidae, total abundance, and number of taxa of macroinvertebrates collected at station AR8 1993-2005.

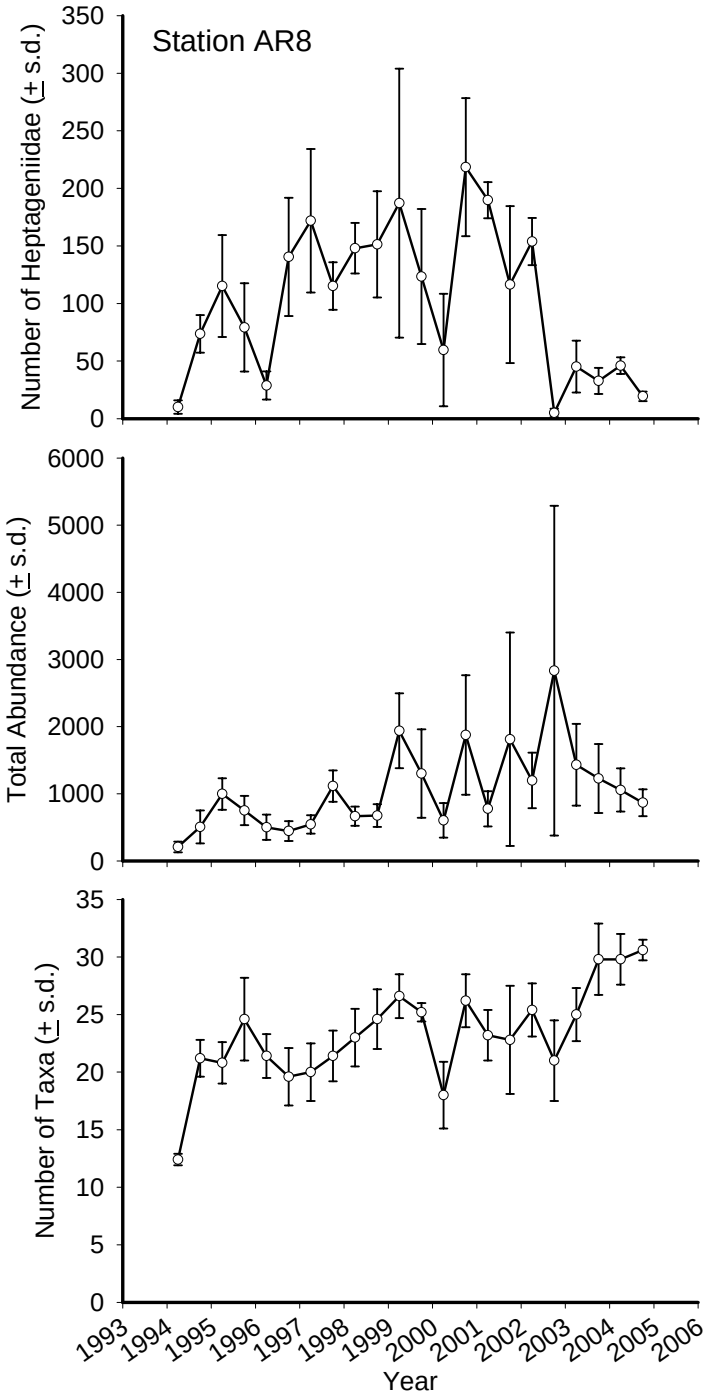


Table 1. Regression analyses of brown trout density (#/ha) as a function of mean spring discharge (Q) for the upper Arkansas River 1994 – 2005.

Station	Type of Fit	Coefficient of determination	P value	Predictive Equation
EF3			p>0.46	Not significant
TC7	Power Function	0.60	p=0.039	$\#/ha=19{,}303*Q^{(-0.649)}$
AR1	Power Function	0.70	p=0.018	$\#/ha=13{,}017*Q^{(-0.491)}$
AR3a	Exponential	0.51	p=0.047	$\#/ha=3391*e^{(-0.0116*Q)}$
AR7			p>0.45	Not significant

Appendix A

Effect of Hardness on the Toxicity of Cadmium to Brown Trout (*Salmo trutta*) Embryos, Larvae, and Fry

Stephen F. Brinkman and Daria L. Hansen

ABSTRACT

Acute and chronic toxicity tests were conducted to determine the toxicity of cadmium (Cd) to different life stages of brown trout at 30, 75, and 150 mg/L water hardness. Increasing water hardness decreased cadmium toxicity. Cadmium was very toxic to post swim-up brown trout fry but embryos and larvae were more tolerant. Median 96 h, lethal concentrations (LC_{50}) were 1.23, 3.90, and 10.1 µg/L, respectively, for swim-up fry exposed to cadmium in 30, 75, and 150 mg/L water hardness. In tests initiated with 30 day post swim-up fry, chronic values (geometric mean of lowest observed effect concentration and no observed effect concentration) were 1.02, 1.83, and 6.54 µg/L at 30, 75, and 150 water hardness, respectively. Chronic values from early life stage (ELS) tests initiated with eyed embryos were 3.52, 6.36, and 13.6 µg/L at 30, 75, and 150 water hardness, respectively. Acclimation during embryo and larval stages is the likely reason for the large differences of chronic values between the ELS and swim-up fry. Cadmium exposure did not affect growth in the ELS tests. A negative impact on growth of swimup fry was detected but was not as sensitive an endpoint as survival. The ratio of Cd exposure concentrations to predicted LC50s can be used to estimate acute mortality of brown trout fry.

INTRODUCTION

An estimated 2080 km of streams in Colorado are impacted by metals (Water Quality Control Division 1988). Cadmium (Cd) is commonly found as a contaminant in the Colorado mineral belt and is often associated with waters impacted by historic mining activities. Brown trout are an important component of Colorado ecosystems in many headwater streams, but their densities are often reduced due to metal contamination (Davies and Woodling 1980). Limited cadmium toxicity data indicate that brown trout is perhaps the most acutely sensitive aquatic species tested (USEPA 2001). Median lethal concentrations (LC_{50}) after 96 hours were 1.4, 2.39 and 1.87 µg/L in water hardnesses of 43.5, 37.6 and 36.9 mg $CaCO_3$/L, respectively (Spehar 1984, Davies and Brinkman 1994). The chronic value was 16.49 µg/L at a water hardness of 250 mg/L $CaCO^3$ from a life cycle test with brown trout (Brown et al. 1994). A brown trout early life stage (ELS) test resulted in a chronic value of 6.67 µg/L at a water hardness of 44 mg/L (Eaton et al.1978). Curiously, hardness-adjusted 96-h LC_{50} values are much lower than chronic values derived from life cycle and ELS tests (USEPA 2001). Life cycle and ELS tests typically start with a tolerant life stage. Acclimation that occurs during a tolerant life stage results in reduced toxicity during a subsequent sensitive life stage (Sinley et al. 1974, Spehar 1976, Davies et al. 2002, Davies et al. 2003). In contrast, acute toxicity tests are usually conducted using unacclimated organisms during a sensitive life stage.

The first objective of this study was to evaluate the acute and chronic toxicity of cadmium to brown trout over an extended range of water hardness. The second objective was to compare toxicity of cadmium in tests initiated with embryo-larval life stages and post swim-up fry. To achieve these test objectives, toxicity tests were conducted using both life stages at water hardnesses of 30, 75 and 150 mg/L.

MATERIAL AND METHODS

Organisms

Brown trout embryos were obtained as newly eyed eggs from the Colorado Division of Wildlife Research Hatchery in Bellevue, Colorado. The source of the eggs was a Colorado Division of Wildlife spawning operation using feral brown trout in the North Delaney Butte Reservoir in Colorado. Ten eggs were placed into each exposure chamber for the ELS tests. Additional eggs were placed in 90 L glass aquaria and hatched and raised for use in the fry toxicity tests. Eggs began hatching about 14 days after initiation of exposure. Brown trout embryos remained as sac fry for approximately 27 days before reaching swim-up stage. Fry were fed appropriately sized trout food (Silver Cup) four times daily (twice daily on weekends and holidays) at an estimated rate of 3% body weight /day upon absorption of the yolk sac. Trout food was supplemented with a concentrated suspension of <24 hr old brine shrimp nauplii (San Francisco brand). The ELS test exposure continued for an additional 14 days post swim-up.

The fry toxicity tests used 34 days post swim-up fry from the same lot of eggs as the ELS tests. Fry were not fed during the initial 96 hours of exposure, but were subsequently fed twice daily (once on weekends and holidays) at an estimated rate of 3% body weight/day. The fry toxicity tests lasted for 30 days.

Exposure Apparatus

Water from an on site well was diluted with either dechlorinated Fort Collins municipal tap water or reverse osmosis water to obtain nominal hardnesses of 30, 75 and 150 mg $CaCO_3$/L (designated 30H, 75H, and 150H, respectively). Consistency of water hardness was maintained using conductivity controllers (Eutech Instruments). Each water hardness supplied identical modified continuous-flow diluters (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components. The diluters delivered five exposures with a 50% dilution ratio, and an exposure control. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 mls/minute each. Exposure chambers consisted of polyethylene containers with a capacity of 2.8 liters. Test solutions overflowed from exposure chambers into water baths which were maintained at 12ºC using temperature-controlled recirculators (VWR Scientific Products). Chemical stock solutions were prepared by dissolving a calculated amount of reagent grade Cadmium sulfate ($CdSO_4$) (Mallinkrodt) in deionized water. The chemical stock solutions were delivered to the diluters via peristaltic pumps (Cole-Parmer model C/L) at a rate of approximately 2.0 mls/minute. New stock solutions were prepared as needed during the toxicity tests. Dim fluorescent lighting provided a 12-h/12-h light-dark photoperiod. Diluters and toxicant flow rates were monitored daily to ensure proper operation. Loading during the ELS test was less than 0.63 g/L of tank volume and less than 0.01 g/L of flow per 24 hrs. During the fry tests, loading never exceeded 2.2 g/L of tank volume and was less than 0.11 g/L of flow per 24 hrs. Loading was well below suggested maximum levels (ASTM 1997).

ELS Test Methods

The number of hatched eggs and mortality of eggs and fry were monitored and recorded daily. Dead fry were blotted dry with a paper towel and total length (to the nearest mm) and weight (to the nearest 0.001 g) measured and recorded. At the end of

the tests, surviving fish from each exposure chamber were terminally anesthetized, blotted dry with a paper towel and total lengths and weights measured and recorded.

Water quality characteristics of exposure water were measured weekly in all treatment levels within a replicate. Different replicates were selected each week for sampling. Hardness and alkalinity were determined according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was used to measure pH and conductivity. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. The conductivity, pH and dissolved oxygen meters were calibrated prior to each use.

Water samples for cadmium analyses were collected weekly from each exposure level with surviving fry. Exposure water was passed through a 0.45 µm filter (Acrodisc), collected in disposable polystyrene tubes (Falcon), and immediately preserved with Ultrex triple distilled nitric acid (JT Baker) to pH <2. Water samples were analyzed using a SH4000 atomic absorption spectrometer with CTF 188 graphite furnace (Thermo Jarrell Ash) and Smith-Hieftje background correction. Dibasic ammonium phosphate (0.1%) was used as a matrix modifier. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard (High Purity Standards, Charleston SC). Sample splits and spikes were collected and analyzed to verify analytical reproducibility and recovery. The cadmium detection limit was < 0.08 µg/L.

Fry Test Methods

Brown trout fry experiments utilized the same exposure apparatus as the ELS tests. Test methods were identical with the following exceptions. Water quality characteristics were determined daily and cadmium concentrations were measured three times during the initial 96h. Fry were not fed during the initial 96 h of exposure but were fed twice daily thereafter (once on weekends). Cadmium exposure lasted for a total of 30 d.

Statistical Analyses

Statistical analyses were conducted using Toxstat version 3.5 software (West Inc. 1996). Analysis of variance (ANOVA) was used to test toxicity endpoints which included hatching success, fry and swim-up survival, biomass at the end of the test, mean time to hatch, and lengths and weights of surviving fish at test termination. Hatching success and survival data were arcsine square root transformed prior to ANOVA (Snedecor and Cochran 1980). Normality and homogeneity of variances were tested using Chi-square and Levene's test, respectively (Weber et al., 1989). Treatment means were compared to the control using William's one-tailed test (Williams 1971, Williams 1972) or Dunnett's one-tailed test (Dunnett 1955, Dunnett 1964), both at $p<0.05$. Steel's Many-One Rank Test was used to compare treatment means when data sets failed assumptions of normality or homogeneity of variance (Weber et al. 1989). The highest cadmium concentration not associated with a treatment effect (e.g. decreased survival, decreased body weight) was designated as the no-observed-effect concentration (NOEC). The lowest concentration of cadmium associated with a treatment effect was designated as the lowest-observed-effect concentration (LOEC). Chronic values were calculated as the geometric mean of the LOEC and NOEC. The inhibition concentration (IC_{20}), the concentration estimated to cause a 20% reduction in organism performance compared with the control (USEPA 1993), was calculated using the combined weight of surviving

organisms from each treatment (biomass or standing crop). Ninety six hour median lethal concentrations (LC_{50}) were estimated by the Trimmed Spearman-Karber technique (Hamilton et al. 1977, 1978) using log transformed cadmium concentrations and 10% trim. The Statistical Analysis System (SAS) Proc Genmod was used for the regression of mortality with hazard quotient.

RESULTS

Hardness of the 30H, 75H and 150H test waters were near target levels (Table A1). Low standard deviations indicated that the water quality characteristics were consistent over the course of the experiments. Temperatures were near or slightly below the 12ºC target. Dissolved oxygen was near saturation. Water quality characteristics were similar between the ELS and Fry tests with the exception of pH which was consistently greater in the ELS tests.

30 mg/L $CaCO_3$ Hardness ELS

Mean time to hatch, hatching success and sac fry survival were not significantly affected by exposure concentrations used. Hatching success exceeded 80% in all treatments. Little mortality occurred during the sac fry stage. Metal-related mortality occurred shortly after fry began exogenous feeding. Survival of swim-up fry was significantly impaired at 4.87 µg/L (Table A2). Based on survival, the no observed effect concentration (NOEC) was 2.54 µg/L and the lowest observed effect concentration was 4.87 µg/L. The chronic value was 3.52 µg/L in the 30 mg/L $CaCO_3$ Hardness ELS test. The highest cadmium concentration appeared to have reduced growth, as measured by lengths and weights of surviving fry, however, this was not significant (Table A2). Mean biomass at termination of the 30 hardness ELS test was significantly reduced at 4.87 (LOEC) but not at 2.54 µg/L (NOEC). The chronic value for the 30 mg/L $CaCO_3$ hardness test was 3.52 µg/L based on biomass. The IC_{20} based on biomass at test termination was 2.22 µg/L. A summary of endpoints for all tests is presented in Table A8.

75 mg/L $CaCO_3$ Hardness ELS

Mean time to hatch, hatching success and sac fry survival were unaffected by cadmium exposure. Metal related mortality was not observed until the yolk sac was absorbed and fry began exogenous feeding and then, only in the highest cadmium concentration tested (8.64 µg/L) which was the LOEC (Table A3). The NOEC was 4.68 µg/L for a chronic value of 6.36 µg/L. Growth was unaffected but biomass was significantly reduced at the highest exposure concentration (Table A3). The chronic values based on biomass and reduced survival were the same. The IC_{20} based on biomass at test termination was 4.71 µg/L.

150 mg/L $CaCO_3$ Hardness ELS

Mean time to hatch, hatching success and sac fry mortality were not significantly affected by cadmium. Survival was significantly reduced at 19.1 but not 9.62 µg Cd/L (LOEC and NOEC, respectively) (Table A4). The chronic value was 13.56 µg Cd/L. Effects of cadmium on growth were not detected. The LOEC, NOEC and chronic value

based on a reduction in biomass were the same as those based on swim-up fry survival. The IC_{20} based on biomass at test termination was 13.6 µg/L.

30 mg/L $CaCO_3$ Hardness FRY

No mortality occurred in the control and lowest exposure concentration during the 96 h acute exposures (Table A5). Mortality increased with increasing cadmium concentration resulting in complete mortality at 5.64 µg/L, the highest concentration. The 96 h median lethal concentration (LC_{50}) for the 30 hardness test was 1.23 µg/L. After the initial 96 h, low levels of mortality occurred in the 0.42µg/L and 0.74 µg/L concentrations. The concentration-response relationship indicated that the mortality in these lower concentrations may be metal-related, however the mortality was not significant at the 0.05 level. Growth, measured by length and weight at test termination was decreased in the single fish surviving at 2.72 µg/L (Table A5). Biomass was significantly reduced at 1.40 µg/L (LOEC) but not 0.74 µg/L (NOEC). The chronic value was 1.02 µg/L based on biomass. The IC_{20} based on biomass at test termination was 0.87 µg/L.

75 mg/L $CaCO_3$ Hardness FRY

Exposure to 8.86 µg/L for 96 h resulted 97.5% mortality (Table A6). During the initial 96 hours, there was no mortality of fry exposed to ≤1.30 µg Cd/L, though minimal mortality occurred by 30 d. The 96-h LC_{50} was 3.90 µg/L. The LOEC was 2.58 µg/L which resulted in 30% mortality in 30 days. Mortality after the initial 96 h was very low and probably not metal-related. NOEC based on 30d mortality was 1.30 µg/L which resulted in a chronic value of 1.83 µg/L. Weights and lengths at test termination were significantly reduced at 4.49 µg/L and 8.86 µg/L, respectively. Reduction of growth was not as sensitive an endpoint as mortality or biomass. The LOEC and NOEC based on biomass were the same as those based on mortality resulting in a chronic value of 1.83 µg/L. The IC_{20} based on biomass at test termination was 2.18 µg/L.

150 mg/L $CaCO_3$ Hardness FRY

All trout exposed for 96 h to cadmium concentrations as high as 4.81 µg/L survived, whereas fish exposed to 8.88 µg/L and 16.4 µg/L had survival rates of 62.5 and10%, respectively (Table A7). The 96 h LC_{50} at 150 mg/L $CaCO_3$ hardness was 10.1 µg/L. Mortality after the initial 96 h was low, 2.5% to 7.5%. Survival of trout exposed to 8.88 µg/L for 30 d was significantly lower than the control (LOEC) but was unaffected at 4.81 µg/L (NOEC). The chronic value based on mortality was 6.54 µg/L. Effects of cadmium exposure on growth were not detected at the highest concentration, 16.4 µg/L, in which near complete mortality occurred (Table A7). Biomass was significantly reduced at the highest two exposure concentrations, primarily as a result of significant mortality. The LOEC, NOEC and chronic value based on biomass was the same as those based on mortality. The IC_{20} based on biomass at test termination was 6.62 µg/L.

DISCUSSION

ELS tests were terminated after 41 days post hatch (14 days post swimup) due to a water line break leading to the Colorado Division of Wildlife Aquatic Toxicology Laboratory. Recommended duration of salmonid ELS tests is 60 days post hatch (USEPA 1985) or at least 30 days post swim-up (ASTM 1997). Often, a majority of metal-related

mortality occurs shortly after swim-up and it is unlikely that significant additional mortality would have taken place had the test continued for an additional 20 days. ELS test results may be suspect if significant mortality occurs near the end of the test (USEPA 1985). If the ELS tests were continued, it is possible that negative effect on growth could have been detected.

Cadmium exposure to brown trout eggs did not affect mean time to hatch. This result differs from zinc exposures which increased time to hatch of brown trout eggs at relatively low concentrations (Davies et al. 2002, Davies et al. 2003). Hatching success and sac fry survival were unaffected by the cadmium concentrations used in the ELS tests. Egg and sac fry life stages of salmonids are generally more tolerant to metal exposure than the subsequent swim-up fry stage (Chapman 1978, Van Leeuwen et al. 1985). Metal-related mortality in the ELS tests occurred shortly after brown trout embryos reached swim-up stage and began exogenous feeding. No effect of cadmium exposure on growth was detected in any of the ELS tests. In contrast, growth in the fry tests were detected at 30 mg/L $CaCO_3$ and 75 mg/L $CaCO_3$ but not 150 mg/L $CaCO_3$. Concentrations of cadmium that negatively impacted growth were greater than those that reduced survival.

Survival and biomass at test termination were equally sensitive at detecting effects of cadmium. Overall, the most sensitive endpoint was the $IC_{20.}$ The inhibitory concentration (IC) is interpolated from a dose-response relationship and provides an estimate of a reduction of biological performance, in this case a reduction of 20% biomass. Biomass at test termination reflects effects of exposure on both survival and growth. Chronic values based on NOEC and LOEC are determined using hypothesis testing and can be influenced by selection of exposure concentrations and variability of the data set. Furthermore, chronic values provide little information on the magnitude of the effect at the LOEC. For fry but not ELS tests, the IC_{20} and the chronic value based on biomass were in close agreement. In contrast, chronic values from the 30 and 75 mg/L $CaCO_3$ hardness ELS tests were considerably greater than the corresponding IC_{20} values. High variability inherent to ELS tests may decrease statistical power to detect reduced survival or biomass.

Chronic endpoints of the ELS tests are consistently greater than the fry and exceeded 96-h LC_{50} values. Exposure of test organisms during cadmium-tolerant egg and larval stages may have resulted in acclimation. Consequently, exposed organisms were more tolerant to lethal effects during the subsequent sensitive fry stage (Sinley et al. 1974, Spehar 1976, Davies et al. 2003).

Chronic water quality criteria are derived from life-cycle, partial life cycle or ELS tests (USEPA 1985). Results from this study found chronic values from the ELS tests were much greater than those from tests initiated with fry and even exceeded 96 h LC_{50}s. This finding is consistent with the cadmium criteria document which reports a Species Mean Acute Value of 1.613 µg/L and a much higher Species Mean Chronic Value of 5.004 µg/L (USEPA 2001). The chronic value was derived from a life cycle test where exposure was initiated with sexually mature adults followed by fertilized eggs, both of which are tolerant life stages where acclimation could occur. Guidelines for deriving water quality criteria require protection of all life stages of an organism (USEPA 1985).

However, chronic criteria derived from tests where acclimation occurred may not protect sensitive life stages. The guidance document notes that an acute-chronic ratio (ACR) less than 2 is probably due to acclimation during the chronic test. In such cases, an ACR of 2 is assumed because acclimation and continuous exposure in field situations cannot be assured.

Acute toxicity of cadmium decreased as hardness increased (Figure A1). Hardness and 96 h LC_{50} values exhibited a ln-ln relationship (USEPA 1985) with a correlation coefficient of 0.95. Included in the regression are LC_{50} values from three previous studies (1.4, 2.39 and 1.87 µg/L at water hardnesses of 43.5, 37.6 and 36.9 mg $CaCO_3$/L, respectively) (Spehar 1984, Davies and Brinkman 1994). The equation estimating the brown trout Cd LC_{50} based on water hardness is:

$$\text{Brown Trout Cd } LC_{50} = e^{(1.258*(\ln(\text{hardness}))-3.999)}$$

Dividing a hardness-adjusted LC_{50} by a factor of 2 can be expected to protect against acute toxicity (USEPA 1985). Therefore a hardness-based equation for protection of brown trout against acute exposures is:

$$\text{Brown trout Acute Cd} = e^{(1.258*(\ln(\text{hardness}))-4.692)}$$

ELS Chronic values from this study and previously reported values from ELS and Life-Cycle tests (Eaton et al. 1978, Brown et al. 1994) show decreasing chronic toxicity with increasing hardness (Figure A2). The ln-ln regression of the ELS chronic values including an ELS test at 44 mg/L (Eaton et al. 1978) and life-cycle test at 250 mg/L hardness (Brown et al. 1994) is a good fit with a correlation coefficient of 0.97. The equation describing the regression line for the ELS/Life Cycle tests is:

$$\text{Brown Trout ELS/Life Cycle Chronic Cd} = e^{(0.7033*(\ln(\text{hardness}))-1.017)}$$

Cadmium concentrations predicted from this equation could be expected to protect brown trout in instances where exposure is constant. Brown trout from clean tributaries or upstream of a cadmium source would not be protected if they migrate into or are washed into contaminated reaches. Brown trout can lose acclimation to metals once exposure to metals is discontinued (Gasser 1998, Davies and Brinkman 1999, Davies et al. 2002). Migration into a clean tributary could lead to a loss of acclimation followed by toxicity on return to a contaminated stream reach. Loss of acclimation could also occur during spring runoff when dilution from spring snowmelt substantially reduces metal concentrations in streams.

Fry chronic values and one from a previous study initiated with post swim-up fry (Davies and Brinkman 1994) are clearly lower than chronic values derived from ELS and life-cycle tests (Figure A2). The equation describing the regression for the fry tests (correlation coefficient=0.97) is

$$\text{Brown Trout Fry Chronic Cd} = e^{(1.093*(\ln(\text{hardness}))-3.734)}$$

Cadmium concentrations predicted by this equation can be expected to protect brown trout fry that are unacclimated or haven't experienced prior exposure to Cd.

The equations described above can be used to normalize cadmium exposure concentrations on the basis of water hardness. Hazard quotient (HQ) for brown trout are expressed as the ratio of exposure concentration and the hardness-based brown trout acute equation. Percent mortality plotted against the HQ exhibits a characteristic sigmoid-shaped curve (Figure A3). Mortality data from the three tests reported here as well as two previous tests (Davies and Brinkman 1994) are included. The fit of the curve is reasonable considering the range of hardness (30-150 mg/L) and size of organisms (0.48-7g). Exposure concentrations and associated mortality were not reported by Spehar and Carlson (1984) and consequently were not used in the regression. That particular study is represented in Figure A3 as a single point with 50% mortality at the reported LC_{50} divided by the hardness-predicted LC_{50}. The equation for the line relating Cd HQ and brown trout mortality is

$$96 \text{ hour Brown Trout Mortality } (\%) = 100/(1+e^{(-2.4011*HQ-5.067)})$$

Figure A3 and the associated regression equation can be used to predict brown trout mortality if the Cd concentrations and hardness are known. Alternatively, hardness–based concentrations of Cd can be calculated for the protection of brown trout, based on an acceptable level of mortality. An HQ value of 1, the level predicted to be protective of brown trout, mortality after 96 hours is expected to be 6.5%.

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Table A1. Mean of water quality characteristics of exposure water during ELS and Fry toxicity tests. Standard deviations are in parentheses.

	ELS			Fry		
	30 H	75 H	150 H	30 H	75 H	150 H
Hardness (mg $CaCO_3$/L)	30.6 (2.1)	71.3 (2.7)	149 (7)	29.2 (0.9)	67.6 (1.5)	151 (2)
Alkalinity (mg $CaCO_3$/L)	22.9 (1.3)	51.5 (1.6)	107 (5)	21.7 (0.8)	47.9 (1.1)	107 (2)
pH (S.U.)	7.72 (0.12)	7.75 (0.14)	7.83 (0.14)	7.54 (0.13)	7.60 (0.10)	7.51 (0.12)
Temperature (ºC)	11.6 (0.4)	12.0 (0.3)	11.8 (0.4)	11.7 (0.1)	11.4 (0.2)	11.8 (0.4)
Conductivity (µS/cm)	52.9 (2.0)	123 (5)	255 (8)	51.5 (0.5)	115 (2)	260 (2)
Dissolved Oxygen (mg/L)	8.49 (0.58)	8.61 (0.67)	8.32 (0.64)	8.61 (0.22)	8.88 (0.17)	8.58 (0.14)

Table A2. Mean dissolved cadmium concentrations (µg/L) and associated survival (%), mean lengths (mm) and weights (g) and biomass (g) of ELS brown trout exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.03)	0.40 (0.04)	0.69 (0.05)	1.31 (0.08)	2.54 (0.22)	4.87 (0.56)
Survival (%)	80.0 (0.0)	82.5 (12.6)	67.5 (9.6)	67.5 (15.0)	65.0 (12.9)	15.0* (17.3)
Length at termination (mm)	28.0 (0.7)	28.1 (0.6)	27.3 (1.5)	27.4 (0.6)	28.0 (0.3)	26.3 (1.0)
Weight at termination (g)	0.170 (0.008)	0.167 (0.003)	0.163 (0.008)	0.167 (0.012)	0.168 (0.008)	0.148 (0.001)
Biomass (g)	1.360 (0.067)	1.378 (0.217)	1.101 (0.159)	1.111 (0.181)	1.087 (0.190)	0.222* (0.256)

*Significantly less than control ($p<0.05$)

IC_{20} (95% Confidence Interval) = 2.22 µg/L (0.61-2.75)

Table A3. Mean dissolved cadmium concentrations (µg/L) and associated survival (%), mean lengths (mm) and weights (g) and biomass (g) of ELS brown trout exposed in 75 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.03)	0.60 (0.05)	1.13 (0.09)	2.46 (0.28)	4.68 (0.17)	8.64 (0.98)
Survival (%)	82.5 (12.6)	77.5 (18.9)	67.5 (12.6)	77.5 (9.6)	72.5 (20.6)	12.5* (12.6)
Length at termination (mm)	28.7 (1.1)	28.6 (0.5)	28.6 (0.7)	28.8 (1.1)	27.8 (0.7)	28.7 (0.6)
Weight at termination (g)	0.183 (0.023)	0.172 (0.012)	0.177 (0.012)	0.183 (0.019)	0.167 (0.012)	0.189 (0.007)
Biomass (g)	1.499 (0.192)	1.349 (0.371)	1.190 (0.187)	1.403 (0.083)	1.207 (0.375)	0.233* (0.230)

*Significantly less than control ($p<0.05$)

IC_{20} (95% Confidence Interval) = 4.71 µg/L (0.95-5.46)

Table A4. Mean dissolved cadmium concentrations (µg/L) and associated survival (%), mean lengths (mm) and weights (g) and biomass (g) of ELS brown trout exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.08)	1.30 (0.14)	2.95 (0.32)	5.47 (0.40)	9.62 (0.79)	19.1 (2.3)
Survival (%)	80.0 (11.5)	90.0 (8.2)	80.0 (8.2)	85.0 (5.8)	90.0 (8.2)	57.5* (17.1)
Length at termination (mm)	28.2 (0.2)	27.7 (0.9)	27.4 (0.5)	27.7 (0.6)	27.1 (0.5)	27.4 (0.2)
Weight at termination (g)	0.168 (0.008)	0.168 (0.005)	0.161 (0.011)	0.165 (0.011)	0.158 (0.008)	0.154 (0.007)
Biomass (g)	1.35 (0.26)	1.52 (0.16)	1.20 (0.08)	1.40 (0.10)	1.42 (0.18)	0.88* (0.25)

*Significantly less than control ($p<0.05$)

IC_{20} (95% Confidence Interval) = 13.6 µg/L (10.8-17.3)

Table A5. Mean dissolved cadmium concentrations (µg/L) and associated acute and 30 day survival (%), lengths (mm), weights (g) and biomass (g) of brown trout fry exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.04)	0.42 (0.05)	0.74 (0.08)	1.40 (0.14)	2.72 (0.23)	5.64 (0.12)
96 hr Survival (%)	100 (0)	100 (0)	97.5 (5.0)	32.5 (15.0)	2.5 (5.0)	0 (0)
30 day Survival (%)	100 (0)	90.0 (8.2)	87.5 (9.6)	32.5* (15.0)	2.5* (5.0)	0* (0)
Length (mm)	39.1 (0.9)	39.8 (0.8)	40.1 (1.0)	40.4 (1.7)	34* --[1]	--
Weight (g)	0.584 (0.019)	0.611 (0.031)	0.612 (0.043)	0.637 (0.088)	0.320* --[1]	--
Biomass (g)	5.84 (0.19)	5.51 (0.57)	5.32 (0.25)	2.08* (0.98)	0.08* (0.16)	0.00* (0.00)

*Significantly less than control ($p<0.05$)

96 hour LC_{50} (95% C.I.) = 1.23 µg Cd/L (1.09-1.38)

IC_{20} (95% Confidence Interval) = 0.87 µg/L (0.82-0.93)

[1]Single surviving fish

Table A6. Mean dissolved cadmium concentrations (µg/L) and associated acute and 30 day survival (%), lengths (mm), weights (g) and biomass (g) of brown trout fry exposed in 75 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.04)	0.69 (0.09)	1.30 (0.16)	2.58 (0.24)	4.49 (0.32)	8.86 (0.75)
96 hr Survival (%)	100 (0)	100 (0)	100 (0)	80.0 (14.1)	35.0 (12.9)	2.5 (5.0)
30 day Survival (%)	92.5 (9.6)	95.0 (5.8)	97.5 (5.0)	70.0* (11.5)	35.0* (12.9)	2.5* (5.0)
Length (mm)	41.0 (1.2)	40.2 (0.8)	40.0 (0.4)	40.6 (0.5)	38.9 (1.3)	38* --[1]
Weight (g)	0.654 (0.066)	0.614 (0.034)	0.602 (0.010)	0.610 (0.023)	0.544* (0.046)	0.490* --[1]
Biomass (g)	6.01 (0.39)	5.82 (0.14)	5.87 (0.26)	4.27* (0.71)	1.94* (0.86)	0.12* (0.24)

*Significantly less than control ($p<0.05$)

96 hour LC_{50} (95% C.I.) = 3.90 µg Cd/L (3.39-4.48)

Table A7. Mean dissolved cadmium concentrations (µg/L) and associated acute and 30 day survival (%), lengths (mm), weights (g) and biomass (g) of brown trout fry exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.05)	1.01 (0.07)	2.44 (0.18)	4.81 (0.36)	8.88 (0.52)	16.4 (1.5)
96 hr Survival (%)	100 (0)	100 (0)	100 (0)	100 (0)	62.5 (5.0)	10.0 (8.2)
30 day Survival (%)	97.5 (5.0)	97.5 (5.0)	97.5 (5.0)	97.5 (5.0)	55.0* (5.8)	7.5* (5.0)
Length (mm)	39.6 (0.7)	40.4 (0.7)	39.9 (1.5)	40.2 (0.8)	40.5 (1.3)	40.3 (0.6)
Weight (g)	0.614 (0.012)	0.621 (0.042)	0.607 (0.033)	0.605 (0.026)	0.617 (0.072)	0.596 (0.013)
Biomass (g)	5.99 (0.41)	6.04 (0.16)	5.91 (0.10)	5.89 (0.18)	3.37* (0.30)	0.447* (0.30)

*Significantly less than control ($p<0.05$)

96 hour LC_{50} (95% C.I.) = 10.1 µg Cd/L (8.95-11.4)

Table A8. Endpoints and associated chronic values (µg/L) of cadmium toxicity tests conducted with brown trout ELS and fry in 30, 75 and 150 mg/L water hardness.

	30 Hardness		75 Hardness		150 Hardness	
	ELS	Fry	ELS	Fry	ELS	Fry
Time to Hatch	>4.87	--	>8.64	--	>19.1	--
Hatch Success	>4.87	--	>8.64	--	>19.1	--
Sac Fry Survival	>4.87	--	>8.64	--	>19.1	--
Swim-up Fry Survival	3.52	1.02	6.36	1.83	13.6	6.54
Length	>4.87	1.95	>8.64	6.31	>19.1	>16.4
Weight	>4.87	1.95	>8.64	3.40	>19.1	>16.4
Biomass	3.52	1.02	6.36	1.83	13.6	6.54
IC_{20}	2.22	0.87	4.01	2.18	13.6	6.62
LC_{50}	--	1.23	--	3.90	--	10.1

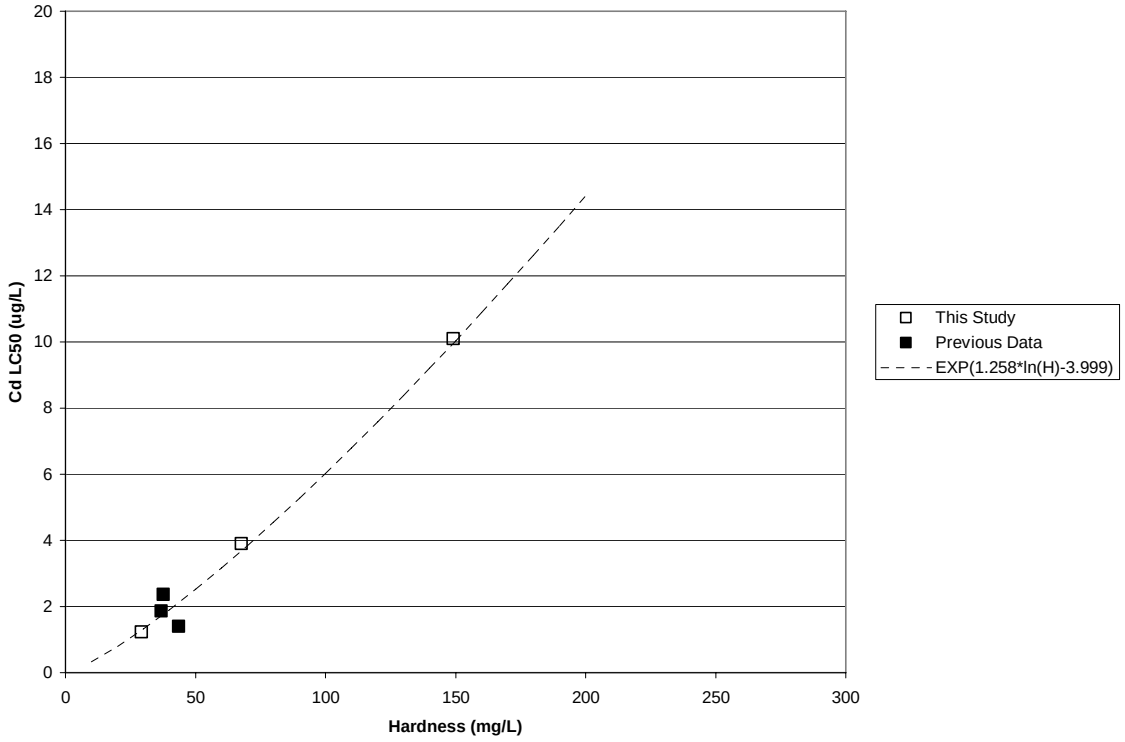

Figure A1. Brown trout Cd LC50 values at different water hardnesses.

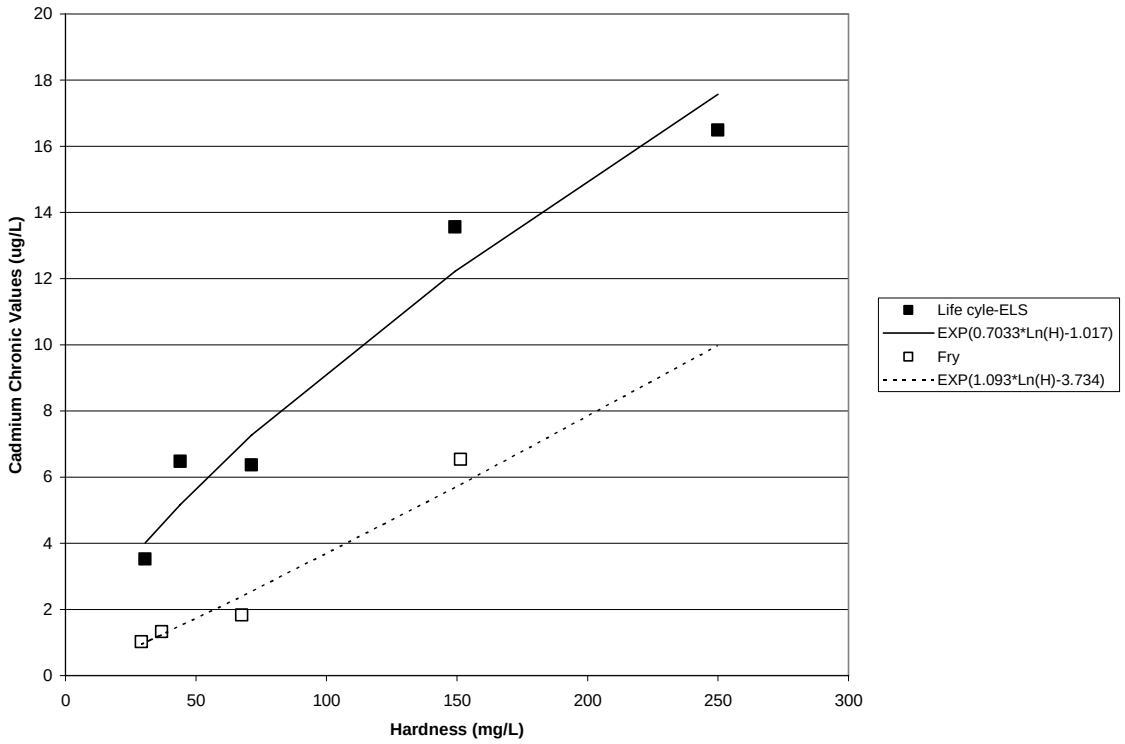

Figure A2. Brown trout Cd Chronic values from brown trout Life Cycle-ELS tests and fry at different water hardnesses.

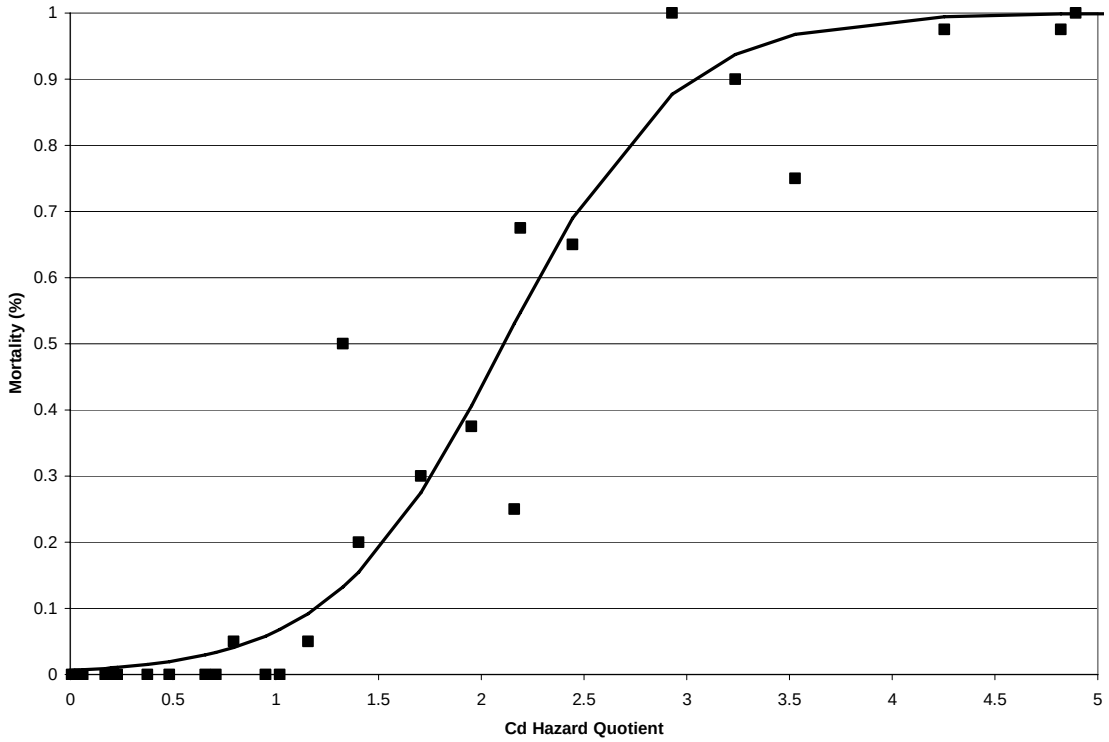

Figure A3. 96 hour brown trout mortality (%) as a function of Cd hazard quotient

Appendix B

Effect of Hardness on the Toxicity of Zinc to Brown Trout (*Salmo trutta*) Embryos, Larvae, and Fry

Stephen Brinkman, Patrick Davies, and Daria Hansen

ABSTRACT

The toxicity of zinc to brown trout at low and high water hardness (30 and 150 mg $CaCO_3$/L) was studied. Tests were conducted at each hardness using early life stage (ELS) and 30 day post swim up fry. In additional, a 96 hour acute test was conducted at 400 mg/L water hardness. Zinc toxicity was negatively related to hardness. Significant effects were observed on early life stage (ELS) time to hatch, survival and termination length and weight. Hatching of eggs was delayed in a dose-dependent manner and chronic values based on delay of hatch were the most sensitive endpoint for the ELS tests (162 and 720 µg Zn/L at 30 and 150 mg $CaCO_3$/L hardness, respectively). Median lethal concentrations (LC_{50}s) to fry were 367, 1104, and 6259 µg Zn/L, at 27, 131, and 410 mg $CaCO_3$/L hardness, respectively. Reduced survival was the primary effect of zinc exposure of swimup fry. Effects on growth were not observed. Chronic values based on reduced survival of fry at low and high hardnesses were 148 and 598 µg Zn/L, respectively. Chronic values from the fry tests were lower than those from the ELS tests suggesting acclimation occurred during the initial stages of the ELS tests. Toxicity test results from previous tests are combined to develop hardness-based equations for the protection of brown trout from zinc toxicity,

INTRODUCTION

An estimated 2080 km of streams in Colorado are impacted by metals (Water Quality Control Division 1988). Brown trout are an important component of Colorado ecosystems in many headwater streams, but their numbers are often reduced due to metal contamination in streams (Davies and Woodling 1980). Data on the toxicity of zinc to brown trout are limited and for the most part exist only for a water hardness of 40 mg/L (Davies and Brinkman 1994, Davies and Brinkman 1999, Davies et al. 2000, Davies et al. 2002). Additional data are needed to assess the effect of hardness on zinc toxicity to assist with development of site specific water quality standards in zinc impacted areas such as the Arkansas River downstream from California Gulch and the Blue River below the confluence with French Gulch. The objective of this investigation was to determine the effect of hardness on the acute and chronic toxicity of zinc to different life stages of brown trout. The effect of water hardness was evaluated by conducting long term flow through toxicity tests at a water hardness of 30 and 150 mg $CaCO_3$/L. Effect of zinc exposure at the two hardnesses on traditional endpoints such as survival, growth and biomass were compared. These endpoints were also used to compare the zinc sensitivity of early life stages (ELS) to the sensitivity of 30 day post swimup fry. Acute toxicity to brown trout fry at a water hardness of 400 mg $CaCO_3$/L was also determined.

MATERIAL AND METHODS

Organisms

Brown trout embryos were obtained as newly eyed eggs from the Colorado Division of Wildlife Research Hatchery in Bellevue Colorado. The source of the eggs was a Colorado Division of Wildlife spawning operation using feral brown trout in the North Delaney Butte Reservoir in Northern Colorado. Ten eyed eggs were placed into each exposure chamber for the ELS tests. Remaining eggs were divided into two lots, placed into a five gallon glass aquaria supplied by the same waters utilized in the 30 and 150 hardness ELS tests and later used for the brown trout fry toxicity tests. Eggs began hatching 12 days after initiation of exposure. Brown trout embryos remained as sac fry for approximately 23 days before reaching swimup stage. The ELS tests continued for an additional 30 days post swimup for a total of 65 days of exposure. The fry toxicity tests were conducted using 34 days post swimup fry. Swimup fry were fed appropriately sized trout food (Silver Cup) four times daily (twice daily on weekends and holidays) at an estimated rate of 3% body weight /day. Swimup fry in the ELS test were fed the trout food diet supplemented with a concentrated suspension of brine shrimp naupalii (San Francisco brand).

Exposure Apparatus

The source water for the 30 mg/L hardness toxicity tests consisted of dechlorinated Fort Collins municipal tap water mixed with reverse osmosis water. The 150 mg/L hardness water was a mixture of well water and dechlorinated Fort Collins municipal tap water. These waters supplied two modified continuous-flow diluters (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components. Chemical stock solutions were prepared by dissolving a calculated amount of reagent grade zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Mallinkrodt) in deionized water. The chemical stock solutions were delivered to the diluters via peristaltic pumps (Cole-Palmer model C/L) at a rate of approximately 2.0 mls/minute. New stock solutions were prepared as needed during the toxicity tests. The diluters delivered five exposures with a 50% dilution ratio, and an exposure control. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 30 mls/minute each. Exposure chambers consisted of polyethylene containers with a capacity of 2.8 liters. Loading during the ELS was less than 1.2 g/L of tank volume and less than 0.08 g/L of flow per 24 hrs. During the fry tests, loading never exceeded 2.9 g/L of tank volume and less than 0.19 g/L of flow per 24 hrs. Loading rates were far below suggested maximum rates (ASTM 1993). Test solutions overflowed from the exposure chambers into water baths which were maintained at 12ºC using temperature-controlled recirculators (VWR Scientific Products). Dim fluorescent lighting provided a 12:12 h light:dark photoperiod. The diluters and toxicant flow rates were monitored daily to ensure proper operation.

ELS Test Methods

The target zinc exposure concentrations were 1600, 800, 400, 200, 100 and 0 µg/ Zn/L for the 30 hardness test. For the 150 hardness test, the target concentrations were 6400, 3200, 1600, 800, 400 and 0 µg/ Zn/L. The number of hatched eggs and mortality of eggs and fry were monitored and recorded daily. Dead fry were blotted dry with a paper towel and total length (to the nearest mm) and weight (to the nearest 0.001 g) measured and recorded. At the end of the tests, surviving fish from each exposure chamber were terminally anesthetized, blotted dry with a paper towel and total lengths and weights measured and recorded.

Water quality characteristics of exposure water were measured weekly in all treatment levels within a replicate. Replicates were alternated each week. Hardness and alkalinity were determined according to Standard Methods (APHA 1985). A Thermo Orion 635 meter measured pH and conductivity. The meter was calibrated with 4.00, 7.00 and 10.00 pH buffers and two conductivity standards prior to each use. Dissolved oxygen was measured using a YSI Model 58 or Orion 1230 dissolved oxygen meter.

Water samples for dissolved zinc analyses were collected weekly from each exposure level with surviving fry. Exposure water was passed through a 0.45 µm syringe filter (Acrodisc), collected in disposable polystyrene tubes (Falcon), and immediately preserved with Ultrex triple distilled nitric acid to pH <2. Samples were analyzed within 24 hours of collection. Analyses were performed using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source (High Purity Standards, Charleston SC). Sample splits and spikes were collected and analyzed to verify analytical reproducibility and recovery. The zinc detection limit was <10 µg/L.

Fry Test Methods

Brown trout fry experiments utilized the same exposure apparatus as the ELS tests. Test methods were identical with the following exceptions. The target zinc exposure concentrations were reduced to 800, 400, 200, 100, 50 and 0 µg/ Zn/L for the 30 hardness test. For the 150 hardness test, the target concentrations were reduced to 3200, 1600, 800, 400, 200 and 0 µg/ Zn/L. Samples for water quality characteristics and zinc analysis were collected daily during the initial 96 hours of exposure and weekly thereafter. Fry were not fed during the initial 96 hours of exposure but were fed twice daily thereafter (once on weekends and holidays). Zinc exposure lasted for a total of 30 days. An acute-only test was conducted at a target water hardness of 400 mg $CaCO_3$/L. Nominal zinc exposure concentrations for the 400 hardness test were 8000, 4000, 2000, 1000, 500, and 0 µg/ Zn/L.

Statistical Analyses

Statistical analyses were conducted using Toxstat version 3.5 software (West Inc. 1996). Analysis of variance (ANOVA) was used to test toxicity endpoints which included hatching success, fry and swimup survival, biomass at the end of the test, mean time to hatch, and lengths and weights of surviving at test termination. Hatching success and survival data were transformed using the arcsine square root prior to ANOVA (Snedecor and Cochran 1980). Normality and homogeneity of variances were tested using Shipiro-Wilk's test and Levene's test, respectively (Weber et al., 1989). Treatment means were compared to the control using William's one-tailed test (Williams 1971, Williams 1972) or Dunnett's one-tailed test (Dunnett 1955, Dunnett 1964), both at $p<0.05$. The highest zinc concentration not associated with a treatment effect (e.g. decreased survival, decreased body weight) was designated as the no-observed-effect concentration (NOEC). The lowest concentration of zinc that was associated with a treatment effect was designated as the lowest-observed-effect concentration (LOEC). Chronic values were calculated as the geometric mean of the LOEC and NOEC. The inhibition concentration (IC_{20}), the concentration estimated to cause a 20% reduction in organism performance compared with the control (USEPA 1993), was calculated using the combined weight of surviving organisms from each treatment. Ninety six hour median lethal concentrations (LC_{50}) were estimated by the Trimmed Spearman-Karber technique (Hamilton et al. 1977, 1978) using log transformed zinc concentrations. The LC_{50} estimations from the 400 hardness fry acute test used 33% trim while all other estimates were obtained using 10% trim.

RESULTS

The average recovery of the external QAQC sample was 99.8% (range 94.8-102.8%). The average spiked sample recovery was 102.2 % (range 96.0-108.3%). The mean percent difference of split sample analyses was 1.2% (range 0.0-4.3%).

30 Hardness ELS

Standard deviations of water quality characteristics during the Early Life Stage test in the 30 hardness test were generally low were generally low and the ranges are narrow indicating the water quality characteristics were consistent over the course of the experiments (Table B1). The mean measured hardness of 26.8 mg/L was slightly lower than the target of 30 mg/L. Mean alkalinity was 19 mg/L and pH was 7.4. Temperatures were maintained in a narrow range around 12ºC. Dissolved oxygen exceeded 6.9 mg/L. Mean conductivity was 46.8 µS/cm.

The time to hatch, hatching success, sac fry and swimup fry survival for the brown trout embryos and the associated zinc exposure concentrations in the 30 hardness ELS test are shown in Table B2. Time to hatch exhibited a generally increasing trend with zinc exposure concentration. The lowest observed effect concentration (LOEC) based on time to hatch was 221 µg/L. The no observed concentration (NOEC) based on time to hatch was 119 µg/L for a chronic value of 162 µg/L. Hatching success exceeded 72 % in all exposure levels and was unaffected by the zinc concentrations used in this experiment.

Substantial mortality occurred during the sac fry stage in the four highest zinc exposures. Sac fry survival in concentrations ≥ 424 µg/L were significantly reduced (LOEC). Sac fry survival at 221 µg/L was only 50% but was not significant at the 0.05 level (NOEC). The chronic value based on sac fry survival is 306 µg/L. The NOEC and LOEC based on survival through the swimup stage were 119 and 221 µg/L, respectively, for a chronic value of 162 µg/L.

Effects of zinc exposure on sublethal endpoints (biomass, mean lengths and weights of surviving fish) are presented in Table B3. Mean length of surviving fish was significantly reduced at zinc concentrations of 798 but not 424 µg/L (LOEC and NOEC, respectively) for a chronic value of 582 µg/L. The NOEC- LOEC values based on surviving weights were lower resulting in a chronic value of 306 µg/L. Mean biomass at the end of the experiment was even more sensitive than surviving weight. The LOEC based on biomass was 221 µg/L and the NOEC was 119 µg/L for a chronic value of 162 µg/L. The 20% inhibitory concentration (IC_{20}) was 180 µg/L. Chronic values and IC20s are summarized in Table B15.

Table B1. Mean, standard deviation and range of water quality characteristics of exposure water used during 30 hardness ELS toxicity test.

	Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
Mean	26.8	19.1	7.45	12.2	46.8	8.04
Std. Dev.	2.2	1.3	0.18	0.2	3.5	0.53
Range	23.4-31.8	17.0-21.4	7.20-7.80	11.9-12.6	42.2-53.2	6.91-8.70

Table B2. Mean dissolved zinc concentrations (µg/L) and associated time to hatch (hrs), hatching success, sac fry and swimup fry survival (%) of brown trout ELS exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (8)	119 (14)	221 (19)	424 (16)	798 (7)	1734 (38)
Time to Hatch (hrs)	306 (8)	324 (13)	356* (4)	383* (13)	411* (19)	373* (11)
Hatching Success (%)	87.5 (9.6)	85.0 (5.8)	92.5 (9.6)	80.0 (14.1)	72.5 (12.6)	77.5 (12.6)
Sac Fry Survival (%)	70.0 (18.3)	75.0 (12.9)	50.0 (11.6)	32.5* (12.6)	27.5* (22.2)	15.0* (23.8)
Swimup Fry Survival (%)	65.5 (12.9)	72.5 (15.0)	47.5* (9.6)	30.0* (14.1)	20.0* (14.1)	5.0* (5.8)

*Significantly less than control ($p<0.05$)

Table B3. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of brown trout surviving 30 hardness ELS test. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (8)	119 (14)	221 (19)	424 (16)	798 (7)	1734 (38)
Mean Length (mm)	34.2 (0.4)	35.4 (1.7)	34.2 (1.1)	32.8 (1.0)	28.3* (3.4)	27.5* (0.7)
Mean Weight (g)	0.326 (0.011)	0.344 (0.036)	0.324 (0.038)	0.260* (0.024)	0.185* (0.048)	0.192* (0.013)
Mean Biomass (g)	2.11 (0.36)	2.46 (0.32)	1.52* (0.27)	0.78* (0.37)	0.41* (0.39)	0.10* (0.11)

*Significantly less than control ($p<0.05$).

150 Hardness ELS

Water quality characteristics measured during the 150 hardness ELS test are presented in Table B4. Mean hardness was very near the 150 mg/L target. Alkalinity at 100 mg/L was about 70% of the hardness, a similar ratio as the 30 hardness ELS test. Conductivity was 256 µS/cm. Temperature, pH and dissolved oxygen were similar to the 30 hardness ELS test.

Time to hatch, hatching success, sac fry and swimup fry survival rates for the brown trout embryos and the associated zinc exposure concentrations in the 150 hardness ELS test are shown in Table B5. The LOECs for each of these endpoints are greater than those from the 30 hardness ELS test demonstrating the well established protective effect of hardness on zinc toxicity. As observed in the 30 hardness ELS test, time to hatch was increasingly delayed with increasing zinc exposure. This delay was significant at a concentration of 983 µg/L but not 528 µg/L. Hatching success was >90% in the controls but significantly reduced at 6402 and 1734 µg/L, but not at 3477 µg/L. Most mortality occurred during the sac fry stage with little or none during the swimup stage. For both sac fry and swimup fry, the NOEC and LOEC were 983 and 1734 µg/L, respectively. The chronic value based on sac fry and swimup fry survival was 1306 µg/L. Surviving length, weights, biomass and associated zinc exposure concentrations are shown in Table B6. The NOEC and LOEC based on surviving lengths and weights was 1734 and 3477 µg/L, respectively. The chronic value for these endpoints is 2455 µg/L. For biomass, 983 µg/L was the NOEC and 1734 µg/L was the LOEC for a chronic value of 1306 µg/L. Chronic values and IC20s are summarized in Table B15 with those from the 30 hardness ELS test for comparison.

Table B4. Mean standard deviation and range of water quality characteristics of exposure water used during 150 hardness ELS toxicity test.

	Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
Mean	153	100	7.53	12.4	256	8.40
Std. Dev.	17	13	0.11	0.4	23.7	0.60
Range	137-201	88.4-133	7.36-7.78	11.7-13.0	235-321	7.25-9.03

Table B5. Mean dissolved zinc concentrations (µg/L) and associated time to hatch (hrs), hatching success, sac fry and swimup fry survival (%) of brown trout ELS exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	528 (44)	983 (88)	1734 (159)	3477 (306)	6402 (524)
Time to Hatch (hrs)	292 (10)	307 (18)	352* (32)	363* (31)	381* (21)	390* (46)
Hatching Success (%)	92.5 (9.6)	85.0 (5.8)	85.0 (10.0)	72.5 (9.6)	87.5 (9.6)	42.5* (12.6)
Sac Fry Survival (%)	90.0 (8.2)	77.5 (5.0)	77.5 (5.0)	45.0* (25.2)	62.5* (17.1)	7.5* (9.6)
Swimup Fry Survival (%)	90.0 (8.2)	75.0 (5.6)	77.5 (5.0)	45.0* (25.2)	60.0* (18.3)	7.5* (9.6)

*Significantly less than control ($p<0.05$)

Table B6. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of brown trout surviving 150 hardness ELS test. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	528 (44)	983 (88)	1734 (159)	3477 (306)	6402 (524)
Mean Length (mm)	35.1 (1.1)	34.7 (0.7)	34.2 (0.6)	33.9 (1.0)	33.2* (1.0)	26.8* (1.8)
Mean Weight (g)	0.335 (0.013)	0.322 (0.020)	0.309 (0.003)	0.307 (0.015)	0.302* (0.036)	0.168* (0.040)
Mean Biomass (g)	2.93 (0.31)	2.40 (0.11)	2.40 (0.16)	1.38* (0.81)	1.79* (0.51)	0.24* (0.06)

*Significantly less than control ($p<0.05$).

30 Hardness Fry

Water quality characteristics for the test conducted with brown trout fry in 30 hardness are presented in Table B7. All characteristics are similar to the test conducted with the brown trout ELS. Table B8 contains the acute (96 hour) and 30 day chronic survival of brown trout fry exposed to zinc in 30 mg/L water hardness. The 96 hour median lethal concentration was 367 µg Zn/L with a 95% confidence interval of 319-421 µg Zn/L. Because of nonzero variances in some treatments, 30 day transformed survival data failed normality tests, but passed Levene's test of homogeneity of variance ($p=0.26$). The results of the ANOVA for 30 day survival are considered reliable because ANOVA is generally considered to be robust with respect to nonnormal data. A single mortality in a control treatment occurred after the initial 96 hours as a result of cleaning operations. Inclusion of this mortality did not affect the results of Williams' means comparison. The LOEC based on 30 day survival was 206 µg Zn/L. The NOEC was 106 µg Zn/L and the chronic value was 148 µg Zn/L. Effects of zinc exposure on growth, as measured by length and weight of surviving fry, were not detected (Table B9). Biomass was significantly reduced for fry exposed to 407 µg Zn/L (LOEC), but not 206 µg Zn/L (NOEC). The chronic value based on biomass was 290 µg Zn/L.

Table B7. Mean, standard deviation and range of water quality characteristics of exposure water used during 30 hardness brown trout fry toxicity test.

	Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
Mean	27.3	20.6	7.49	11.8	50.1	8.04
Std. Dev.	3.2	3.1	0.21	0.2	6.7	0.39
Range	24.0-32.4	17.2-26.6	7.12-7.89	11.6-12.3	43.4-60.8	7.23-8.74

Table B8. Mean dissolved zinc concentrations (µg/L) and associated acute and 30 day survival (%) of brown trout fry exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (2)	56 (2)	106 (5)	206 (8)	407 (13)	879 (9)
96 hr Survival (%)	100 (0)	100 (0)	97.5 (5.0)	90.0 (8.2)	40.0 (8.2)	0 (0)
30 day Survival (%)	97.5 (5.0)	100 (0)	97.5 (5.0)	90.0* (8.2)	40.0* (8.2)	0* (0)

LC_{50} (95% C.I.)=367 µg Zn/L (319-421)
*Significantly less than control ($p<0.05$)

Table B9. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of brown trout fry exposed in 30 mg/L hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (2)	56 (2)	106 (5)	206 (8)	407 (13)	879 (9)
Mean Length (mm)	42.5 (1.0)	41.9 (0.6)	42.8 (1.1)	41.9 (1.9)	40.1 (2.8)	--
Mean Weight (g)	0.729 (0.046)	0.717 (0.041)	0.744 (0.058)	0.736 (0.094)	0.663 (0.145)	--
Mean Biomass (g)	7.11 (0.61)	7.17 (0.41)	7.24 (0.51)	6.63 (1.15)	2.68* (1.01)	0* (0)

*Significantly less than control ($p<0.05$).

150 Hardness Fry

Water quality characteristics during the 150 hardness brown trout fry exposures are shown in Table B10. Mean hardness was 131, lower than the 150 hardness ELS test. Alkalinity was similarly reduced. Other characteristics were nearly identical to the 150 ELS test. Acute (96 hours) and chronic (30 day) survival are presented in Table B11. The LC_{50} was 1104 µg Zn/L with a 95% confidence interval of 951-1281. The NOEC and LOEC based on survival was 436 and 819 µg Zn/L, respectively, for a chronic value of 598 µg Zn/L. As in the 30 hardness fry test, there was no detected effect of zinc exposure on length or weight (Table B12). The NOEC and LOEC based on biomass was the same as survival with a chronic value of 598 µg Zn/L.

Table B10. Mean, standard deviation and range of water quality characteristics of exposure water used during 150 hardness brown trout fry toxicity test.

	Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
Mean	131	90.8	7.57	12.3	243	8.25
Std. Dev.	4.1	4.1	0.10	0.5	7.6	0.55
Range	123-141	84.2-97.8	7.41-7.76	11.6-13.3	231-259	6.85-8.96

Table B11. Mean dissolved zinc concentrations (µg/L) and associated acute and 30 day survival (%) of brown trout fry exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	222 (21)	436 (38)	819 (62)	1501 (96)	3040 (141)
96 hr Survival (%)	100 (0)	100 (0)	95.0 (5.8)	72.5 (12.6)	27.5 (9.6)	0 (0)
30 day Survival (%)	95.0 (5.8)	100 (0)	92.5 (5.0)	65.0* (12.9)	22.5* (9.6)	0* (0)

96 hour LC_{50} (95% C.I.)=1104 µg Zn/L (951-1281)
*Significantly less than control ($p<0.05$)

Table B12. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of brown trout fry exposed in 150 mg/L hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	222 (21)	436 (38)	819 (62)	1501 (96)	3040 (141)
Mean Length (mm)	40.1 (1.2)	40.3 (1.9)	40.7 (1.0)	38.8 (0.7)	38.2 (2.2)	--
Mean Weight (g)	0.628 (0.052)	0.659 (0.093)	0.663 (0.055)	0.610 (0.044)	0.548 (0.120)	--
Mean Biomass (g)	5.95 (0.40)	6.59 (0.93)	6.11 (0.29)	3.94* (0.68)	1.31* (0.72)	0* (0)

*Significantly less than control ($p<0.05$).

400 Hardness Fry

Water quality characteristics for the seven day acute test were consistent over the duration of the exposure (Table B13). Temperature, pH and dissolved oxygen were similar to previous tests. Table B14 shows zinc exposure concentrations as well as associated 96 hour and 7 day survival. The 96 hour LC_{50} concentration was 6259 µg Zn/L with a 95% confidence interval of 5073-7720. After 7 days, the LC_{50} decreased slightly to 6014 with a 95% confidence interval between 5022-7202. Mean length and weight of test organisms was 47.9 mm and 1.062 g, respectively.

Table B13. Mean, standard deviation and range of water quality characteristics of exposure water used during the brown trout fry toxicity test at 400 hardness.

	Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
Mean	411.4	295.7	7.34	12.2	692	7.86
Std. Dev.	12.5	5.2	0.08	0.4	12.5	0.40
Range	392.8-434.6	284.8-302.6	7.60-7.88	11.8-13.0	678-713	7.35-8.39

Table B14. Mean dissolved zinc concentrations (µg/L) and associated 96 hour and 7 day survival (%) of brown trout fry exposed in 400 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	540 (119)	1114 (226)	1629 (23)	3730 (340)	8107 (750)
96 hr Survival (%)	100 (0)	100 (0)	100 (0)	100 (0)	83.3 (13.6)	33.3 (13.6)
7 day Survival (%)	95.8 (5.8)	100 (0)	100 (0)	100 (0)	83.3 (13.6)	29.2 (21.0)

96 hour LC_{50} (95% C.I.)=6259 (5073-7720) µg Zn/L
7 day LC_{50} (95% C.I.)=6014 (5022-7202) µg Zn/L

Table B15. Chronic values (µg/L) and endpoints for zinc toxicity tests conducted with brown trout ELS and fry in 30 and 150 mg/L water hardness.

Endpoint	30 Hardness		150 Hardness	
	ELS	Fry	ELS	Fry
Time to Hatch	162	--	720	--
Hatch Success	>1734	--	4718	--
Sac Fry Survival	306	--	1306	--
Swimup Fry Survival	162	148	1306	598
Length	582	>407	2455	>1501
Weight	306	>407	2455	>1501
Biomass	162	290	1306	598
IC_{20}	180	251	1034	629
LC_{50}	--	367	--	1104

DISCUSSION

Early life stage tests at 30 and 150 hardness found a positive relationship between zinc exposure concentration and time to hatch. In fact, this was among the most sensitive endpoints in both ELS tests. This phenomenon has been previously reported for brown trout eggs exposed to zinc (Davies et al. 2002). Altered time to hatch is not a common endpoint for metal toxicity. Manganese accelerated hatching in brown, brook and rainbow trout eggs (Stubblefield et al. 1997, Davies et al. 1998) and exposure to silver resulted in premature hatching of rainbow trout eggs (Davies et al. 1978). Changes of the timing of egg hatch could have important consequences in terms of survival of young of the year and their ability to recruit. The effect of a delay of 100 hours, as we observed, is probably insignificant. Zinc exposures were initiated with eyed eggs and the temperature was maintained near 12ºC. Brown trout spawn in the fall and the eggs remain in redds over the winter months before hatching in the spring. The relatively minor delay of hatching observed in this experiment could be expected to be much greater if zinc exposure were initiated at fertilization and incubated at lower temperatures typical of streams in the winter. The effect of zinc exposure on brown trout eggs starting at fertilization and using colder temperatures deserves further study.

Early life stage tests were more sensitive than fry tests at detecting effects of zinc exposure on growth. This finding may be a result of the longer duration ELS tests compared to the fry tests (65 versus 30 days). Also, relative growth is greater during an ELS test relative to swimup fry. Variability of termination lengths and weights in the fry tests also reduced statistical power to detect effects on growth. In ELS tests, reduction of growth is often a more sensitive endpoint than survival. This was the case in the 30 but not the 150 hardness ELS test. In both tests, biomass at test termination was the most sensitive endpoint. Because biomass was a measure of both weight and survival, small effects on growth and survival became magnified. This compounded effects of each and led to a greater ability to detect effects of zinc exposure.

The results of this study confirmed the well established negative relationship between hardness and zinc toxicity. Previous toxicity tests with brown trout have been conducted over a narrow range of hardnesses precluding an analysis of the effect of hardness on zinc toxicity for this species. The LC_{50}s for the three acute tests presented here and other data from this project (Davies and Brinkman 1994, Davies and Brinkman 1999, Davies et al. 2000, Davies et al. 2002, Brinkman and Hansen unpublished, Hoff and Wall unpublished) allows development of a relationship between hardness and brown trout LC_{50}. Data used for the regression are summarized in Table B16. The log-log regression typical of metal toxicity-hardness relationships resulted in a coefficient of determination = 0.79. Dividing a predicted LC_{50} by a factor of 2 can be expected to protect brown trout from acute exposures to zinc (EPA 1985). The resulting hardness-based equation expected to protect brown trout from acute zinc exposure is

$$\text{Brown Trout Zn acute} = e^{(0.9634*(\ln(\text{hardness}))+1.986)}$$

Chronic zinc toxicity tests with brown trout have been conducted over a narrower range of water hardness than acute tests. In order to develop a hardness-based equation for the protection of brown trout, acute-chronic ratios were used. Toxicity tests with paired LC_{50}s and chronic values were used to calculate acute-chronic ratios (Table B17). Two tests were excluded because of excessive mortality in control treatments and one test was excluded because of toxicant pump failure during the test (see Table B16). Acute-chronic ratios do not appear to be affected by hardness, although values over a range of hardness are limited. Acute-chronic ratios were between 1.57 and 5.52, although most values fell between 2 and 3.5. The arithmetic mean of the acute chronic ratio is 2.51. The resulting hardness-based equation expected to protect brown trout from chronic zinc exposure is

$$\text{Brown Trout Zn chronic} = e^{(0.9634*(\ln(\text{hardness}))+1.759)}$$

Zinc values from this equation can be expected to protect unacclimated brown trout fry. While acclimated brown trout are capable of tolerating higher levels of zinc, it is important that water quality standards protect unacclimated organisms. Unacclimated fry from clean tributaries may wash into contaminated stream reaches. Protection of unacclimated individuals is also necessary because acclimation to metals can be quickly lost once exposure to metals is removed (Gasser 1998, Davies and Brinkman 1999, Davies et al. 2002). Migration into a clean tributary would lead to a loss of acclimation

followed by toxicity on return to a contaminated stream reach. Loss of acclimation can also occur during spring runoff when dilution from spring snowmelt substantially reduces metal concentrations in streams.

The hardness-based equations were developed using toxicity tests conducted with brown trout that weighed between 0.4 and 9 grams. It is generally agreed that smaller organisms are more sensitive to metals than larger organisms. However, examination of toxicity test endpoints as they related to organism weight did not reveal a trend, over the range of organisms used.

ELS tests are generally considered to encompass the most sensitive life stage of fish. Results from ELS tests are comparable to results from life cycle tests (Macek and Sleight 1997, McKim 1997). However, toxicant exposure initially occurs during embryonic stage, a life stage relatively tolerant to zinc. Exposure during a tolerant life stage provides an opportunity for the exposed organisms to become acclimated and more tolerant to lethal effects during a subsequent sensitive life stage (Sinley et al. 1974, Spehar 1976, Davies et al. 2002). Acclimation of rainbow trout to zinc is a well documented phenomenon (Sinley et al. 1974, Bradley et al. 1985, Stubblefield 1988, Anadu et al. 1989). Brown trout are also able to acclimate to zinc (Davies and Brinkman 1999, Appendix C) and a combination of zinc and cadmium (Gasser 1998). In some cases, toxicity endpoints from ELS tests are greater than median lethal concentrations derived from 96 hour exposures (Table B15). Basing chronic criteria on ELS test results can lead to acute-chronic ratios that are less than one; in instances where embryonic exposure produces an acclimation response. Consideration should be given to tests conducted with the most sensitive life stage when calculating biological criteria. Failure to due so will result in the underestimation of chronic toxicity.

Table B16. Data used for regression of hardness and 96 hour LC50 of zinc to brown trout.

Reference	Test	Hardness (mg/L)	Mean Weight (g)	LC_{50} (µg/L)
Davies and Brinkman 1994	Acclimation test-pre-exposure control	37.6	0.48	642[a]
Davies and Brinkman 1999	Acclimation test-pre-exposure control	51.8	1.4	392
Davies and Brinkman 1999	Acclimation test-pre-exposure control	51.9	0.53	871
Davies and Brinkman 1999	Low Hardness-Low Alkalinity	54.4	2.5	1033
Davies and Brinkman 1999	High Hardness-High Alkalinity	207.2	3.0	>2260
Davies and Brinkman 1999	High Hardness-Low Alkalinity	206.7	2.7	2267
Davies and Brinkman 1999	Low hardness-High Alkalinity	54.0	2.6	690
Davies et al. 2000	Animas R Spring 2000	81.3	0.85	2161[b]
Davies et al. 2000	Animas R Spring 1999	42.3	2.8	476
Davies et al. 2000	Lab-Animas R comparison	54.6	2.3	603 [a]
Davies et al. 2000	Lab-Animas R comparison	52.6	8.9	484
Davies et al. 2002	Zn-Cu interaction (Zn only)	45.3	0.41	382
Davies et al. 2002	Zn-Cu interaction (Zn only)	49.5	0.41	508
Davies et al. 2003	Hardness test	131	0.64	1104
Davies et al. 2003	Hardness test	411	1.1	6259
Davies et al. 2003	Hardness test	27.3	0.73	367
Brinkman and Hansen	2004 Lab-Arkansas R comparison	50.3	0.40	1511
Brinkman and Hansen	9ºC incubation	43.7	0.62	642
Brinkman and Hansen	6ºC incubation	44.5	0.54	381
Brinkman and Hansen	12ºC incubation	45.6	0.53	617
Brinkman and Hansen	3ºC incubation	54.2	0.47	757
Hoff and Wall	2005 Arkansas R field test	46		791

[a]Data excluded from regression-excessive mortality in control
[b]Data excluded from regression- toxicant pump failure

Table B17. Chronic values, LC50s, water hardness and duration of zinc toxicity tests used to calculate acute-chronic ratios for brown trout.

Reference	Hardness (mg/L)	Duration	LC_{50} (µg/L)	Chronic Value (µg/L)	Acute-Chronic Ratio
Davies and Brinkman 1999	51	31 d	871	303	2.87
Davies and Brinkman 1999	52	18 d	392	194	2.02
Davies and Brinkman 1999	54.4	30 d	1033	187	5.52
Davies and Brinkman 2000	52.6	7 d	484	234	2.07
Davies and Brinkman 2002	45.3	7 d	382	151	2.53
Davies and Brinkman 2002	49.5	7 d	508	147	3.46
Davies and Brinkman 2003	27.3	30 d	367	148	2.48
Davies and Brinkman 2003	131	30 d	1104	598	1.85
Brinkman and Hansen (3ºC)	54.2	7 d	757	329	2.30
Brinkman and Hansen (6ºC)	44.5	7d	381	<238	1.60
Brinkman and Hansen (9ºC)	43.7	7d	642	354	1.81
Brinkman and Hansen (12ºC)	45.6	7d	617	392	1.57

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Appendix C

Acclimation and Deacclimation of Brown Trout (*Salmo trutta*) to Zinc and Copper Singly and Zinc in Combination with Cadmium or Copper.

Stephen Brinkman and John Woodling

Abstract. Brown trout (*Salmo trutta*) were acclimated from eyed eggs through fingerling stage to two levels of zinc singly, copper singly, zinc and copper in combination and zinc and cadmium in combination. Acclimation resulted in increased tolerance to metals, as measured by increases in median lethal concentrations compared to unexposed controls. The increase in tolerance was rather modest and never exceeded more than an 84% increase compared to unexposed controls. Acclimation came at a metabolic cost, as growth was often reduced as a result of the sublethal acclimation exposure concentrations. Deacclimation occurred within a few weeks following the return to clean water.

Introduction

Throughout western North America, discharges from mine drainage tunnels and surface runoff from milling operations introduce metals, such as cadmium, copper, and zinc, into many lotic waters. Many of these discharges date back to 1800's mining activities. In Colorado, about 2,120 km of lotic habitat receive metal loadings (Colorado Water Quality Control Division 1998). Although trout are often present in lotic waters downstream from metal contaminant sources, densities may be reduced in comparison to adjacent uncontaminated areas (Davies and Woodling 1980). Brown trout (*Salmo trutta*) are often the dominant salmonid species in Colorado waters contaminated by cadmium, copper and zinc.

Compensatory responses by fish may result in acclimation to manipulations of an environmental variable, such as metal concentrations (Fry 1947). Increased tolerance results if fish survive permanently in elevated concentrations following acclimation. In contrast, increased resistance results if the fish survive longer but eventually die at elevated concentrations following acclimation (Sprague 1985).

Acclimation to cadmium reduced the toxicity of cadmium to fish, compared to unexposed fish (Pascoe and Beattie 1979; Kito et al. 1982; Davies and Brinkman 1994; Stubblefield 1999). Acclimation to zinc likewise reduced the toxicity of zinc to rainbow trout (*Oncorhynchus mykiss*). where fish exposed to sub-lethal zinc concentrations subsequently withstood higher levels, compared to fish with no acclimation (Sinley et al. 1974; Bradley et al. 1985; Stubblefield 1999). Juvenile rainbow trout pre-exposed to sub-lethal zinc survived in higher concentrations of cadmium, copper and zinc than unexposed fish (Anadu et al. 1989).

Increased resistance or tolerance did not result from all acclimation regimes. Acclimation of rainbow trout for seven d to 100 µg/L zinc increased tolerance, while continued acclimation of fish to 100-500 µg/L zinc for three weeks did not further increase tolerance (Anadu et al. 1989). In a seemingly random manner, some acclimation concentrations of copper induced tolerance in rainbow trout while other concentrations had no effect or reduced tolerance (Dixon and Sprague 1981). In the same study, acclimation of rainbow trout to copper resulted in reduced zinc resistance. Aluminum acclimation of rainbow trout in low pH water increased aluminum tolerance but not copper (Wilson et al. 1994a).

Acclimation was not permanent, but was lost 7- to 21-d after the return of rainbow trout to copper-free and zinc-free water (Dixon and Sprague 1981; Bradley et al. 1985; Anadu et al. 1989). Zinc acclimation in fathead minnows (*Pimephales promelas*) increased significantly after a two-week acclimation but was not retained, returning to control levels by 21-d and remained stable until the end of the 35-d test (Hobson and Birge 1989). Acclimation to copper in coho salmon (*Oncorhynchus kisutch*), induced by a one- to two-week acclimation to sub-lethal copper concentrations, declined with time until the acclimation experiment ended at eight weeks (McCarter and Roch 1983). As the duration of a zinc acclimation program to rainbow trout increased from 5-d, 12-d to 20-d, LC50's decreased although the trend was not significant (Bradley et al. 1985).

We exposed brown trout eggs and fry for up to six months to two different levels of a single metal, zinc(ZN) or copper (CU) or to the metal mixtures of zinc and cadmium (ZNCD) or zinc and copper (ZNCU). The first objective was to determine if acclimation ,indicated by increased tolerance, resulted by conducting 96-h toxicity tests and comparing the median lethal concentrations (LC50s) to unacclimated, naive controls. The second objective was to determine whether increased tolerance was retained or lost after the cessation of the acclimation regime .

MATERIALS AND METHODS

Overview

Four sets of toxicity tests were performed over the course of four years, one each year. The cadmium and zinc combination exposure study (ZNCD) was performed in 1996. The zinc study (ZN) was performed in 1998, the copper and zinc combination study (ZNCU) in 1999 and the copper study (CU) in 2000. The tests were conducted over a period of four years and some changes occurred in the toxicity laboratory equipment. These changes did not change the over-all procedure but are noted in the following paragraphs where appropriate.

The basic design was to expose brown trout embryos to a low and a high level of metal(s) and an exposure control. Acute toxicity tests were later conducted. The 96 h median lethal concentrations (LC_{50}s) of exposed juveniles were compared to the unexposed controls to assess degree of acclimation. The juveniles were then transferred to clean water for a period of time and acute toxicity tests were again conducted to measure deacclimation.

Test organisms

Eyed brown trout eggs were obtained from the Colorado Division of Wildlife Bellvue Research Hatchery for the ZN, CU, and ZNCU tests and from the Wyoming State Game and Fish Department at DuBois, Wyoming for the ZNCD tests. Eggs were tempered to dechlorinated Fort Collins, Colorado municipal tap water for 1-d to 14-d

prior to initiating the acclimation exposures (Table C1). Egg hatch began from 3-d to 10-d after the acclimations were initiated. Yolk sac absorption occurred from approximately 22-d to 27-d after hatch. The swimup fry were initially fed Biokyowa starter upon yolk sac absorption, followed by appropriately sized Silver Cup fish food (Piper et al 1982). Brine shrimp naupalii (San Francisco Bay) supplemented the Silver Cup diet during the copper acclimation.

Toxicants

Zinc was added as reagent grade zinc sulfate heptahydrate ($ZnSO_4 \bullet 7H_20$) (Mallinckrodt). Copper was added as reagent grade copper sulfate pentahydrate ($CuSO_4 \bullet 5H_20$) (Mallinckrodt). Cadmium was added as reagent grade cadmium sulfate ($CdSO_4$) (JT Baker Chemical Company). Stock solutions for each acute test and the acclimation exposures were prepared as needed by dissolving a measured amount of dried chemicals in deionized water to achieve desired concentrations. Stock solutions were delivered to test diluters using a diaphragm pump (Cole-Palmer) for the intermittent flow diluters (Mount and Brungs 1967) and a peristaltic pump (Cole-Palmer C/L) for the Benoit continuous flow diluters.

Acclimation

ZN, ZN/CD, and ZN/CU acclimation exposure solutions were delivered to brown trout embryos and fry via a Mount and Brungs diluter (1969) modified to deliver two levels of toxicant and a control. Two liters of dechlorinated Fort Collins, Colorado municipal tap water were delivered approximately every five minutes to 90 liter glass aquaria. Tap water was dechlorinated with an activated carbon filter. Hardness and alkalinity were not adjusted in any way prior to use. CU acclimations used a Benoit continuous flow diluter (Benoit et al. 1982) to polypropylene exposure chambers. Toxicant delivery and diluter performance was monitored daily. Target exposure concentrations are shown in Table 2. Acclimation exposures were replicated except for ZN

Mortality was monitored and recorded daily. Aquaria were siphoned to remove uneaten food and feces as needed. Aliquots of water from each aquarium were collected daily and combined into a weekly composite sample for metal analysis. Metals samples were acidified with Ultrex nitric acid (JT Baker) and refrigerated until analysis. Water quality analyses were conducted weekly in all aquaria during the acclimation phase. Hardness and alkalinity were determined as per Standard Methods (APHA 1985). The pH was determined using an Orion Research pH meter 811 calibrated prior to each use with pH 4.0 and pH 7.0 buffers. Conductivity was measured using a YSI model 35 conductance meter. Dissolved oxygen was measured using a YSI model 58 dissolved oxygen meter calibrated prior to each use.

Challenge tests following acclimation

A series of 96-h, acute flow-through toxicity tests were conducted on control fish, the high acclimation group and the low acclimation group; except for the high ZNCU

exposure where an insufficient number of fish survived to conduct tests. The acute challenges were conducted using the same source water and, in the case of metal mixtures, the same metal ratios of the acclimation phase.

Acute challenge tests were conducted after 116-d and 215-d of acclimation to the zinc and cadmium mixture (ZNCD). Tests were conducted after 126-d and 210-d of acclimation for the zinc and copper acclimation (ZNCU). Acute challenge tests were conducted after 80-d and 70-d of acclimation for the zinc-alone (ZN) and copper-alone (CU) acclimations, respectively. Continuous flow serial diluters (Benoit et al. 1982) with a dilution ratio of 0.5 were used for the ZN, CU, and ZNCU challenge tests. Intermittent flow diluters (Mount and Brungs 1967) were used in the ZN/CD challenge tests. Each aquarium received ten randomly assigned fish. Dead and moribund organisms were removed, measured for total length to the nearest mm and weighed, after being blotted dry, to the nearest 0.001 g with an OHAUS electronic balance. Mortality was monitored hourly during the 96-h acute tests to determine accurate time-to-death comparisons among the acclimation treatments.

Water quality samples were collected at least twice during each 96-h toxicity test from each aquarium using the procedures outlined in the preceding section. Grab samples for metal analyses were collected in high density polyethylene bottles at least three times during each test from each aquarium immediately preserved in Ultrex nitric acid and refrigerated until analyzed. At the end of the test, all surviving fish were terminally anesthetized with MS222 and their lengths and weights measured and recorded. Estimates of lengths and weights of brown trout at the end of each acclimation were based on fish utilized in the acute acclimation tests.

Deacclimation Tests

To assess loss of acclimation, all remaining pre-exposed trout not used in the acclimation challenge tests were returned to clean, undosed 90-L aquaria. Following deacclimation time periods, 96-h acute challenge toxicity tests were conducted using the same procedures used to assess acclimation. The fish from the ZN/CD acclimation exposure groups were tested after 1, 2, and 4 weeks of deacclimation. The ZN acclimations were tested after 3 weeks of being returned to clean water. The ZN/CU and CU acclimations were tested after 5 weeks. The length of the deacclimation period changed based on information learned through the four year program.

Metal Analyses

Zinc concentrations were determined using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame. Cadmium and copper concentrations were determined using a Thermo-Jarrell Ash SH 40000 spectrometer with a CTF 188 graphite furnace atomizer. Both spectrometers utilized Smith-Hieftje background correction. The spectrometers were

calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source.

Statistical Analyses

Statistical analyses were conducted using Toxstat version 3.5 software (West Inc. 1996). Analysis of variance (ANOVA) was used to test for differences of hatching success, fry and swim-up survival, and lengths and weights. Hatching success and survival data were arcsine square root transformed prior to ANOVA (Snedecor and Cochran 1980). Normality and homogeneity of variances were tested using Chi-square and Levene's test, respectively (Weber et al., 1989). Acclimation groups were compared to the respective acclimation control using Dunnett's one-tailed test ($p<0.05$) (Dunnett 1955, Dunnett 1964). Median lethal concentrations (LC50s) were estimated using the trimmed Spearman-Karber method (Hamilton et al. 1997, 1998). LC50s were considered to be different if estimated 95% confidence intervals did not overlap.

RESULTS

Acclimation

Water quality characteristics were comparable among tests though the pH fluctuated between 6.5 and 8.0. Temperature varied between 10.3 and 14.6°C. The mean dissolved oxygen was near saturation and did not fall below 5.5 mg/L. Mean concentrations were near target concentrations except for the CU acclimation periods, which were about 75 % of nominal (Table 2). Water quality parameters were consistent during the acclimation exposures.

Hatching success was unaltered by acclimation exposures except for the high ZN/CU exposure which resulted in a significantly lower hatch (80.4%) compared to the corresponding control fish (89.2%)(Table 3). Hatching success in all other acclimation treatments was greater than 85.8%. Sac fry stage and the swim up fry stage survival was also significantly lower in the high ZN/CU exposure compared to corresponding control fish (28.4% compared to 80.2% and 1.5% compared to 54.2%, respectively). Survival was similar through the sac fry stage and through the swim up fry stage among all other acclimation levels compared to controls. Relatively high mortality occurred in the control fish and both acclimation levels of the ZNCU test through the sac fry and swim up stage as a result of failure to adapt properly to exogenous food.

A significant, sub-lethal chronic response of reduced growth resulted by the end of all acclimation exposures except zinc. Except for ZN, all acclimation exposures reduced growth relative to unexposed controls. The pattern of reduced growth varied among the different acclimation programs. The ZNCU and CU acclimation program resulted in significant decreased growth as shown by both length and weight measurements (Table 4). Growth was not lower after 116 days of acclimation in the zinc

and cadmium in combination toxicity test program, but was observed after 215 days of acclimation in both the high and low acclimation regimes. Survival after 126 days was insufficient to perform a 96-h acute challenge test in the high ZNCU test, so remaining fry were terminally anesthetized and lengths and weights measured.

Challenge tests

Exposure water characteristics did not vary during any of the four challenge test series. Measured metal concentrations were consistent for the duration of all tests and were close to the desired nominal concentrations.

Acclimation was observed in all four of the metal(s) in the form of increased tolerance, as measured by increased LC50s. As with reduced growth in the acclimation programs, different patterns of increased tolerance were observed in each of the four test series. The 96-h LC50s for both the low and high ZN acclimations were both significantly greater than the control following the 80-d acclimation regime but did not differ significantly from each other (Table 5). The same was true for both of the ZN/CD acclimations after 116 and 215 days of acclimation. The LC50s of the low ZN/CU acclimation was significantly greater for the 126 days of acclimation but not for the 210-d acclimation test. Adequate numbers of brown trout did not survive the high ZN/CU acclimation to allow a 96-h toxicity test after 126-d or 210-d of acclimation to 405 μg/L zinc and 16 μg/L copper. The LC50 of the low CU acclimation group was similar to controls. Increased tolerance did not develop in the brown trout in the low CU acclimation program. However, the LC50 of the high CU acclimation group was significantly greater than the controls after the 67 day acclimation program, indicating increased tolerance.

Deacclimation tests

Exposure water characteristics did not vary during any of the four deacclimation test series. Measured zinc concentrations were consistent for the duration of all tests and were close to the desired nominal concentrations.

Deacclimation was observed in all four of the metal(s) exposures. As with reduced growth in the acclimation programs different patterns of acclimation were observed in each of the four test series. The 96-h LC_{50}s for both the low and high ZN acclimation groups decreased significantly after two weeks deacclimation, but the 96-h LC_{50} for the low acclimation group was still greater than the controls (Table C6). The 96-h LC_{50} values for both the low and high ZN acclimation continued to decrease after three weeks deacclimation, although the difference was not significant. A different pattern of tolerance loss was observed in the ZN/CD deacclimation program. The LC_{50}s of the low ZN/CD but not the high acclimation decreased significantly in comparison to controls after one-week deacclimation. The LC_{50} determinations decreased for both the low and high ZN/CD acclimations and no difference existed in comparison to the

controls following two weeks of deacclimation. Deacclimation periods were extended to 35-d for both the ZN/CU exposure program and the CU singly program. No difference was observed between LC_{50}s of the group acclimated to the low ZN/CU metal regime and the controls following the 35-d deacclimation. Adequate numbers of brown trout did not survive the high ZN/CU acclimation regime to allow a 96-h toxicity test after 35 days deacclimation. The LC_{50}s of the low and high CU acclimation group were similar to controls following 35-d deacclimation. Increased tolerance was lost in the high CU acclimation group.

DISCUSSION

The toxicity of cadmium, copper and zinc is not well studied in brown trout. In general, the toxicity results derived from control fish in this study conform to limited available data. The 96-h zinc LC50s ranged for control fish from a high of 871 μg/L to a low of 392 μg/L after the 80-d acclimation program and the 21-d deacclimation program, respectively. Nehring and Goettl (1974) reported a 9-day LC50 of 640 μg/L for brown trout. A series of studies reported 96-h brown trout LC50s ranged from 382 μg/L zinc to 1,033 μg/L zinc (Davies and Brinkman 1994, Davies et al. 1999, Davies et al. 2000 and Davies et al. 2002) using water of similar characteristics as the current effort. The brown trout utilized in the current test series had the same toxic response to zinc as reported in other studies.

Similar observations were made for copper and cadmium. The copper LC50s for naive (control) brown trout were 30.2 μg/L and 39.4 μg/L, similar to brown trout values of 35.8 μg/L and 29.4μg/L reported by Davies et al. (2002). A 96μg/L brown trout, cadmium LC50 of 1.4 μg/L was reported by Spehar and Carlson (1984). The 96-h LC50 cadmium concentrations of 1.17 μg/L and 1.31 μg/L reported in this test series were lower, likely to due to the presence of zinc in the toxicant mixture. The brown trout used in these studies had similar sensitivity to metals as reported in other tests.

Brown trout acclimated to zinc singly (ZN), copper singly (CU) and zinc in combination with both copper (ZN/CU) and cadmium (ZN/CD). The acclimation observed was an increase in tolerance determined by the 96-h challenge tests. Zinc and copper in combination resulted in the greatest increases in tolerance, 88.9% increased tolerance to zinc and 80.1% increased tolerance to copper. The lowest rate of increased tolerance was 46.7% measured in the high CU acclimation group. Increased tolerance induced by acclimation never resulted in a doubling of tolerance. Increased tolerance relative to controls was fairly modest and never resulted in even a doubling of tolerance at any amplitude or duration of exposure. More than one acclimation pattern was observed.

The low and high acclimation exposures did not result in a tiered acclimation response. The high-level acclimation program did not result in any further increased tolerance compared to the low acclimation program when both acclimation regimes

induced an increase on tolerance. Anadu et al. (1989) found the same observation where acclimation of rainbow trout at 100 μg/L zinc produced an increased tolerance while continued exposure from 100 μg/L to 500 μg/L zinc did not further increase tolerance. Acclimation apparently has a plateau in brown trout beyond which no further increases in tolerance result even if a higher acclimation concentration is utilized.

Not all acclimation exposures resulted in increased tolerance. The low CU acclimation regime (7.5 μg/L Cu) did not result in increased tolerance. Dixon and Sprague (1981) found the same outcome in rainbow trout where the low-level acclimation exposure to copper failed to elicit an acclimation response. In contrast, the acclimation exposure of 210 μg/L zinc and 8.7 μg/L copper resulted in an 81% increase in copper tolerance. The combination of zinc and copper resulted in acclimation when exposure to copper singly did not result in acclimation. Exposure to zinc has been shown to induce acclimation to other metals (Anadu et al. 1989). Increased acclimation in wild brown trout populations may not occur depending on the metal concentrations present and the combination of metals in the water. Acclimation in wild trout may be more likely in waters where zinc is the predominate metal present in the water column.

Exposure of brown trout eggs and emerging fish induced a toxic response, not acclimation, when the acclimation concentrations exceeded levels to which brown trout could acclimate. Upper limits to acclimation were observed in the ZN/CU exposure program. A reduced hatch and growth of brown trout eggs resulted when acclimation levels were too high. The high acclimation regime of the ZN/CU in combination (400 μg/L zinc and 15 μg/L copper) exceeded the levels to which the brown trout could acclimate. In contrast, brown trout developed an increased tolerance when acclimated to a concentration of 400 μg/L zinc singly and 15 μg/L copper singly. The combination of these two concentrations of metals was greater than the levels to which brown trout eggs and alevins could acclimate. In this instance, exposure to zinc did not induce the acclimation to copper as noted by Anadu et al. (1989). Metal concentrations in portions of Clear Creek in Colorado, USA often exceed 400 μg/L zinc and 15 μg/L copper in the late winter months when brown trout eggs are developing in redds. In contrast to acclimation, this exposure regime may well be fatal for developing brown trout eggs throughout those reaches of Clear Creek.

Increased tolerance did not remain for the length of all acclimation exposures. The increased tolerance observed in the ZN/CU acclimation exposure at 126-d disappeared at 210-d. Acclimation loss has been noted in prior studies of zinc and fathead minnows (Hobson and Birge 1989) and copper and coho salmon (McCarter and Roch 1983). The combination of zinc and copper appeared to present a more rigorous challenge to brown trout verses the combination of zinc and cadmium, at least at the ratios used in these toxicity tests. The induction of increased tolerance to metals during embryonic development and in the first months post-hatch may well depend on both the metal(s) to which the fish are exposed, the ratio of metals present, and the magnitude of exposure.

Increased tolerance was lost when acclimation exposures were terminated. The loss of acclimation was not immediate. Increased tolerance remained in the high ZN/CD regime after a 7-d deacclimation period compared to controls but disappeared after 14-d. Acclimation in wild brown trout may well remain if temporal decreases in metal concentrations do not extend for time periods of about one week. Metal concentrations decrease annually in the Eagle River and Clear Creek, Colorado during the spring and early summer periods due to melting of the snow pack in the mountains (Colorado Division of Wildlife unpublished data bases). The resulting metal concentrations are less than the acclimation regimes in the current study. Data do not exist that demonstrate increased tolerance will again develop in wild brown trout populations after several months of exposure to decreased metal concentrations.

Acclimation exerted a physiological cost on individual brown trout during the acclimation phase of the current study when only zinc was used in the acclimation regimes. Significantly decreased weights and lengths resulted following the acclimation exposures to ZN/CD, ZN/CU and CU singly. The pattern was not the same for all acclimation exposure regimes. Brown trout exposed to zinc and copper in combination were significantly shorter and weighed less than control fish following 126-d exposure to both the high and low acclimation regimes. The brown trout exposed to zinc and cadmium in combination experienced significantly reduced growth (length and weight) after a 215-d acclimation but not after 116-d acclimation. Brown trout exposed to copper singly were significantly shorter and weighed less than control fish after 76-d, while no effects were observed in control fish during the zinc acclimation. The reasons for the difference are not known. Inclusion of copper into any acclimation appeared to increase the magnitude of reduced growth during acclimation response in fish.

A sub-lethal chronic growth response of brown trout is not limited to brown trout. Woodward et al. (1995) observed decreased growth following an 88-d exposure of young of the year brown trout and rainbow trout to elevated metals including arsenic, cadmium, copper and lead. Although the exposure route was different, foodborne compared to water column metal, the result was the same, reduced growth. Reduced growth over extended time periods has been documented along with acclimation to copper (Dixon and Sprague 1981a, Collvin 1984 and Sprague 1985) and zinc (Hobson and Birge 1989).

Laboratory induced growth reduction is relevant to wild brown trout populations. Brown trout two years old and older weighed less in stream reaches of the Eagle River and Clear Creek, Colorado contaminated by metals (including cadmium, copper and zinc) than fish from adjacent reference stream reaches (Albeke et al. 2001). The Eagle River and Clear Creek wild fish were older than fish used in the course of the current study or by Woodward et al. (1995), demonstrating reduced growth first observed in fingerling fish in laboratory situations can extend to older fish in metal contaminated stream reaches.

Significantly lower growth (length and weight) was observed in the low copper acclimation exposure (5.8 μg/L) even though acclimation was not induced. Exposure to

low level of metals ,in this case copper, did not always induce an acclimation result; but did induce a chronic toxic response of significantly decreased growth.

The ecological relevance of increased tolerance to wild brown trout populations in metal contaminated Colorado waters such as Clear Creek and the Eagle River appears to be dependent on both the length of acclimation and the metal combination tested. Acclimation to the low and high acclimation regimes resulted in increased tolerance to ZN/CD up to 215-d. Increased tolerance to the combination exposure of the low ZN/CU acclimation regime was present following 126-d concentrations but disappeared after 210-d acclimation. The acclimation exposure levels and ratios were selected based on measured concentrations in the Eagle River and Clear Creek. Eagle River and Clear Creek metal concentrations equivalent to those used in acclimation programs are present annually during fall and winter low flow conditions. Low-flow, high-metal periods last for six to seven months each year. The brown trout populations in the two waters may not have net benefit from acclimation. Although individual tolerance was increased, most of the acclimation regimes exacted a cost of reduced growth. Acclimation may have another cost to the brown trout populations. Low level exposure selected against sensitive individuals. Metal exposure may alter or decrease genetic diversity. The tolerance developed by exposure of brown trout to metal concentrations may extend the survival of some or all individual fish but decrease the fitness of the population as a whole. Acclimation is not a panacea for the population but a benefit for the individuals that can acclimate to certain ratios and concentrations of metals.

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Table C1. Brown trout development and hatching information

Test	Days egg tempered Prior to acclimation	Days to hatch during acclimation	Yolk absorption days post-hatch
Zinc tests	7	3	26
Zinc and cadmium	1	10	27
Zinc and copper	4	6	24
Copper	14	3	22

Table C2. Nominal and average measured acclimation concentrations (µg/L) for the 1996 (Zinc), 1998 (Zinc and cadmium), 1999 (zinc and copper) and 2000 (copper) toxicity tests. Standard deviations in parentheses.

Test		Control	Low	High
Zn	Nominal	<10	200	400
	Actual	<10 (3.1)	192 (7.7)	416 (17)
Zn/Cd	Nominal	<10/0.1	200/0.5	400/1
	Actual	<10(8))/<0.1(0.01)	195(18))/0.44(0.07)	436(29)/1.01(0.08)
Zn/Cu	Nominal	<10/1.0	200/7.5	400/15
	Actual	<10(4.5)/<1.0(0.3)	210(20)/8.7(0.7)	405(12.5)/16(1.3)
Cu	Nominal	<1	7.5	15
	Actual	<1.0(0.8)	5.8(2.3)	11.3(1.0)

Table C3. Hatching success, survival through sac fry and survival through swim up fry stage (%) of brown trout pre-exposed to Zinc (Zn), zinc and cadmium in combination (ZnCd), zinc and cadmium in combination (Zn/Cu) and copper (Cu). Standard deviations in parentheses. Zinc acclimation not replicated. *Significantly less than control ($p<0.03$). **Significantly less than control ($p<0.05$)

Test	Control	Low	High
Hatching success			
Zn	89.5	89.6	87.7
Zn/Cd	95.7 (0)	94.3 (0.5)	95.2 (0.7)
Zn/Cu	89.2 (1.1)	85.8 (2.1)	80.4 (1.5)*
Cu	98.8 (1.0)	97.8 (2.1)	99.1 (0.6)
Survival through sac fry			
Zn	82.1	79.1	76.4
Zn/Cd	91 (0.9)	93 (0.5)	91.4 (0.9)
Zn/Cu	80.2 (1.2)	75.2 (3.4)	28.4 (0.1)**
Cu	95.9 (2.1)	95.3 (1.9)	98.8 (1.0)
Survival through swim up			
Zn	82	77.1	74.5
Zn/Cd	81.6 (6.4)	87.6 (0.8)	83.8 (2.6)
Zn/Cu	54.2 (1.6)	48.6 (2.5)	1.5 (2.5)**
Cu	89.1 (3.1)	80.6 (7.8)	90.3 (3.3)

Table C4. Mean lengths (mm) and weights (g) of brown trout pre-exposed to metals and metal mixtures for the different lengths of time. Standard deviations in parentheses. *Significantly less than control ($p<0.05$).

Acclimation	Duration (days)	Control	Low	High
Length mm				
Zn	80	46.2 (7.9)	47.7 (8.2)	47.9 (7.9)
Zn/Cd	116	50.6 (4.9)	51.8 (5.9)	52 (5.8)
	215	83.1 (9.8)	78.4 (1.2)*	76.6 (10.2)*
Zn/Cu	126	47.3 (5.3)	42.7 (4.5)*	31.1 (6.0)*
	210	79.8 (9.8)	74 (9.2)*	--
Cu	67	31.9 (2.4)	30.7 (2.4)*	29.6 (2.3)*
Weight				
Zn	80	0.99 (0.58)	1.1 (0.66)	1.1 (0.59)
Zn/Cd	116	1.16 (0.34)	1.28 (0.43)	1.3 (0.44)
	215	5.9 (2.26)	4.88 (1.95)*	4.42 (1.95)*
Zn/Cu	126	0.91 (0.34)	0.64 (0.22)*	0.25 (0.19)*
	210	4.83 (1.96)	3.85 (1.62)*	--
Cu	67	0.25 (0.06)	0.21 (0.06)*	0.19 (0.06)*

Table C5. Median 96 h LC50 concentrations (ug/L) of metals and combinations of metals to brown trout following four acclimation programs, Zinc singly = Zn, Zinc and Cadmium combination = Zn/Cd, zinc and copper = Zn/Cu and Copper=Cu. Metal concentrations in μg/L. 95% confidence intervals in parentheses. * = Significantly more than control (p,0.05).

Test	Acclimation Duration (Days)	Metal	Control	Low	High
Zn	80	Zn	871 (729-1041)	1397 (1321-1477)*	1578 (1430-1742)*
Zn/Cd	116	Zn/	725 (574-916)	1179 (1060-1321)*	1282 (1104-1489)*
		Cd	2.01 (1.60-2.52)	3.61 (3.20-4.07)*	3.47 (2.88-4.19)*
	215	Zn/	412 (257-661)	1066 (916-1240)*	1245 (1038-1494)*
		Cd	1.17 (0.83-1.64)	2.55 (2.23-2.92)*	2.95 (2.52-3.45)*
Zn/Cu	126	Zn/	571 (540-603)	1,079 (967-1,204)*	--
		Cu	20.2 (19.2-21.2)	36.5 (32.9-40.4)*	--
	210	Zn/	523 (450-608)	619 (572-671)	--
		Cu	19.7 (17.0-22.7)	23.4 (21.4-25.5)	--
Cu	67	Cu	30.2 (27.3-33.5)	27.2 (23.2-31.9)	44.3 (36.6-53.5)*

Table C6. Median 96 h LC50 concentrations (ug/L) of metals and combinations of metals to brown trout following four deacclimation programs, Zinc singly = Zn, Zinc and Cadmium combination = Zn/Cd, zinc and copper = Zn/Cu and Copper=Cu. Metal concentrations in μg/L. 95% confidence intervals in parentheses. * = Significantly more than control (p,0.05). ** = Not enough fish available to perform toxicity test. ***Unable to calculate 95% Confidence Interval.

Test	Deacclimation Duration (Days)	Metal	Control	Low	High
Zn	14	Zn	392 (332-464)	561 (495-635)*	506 (437-585)
	21	Zn	**	438 (366-524)	384 (305-483)
Zn/Cd	7	Zn/	515 (424-628)	721 (***)	1,143 (992-1,318)*
		Cd	1.11 (0.92-1.35)	1.53 (***)	2.46 (2.13-2.84)*
	14	Zn/	632 (415-962)	730 (630-846)	807 (700-931)
		Cd	1.31 (1.09-1.57)	1.62 (1.41-1.86)	1.72 (1.47-2.01)
	28	Zn/	204 (103-406)	258 (126-524)	587 (459-751)
		Cd	0.55 (0.34-0.87)	0.68 (0.42-1.11)	1.22 (0.97-1.54)
Zn/Cu	35	Zn/	412 (352-482)	442 (375-521)	**
		Cu	15.4 (13-18.2)	16.7 (14-19.8)	**
Cu	35	Cu	39.4 (32.1-48.5)	36.4 (27.8-47.6)	45.4 (30.6-44.7)