



Metal exposures to native populations of the caddisfly *Hydropsyche* (Trichoptera: Hydropsychidae) determined from cytosolic and whole body metal concentrations

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Abstract

Metal concentrations of the soluble fraction of the cytoplasm (cytosol) and the whole body were determined in the caddisfly *Hydropsyche* spp. (Trichoptera). Metal accumulation in the cytosol and the whole body were compared in samples collected along 380 kms of a contamination gradient in the *Clark Fork river* in four consecutive years (1992–1995), and from a contaminated tributary (Flint Creek). Samples from the contaminated sites were compared to an uncontaminated tributary (*Blackfoot River*). Relations between cytosolic metal concentration and cytosolic protein (used as a general biomarker of protein metabolism) also were examined in 1994 and 1995. Relative to whole body concentrations, cytosolic metal concentrations varied among metals and years. Spatial patterns in whole body and cytosolic Cd, Cu and Pb concentrations were qualitatively similar each year, and these concentrations generally corresponded to contamination levels measured in bed sediments. The proportions of metals recovered in the cytosol ranged from 12 to 64% for Cd and Cu and from 2 to 38% for Pb. Zinc in the whole body also was consistent with contamination levels, but cytosolic Zn concentrations increased only at the highest whole body Zn concentrations. As a result, the proportion of Zn recovered in the cytosol ranged from 16 to 63% and tended to be inversely related to whole body Zn concentrations. The proportions of cytosolic metals varied significantly among years and, as a result, interannual differences in metal concentrations were greater in the cytosol than in the whole body. The results demonstrated that *Hydropsyche* in the river were chronically exposed to biologically available metals. Some features of this exposure were not evident from whole body concentrations. In general, protein levels did not correspond to cytosolic metal concentrations. A variety of environmental factors could interact with metal exposures to produce complex responses in protein metabolism. Systematic study will be necessary to differentiate the effects of multiple environmental stressors on organisms living in contaminated ecosystems.

Introduction

Metal concentrations have been measured in a wide variety of aquatic organisms to identify exposure to biologically available metals (Phillips and Rainbow, 1993), to validate models of metal bioavailability and bioaccumulation (Luoma et al., 1992; Hare and Tessier, 1996; Wang et al., 1996), and to support assessments of metal effects on biological communities (Bryan and Hummerstone, 1971; Clements and Kiffney, 1994; Hare and Shooner, 1995). In fresh-

waters, insects have been used in metal contaminant studies because of their wide distribution and ecological significance (Hare, 1992). However, with a few exceptions (e.g. Hare et al., 1991a), bioaccumulation data for naturally-exposed populations of insects are based on whole body concentrations.

Whole body metal concentrations may not be indicative of intracellular tissue concentrations. Sorption to external body parts can be important for some elements, for example, As, Fe, Mn or Pb (Krantzberg & Stokes, 1988; Hare et al., 1991a; Cain et al., 1992).

Whole body concentrations also can include substantial quantities of metal in unassimilated gut content (Hare et al., 1989; Cain et al., 1995). These extracellular metals could confound interpretations of metal exposure by contributing to correlations of metal concentrations between the whole body and environmental media. Furthermore, non-absorbed metals probably represent little toxic risk to the organism, and might not be efficiently assimilated by predators.

Absorption and intracellular accumulation of metals is an unequivocal indicator of the internal bioavailability of a metal. Metals can be associated with the cell cytoplasm, bound to cell membranes, and contained in organelles and insoluble granules. If metal toxicity manifests through non-specific, unsuitable binding to intracellular ligands that are functionally critical to cellular metabolism (Mason & Jenkins, 1993), then relative concentrations of metals associated with intracellular fractions containing molecules susceptible to metal binding could provide a basis for assessing toxic risk. The soluble portion of the cytoplasm (cytosol) contains proteins that are active in many biochemical pathways. Excessive metal accumulation could saturate protective, metal-binding cytosolic proteins (e.g. metallothionein), leading to metal 'spillover' to other functional proteins. Sublethal effects accompanied increased Cd and Cu accumulation in the cytosol and redistribution of metals among cytosolic ligands (Sanders et al., 1983; Jenkins & Mason, 1988). Differences in species sensitivity to metals have been related to differences in metal accumulation in the cytosol (Thorpe and Costlow, 1989). Cytosolic metal also appears to play an important role in the trophic transfer of metals. Several studies have shown that cytosolic metals are assimilated efficiently by predators (Reinfelder & Fisher, 1991; 1994; Wallace & Lopez, 1997); whereas, metals stored in insoluble, intracellular granules are passed unassimilated through the digestive tract (Nott & Nicolaidou, 1990).

In this study we characterize metal partitioning between cytosolic and non-cytosolic fractions in naturally-exposed populations of the caddisfly *Hydropsyche*. We examine the conformity in spatial and year-to-year variation between whole body and cytosolic metal concentrations in samples collected along an extensive contamination gradient. We also test whether cytosolic metal concentrations correlate with cytosolic protein concentrations in a subset of samples. Protein concentrations represent a non-specific biomarker that can be used to make inferences about protein metabolism and growth (Variengo et al.,

1980, 1982; Maroni & Watson, 1985; Hylland et al., 1994).

Methods

Study site

The study was conducted at the Clark Fork river, located in western Montana (Figure 1). The mine at Butte and the ore smelter at Anaconda, Montana, both located in the headwater's of the Clark Fork, once comprised the largest copper-producing complex in the United States (Moore & Luoma, 1990) (Figure 1). Metals no longer enter the river from the mine. Presently, metal inputs to the river are derived from contaminated sediments stored within the river channel and its floodplain. The highest inputs per river kilometer occur within the upper 40 km (Hornberger et al., 1997). A gradient of metal contamination in bed sediments extends for at least 380 km downstream of the Butte/Anaconda mining complex (Brook & Moore, 1988; Luoma et al., 1989; Moore & Luoma, 1990; Axtmann & Luoma, 1991).

Elevated metal concentrations in aquatic insects and fish indicate that resident fauna are exposed to biologically available metals (Luoma et al., 1989; Cain et al., 1992, Farag et al., 1995; Lambing et al., 1995;

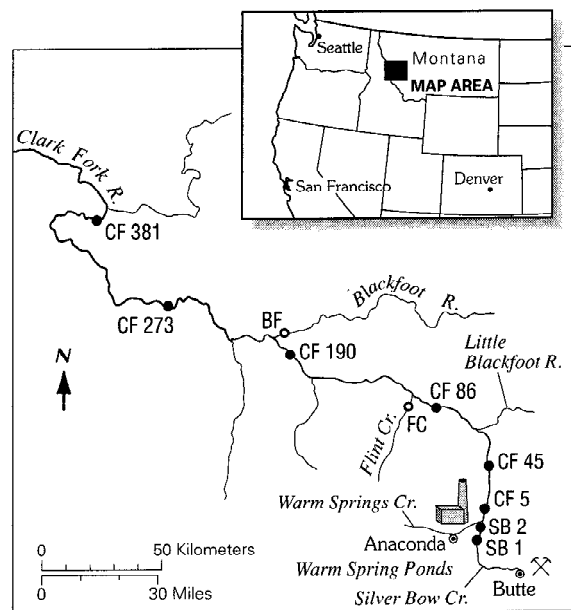


Figure 1. The Clark Fork river (CF), Silver Bow Creek (SB), the Blackfoot River and Flint Creek showing sampling stations. Stations in the Clark Fork are designated by distance (km) downstream of the confluence of Silver Bow and Warm Springs Creeks.

Axtmann et al., 1997). Metal exposure also has been implicated in adverse effects on fish and macroinvertebrate communities (Farag et al., 1995; McGuire, 1995; Woodward et al., 1995). Woodward et al. (1995) concluded that ingestion of metal-contaminated invertebrates may be a principal route of exposure to fish in the Clark Fork.

Sample collection

The net spinning caddisfly *Hydropsyche* (Trichoptera) was collected over 380 km of the metal contamination gradient in four consecutive years (Figure 1). *Hydropsyche* is well suited as a biomonitor because of its sedentary larval life stage, wide distribution and relatively high tolerance to metals (Cain et al., 1992; Clements et al., 1992; Vuori & Kukkonen, 1996). The genus is present at all stations in the Clark Fork and has been used to monitor trends in metal concentrations since 1986 (Hornberger et al., 1997). Stations on the Clark Fork (CF) are designated by river kilometers downstream of the confluence of Silver Bow and Warm Springs Creeks (Figure 1). Silver Bow Creek was sampled at two locations (SB1 and SB2, located approximately 2.1 km and 0.1 km upstream of the confluence of Silver Bow and Warm Springs Creeks). These stations are immediately downstream of a series of settling ponds designed to trap contaminated sediment from upper Silver Bow Creek. Samples also were collected in two tributaries, Flint Creek and the Blackfoot River (Figure 1). Tributaries are affected to different degrees by mining within the basin (Axtmann and Luoma, 1991). Sediment metal concentrations at the Flint Creek station are significantly enriched compared to sediments in the Blackfoot River, but, with the exception of Pb, are lower than sediments in the upper (0–190 km) Clark Fork (Axtmann & Luoma, 1991). A number of small mines, adits and waste piles occur in the Blackfoot drainage. However, contamination in the Blackfoot River is greatly attenuated downstream of these sources and is low where our samples were collected (Moore et al., 1991). Therefore, this station served as the regional contamination reference site.

Sources of metal to *Hydropsyche* have not been quantified in the Clark Fork. The animal feeds on seston (Mecom, 1972; Wallace, 1974), and thus, dietary metal exposures are probably linked to suspended and settling organic material (Snyder & Hendricks, 1995). Previous studies showed that spatial patterns in whole body metal concentrations generally followed metal concentrations in fine bed sediments and total suspended particulates (Cain et al., 1992; Axtmann et al.,

1997; Lee et al., unpublished). In this study, bed sediments (<63 μm) were collected simultaneously with *Hydropsyche* following procedures reported by Axtmann et al. (1997) as an independent indicator of the contamination gradient.

Collections were made during times of base discharge in the summer. In 1992 (July 21–23), samples were collected from stations along a reach extending from Silver Bow Creek (SB1) to CF273. Most of these stations were resampled in 1993 (August 1–5) and 1995 (August 14–17), and coverage was extended to 381 km (CF381). In 1994 (August 9–13), a subset of stations in the upper river (CF86, CF190 and CF381) was sampled.

Hydropsyche larvae were collected with large kick nets and by hand from a single, wadeable (<0.5 m deep) riffle at each station. The insects were sorted on site and placed in plastic containers (previously acid washed) with stream water. Water in the containers was freshened periodically. During collection, specimens were held temporarily (usually 1–2 h) in a cooler, and then transferred from the containers to plastic, sealable bags and frozen on dry ice in a small volume of river water. The samples were moved to the laboratory where they were stored at $-70\text{ }^{\circ}\text{C}$ until analysis.

Isolation of cytosol, pellet, whole body

Specimens were partially thawed, rinsed with cold deionized water to remove adhering particles, then transferred to a sorting dish that was placed on a bed of ice. Species were identified (Alstad, 1980; Scheffer & Wiggins, 1986) with the aid of a stereomicroscope, sorted and immediately transferred to an ice cooler. Instars were not sorted, although smaller specimens that could not be identified were discarded. Specimens were blotted dry with tissue paper and pooled into subsamples weighing at least 400 mg wet weight (or approximately 100 mg dry weight). Samples consisted of 1–5 subsamples. The sample size generally reflected the relative abundance of a species at a given station and time.

Subsamples were homogenized with a stainless-steel, high speed tissue homogenizer in ice cold 0.05 M Tris-HCl buffer (pH 7.4, previously degassed and bubbled with N_2), under a nitrogen atmosphere for 1 min. The homogenate was split into 'total' (whole body) and cytosol subsamples. The whole body subsample was pipetted into a 20 ml screw cap glass vial, and frozen at $-40\text{ }^{\circ}\text{C}$. The cytosol subsample was immediately centrifuged at $100\ 000\times g$ at $5\text{ }^{\circ}\text{C}$.

(Samples collected in 1992 were centrifuged for 4 h. This time was reduced to 1 h for samples collected in later years after it was determined that cytosolic metal concentrations in replicate samples centrifuged for 4 h and 1 h were the same.) The supernatant (cytosol) and pellet were collected, transferred to separate 20 ml screw cap glass vials and then frozen. All samples were subsequently freeze-dried, weighed, digested in a hot 16 N HNO₃ reflux and then evaporated to dryness. The 1992 samples were reconstituted in 5% HCl, filtered (arcrodisk 0.45 μm), and then analyzed by inductively coupled argon plasma optical emission spectrophotometry (ICPAES). Concentrations of Cd and Pb were below the detection limits of ICPAES in some samples. Replicates of these samples were digested, as above, then reconstituted in 1% HNO₃, filtered, and analyzed for Cd and Pb by graphite furnace atomic absorption spectrophotometry (GFAAS). In 1993–95, the samples were reconstituted in 1% HNO₃ and analyzed by either ICPAES or GFAAS. In 1993, Pb was analyzed by ICPAES only. Concentrations of Pb in many of these samples were below the limit of quantitation, however. Therefore, these results are not presented.

All plastic and glassware was cleaned by soaking overnight in a detergent solution (Micro[®]), rinsed with deionized H₂O, then washed in 5% HCl and rinsed with deionized H₂O. The tissue homogenizer was cleaned by soaking overnight in a detergent (RBS[®]) and rinsed in deionized H₂O.

Protein analysis

Total protein was determined in the cytosol of samples collected in 1994 and 1995. The cytosol was subsampled after centrifugation and frozen at -70 °C until analysis. Samples were analyzed using a commercial protein assay kit (Bio-Rad) that is based on the method described by Bradford (1976). Bovine gamma globulin was used as the standard (Bio-Rad catalog number 500-0005). Protein concentration in the cytosol was calculated as mg protein g⁻¹ dry weight of the sample.

Quality assurance

Procedural blanks, metal-spiked samples and NIST standard reference material (SRM 1566a, oyster tissue) were analyzed for quality assurance. The metal contents of the cytosol and pellet were summed and compared to the homogenates of the whole insect to determine metal recoveries for samples and the standard reference material. The median and mean

recovery in unspiked samples was 106% and 102% for all metals, respectively. For the standard reference material, the median and mean recovery was 112%. Pre-digestion spike recoveries of Cd, Cu and Zn ranged from 91 to 125% with mean recoveries of 99% for Cd and Zn, and 107% for Cu.

Data analysis

Two species of *Hydropsyche* were collected for this study. *H. occidentalis* and *H. cockerelli* have similar life history patterns and longitudinal distributions, however, their relative abundance varies spatially and temporally (Hauer & Stanford, 1982; McGuire, 1995). In some samples, one or the other species was not represented because too few individuals were collected to satisfy biomass requirements for metals analysis (see above). Both species were represented in approximately 40% of the samples, but neither species was represented in all of the samples collected over the four years of the study. Therefore, data for both species were combined to generally characterize the spatial and temporal variation in metal concentrations. Previous studies have shown that species-specific differences in metal bioaccumulation are greatest at stations upstream of CF190 (Cain et al., 1992). In this segment of the river, temporal or spatial changes in metal bioaccumulation were clarified by comparing metal concentrations in individual species.

Correlation between whole body and cytosolic metal concentrations were determined from either the Pearson product moment correlation coefficient or the Spearman rho. Differences in the proportion of total metal recovered in the cytosol among years were tested by single factor (year) ANOVA after arcsine transformation of the data. Each station was analyzed separately for each element. Differences in protein concentrations among stations were tested by the Kruskal–Wallis ANOVA. All differences were considered significant if $\alpha < 0.05$.

Results

Spatial and yearly variation

The variation in whole body and cytosolic metal concentrations among stations is shown relative to the sediment contamination gradient in Figure 2 for samples collected in 1993 for Cd, Cu and Zn, and 1992 for Pb. Peak concentrations in the whole body metal concentrations occurred at CF5, 5 km downstream of the highest sediment metal concentrations

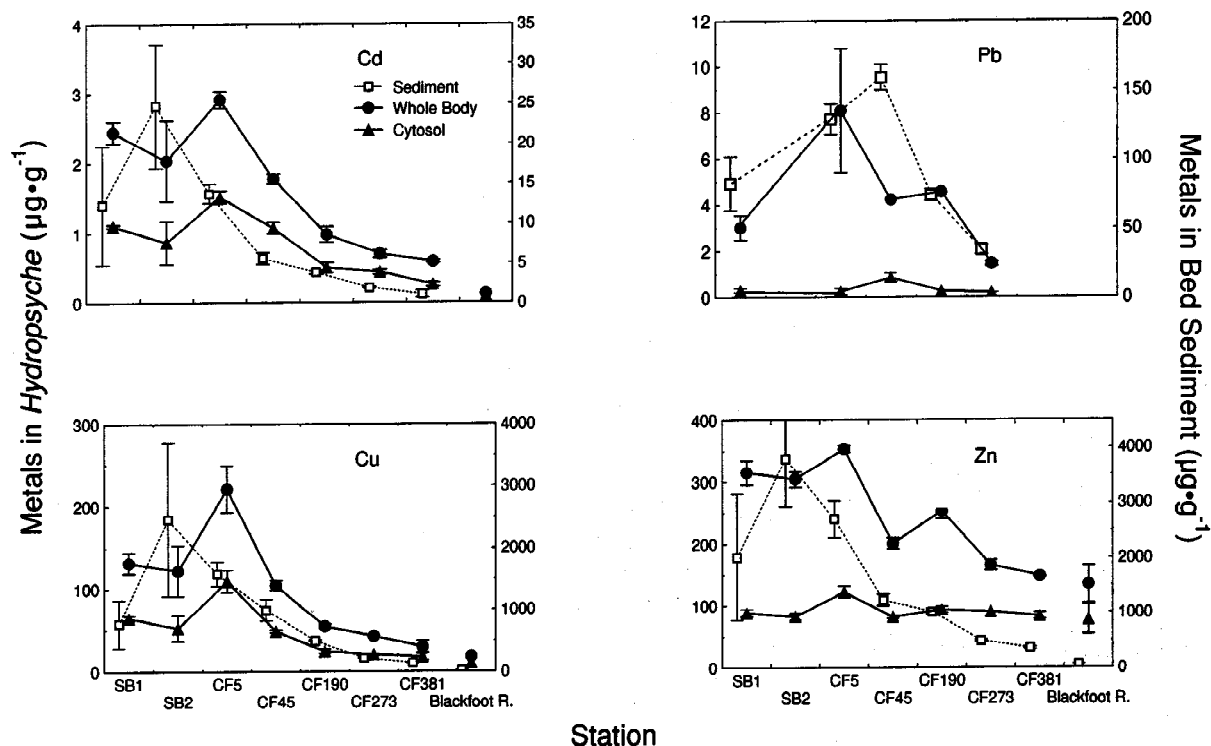


Figure 2. Metal concentrations (mean \pm std) in the whole body (\bullet) and the cytosol (\blacktriangle) of *Hydropsyche* (left Y axis) plotted relative to metal concentrations (mean \pm std) of fine-grained ($<64\ \mu\text{m}$) bed sediments (\square) (right Y axis). Cadmium, Cu and Zn data are for *H. cockerelli* collected in 1993, except at CF190 and the Blackfoot River (*H. occidentalis*). Lead data are for *H. occidentalis* collected in 1992.

(Figure 2). Downstream of this station, metal concentrations in *Hydropsyche* generally conform to declining contamination levels present in sediments (data in Figures 2–4 are plotted by station to facilitate comparisons; however, concentrations decline exponentially downstream of CF5 when plotted as a function of river km). All concentrations in the mainstem of the Clark Fork and in Silver Bow Creek were elevated relative to concentrations of samples from the reference station on the Blackfoot River (also see Figure 3).

Spatial patterns of cytosolic metal concentrations were metal-specific. Cadmium and Cu concentrations of the cytosol were generally consistent with the whole body concentrations (Figure 2). In contrast, cytosolic Zn and Pb concentrations varied little among stations compared to whole body concentrations (Figures 2, 4). Cytosolic Pb concentrations were much lower than the whole body concentrations.

Whole body metal concentrations varied among years. Yearly differences were similar for all metals. The greatest interannual variation occurred at stations upstream of CF86. In the last year of the study, the spatial pattern in bioaccumulation within this reach

changed markedly. For example, Cd concentrations between 1992 and 1993 differed by about 2 fold along the entire 381 km reach (Figure 3). Little change was indicated between 1993 and 1995 downstream of CF86, but at CF5 the mean Cd – as well as Pb and Zn – concentration occurred at CF86 rather than at CF5 as in previous years. Copper concentrations also declined substantially at CF5 between 1993 and 1995. As a result, the downstream gradient of Cu bioaccumulation was weaker in 1995 than in earlier years.

Concentrations of cytosolic metals also generally reflected the yearly changes observed in whole body metal concentrations. However, differences in cytosolic Cd and Zn (Figure 4) were more pronounced than whole body Cd and Zn (Figure 3), especially between 1992 and later years. For example, the mean cytosolic Cd concentrations ranged between 0.30 and $1.52\ \mu\text{g}\ \text{g}^{-1}$ (the coefficient of variation, CV, is 89%) at CF5, while whole body concentrations ranged between 1.2 and $2.9\ \mu\text{g}\ \text{g}^{-1}$ (CV is 43%). Interannual differences in Pb concentrations also were more evident in the

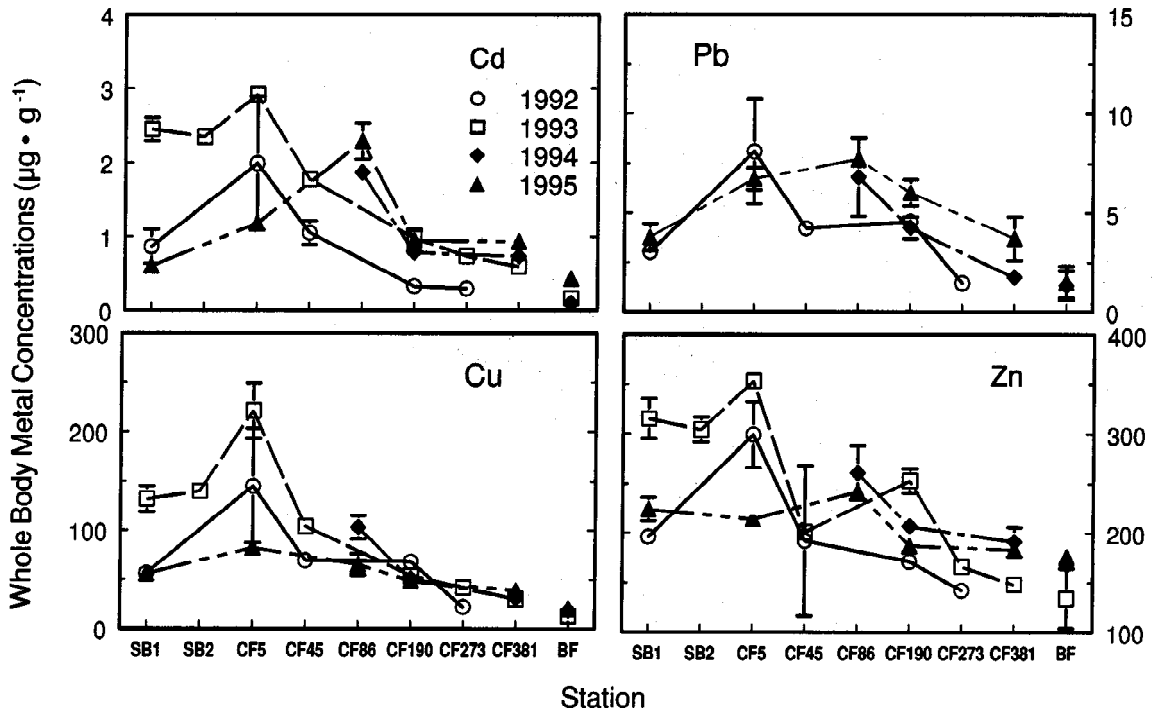


Figure 3. Metal concentrations (mean±std) in the whole body of *Hydropsyche* spp. collected from Silver Bow Creek and the Clark Fork river in 1992 (○), 1993 (□), 1994 (◆) and 1995 (▲). The Blackfoot River is initialed BF.

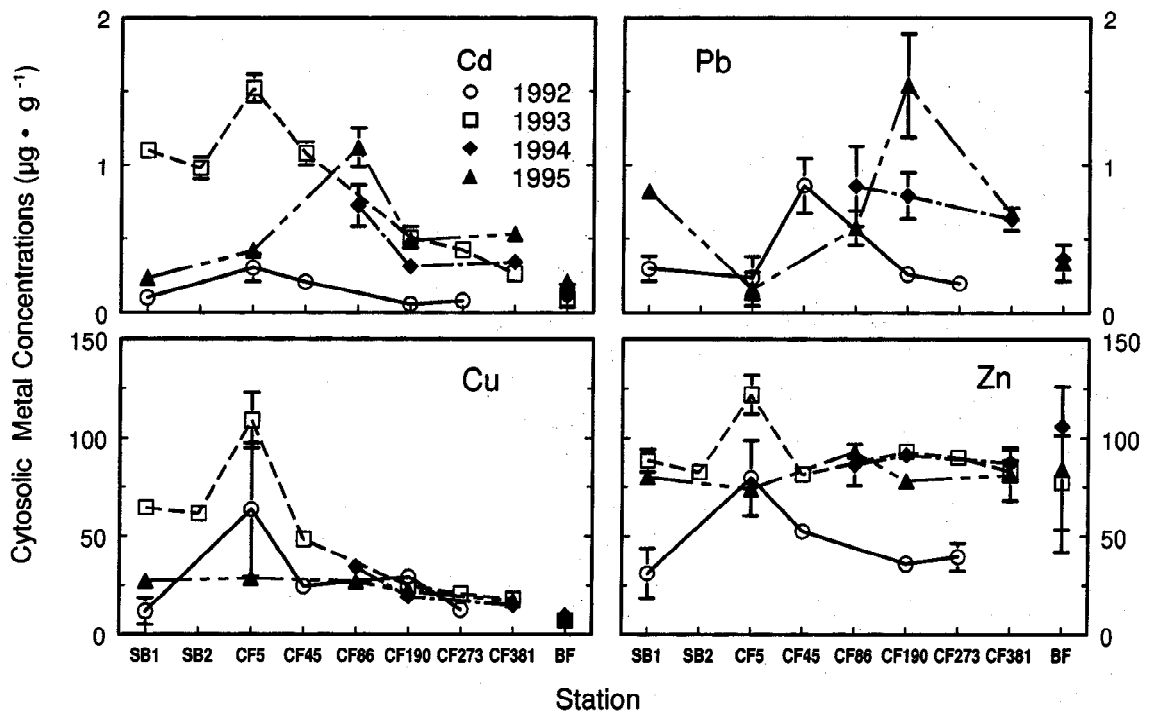


Figure 4. Metal concentrations (mean±std) in the cytosol of *Hydropsyche* spp. collected from Silver Bow Creek and the Clark Fork river in 1992 (○), 1993 (□), 1994 (◆) and 1995 (▲). The Blackfoot River is initialed BF.

Table 1. Whole body and cytosolic Cd concentrations (mean \pm 1 std; $n = 1-4$; $\mu\text{g g}^{-1}$) of *Hydropsyche* spp. from Silver Bow Creek and the upper Clark Fork river (ns means not sampled)

Station	Year	<i>H. cockerelli</i>		<i>H. occidentalis</i>	
		Whole body	Cytosol	Whole body	Cytosol
SB1	1992	0.98 \pm 0.15	0.10 \pm 0.04	0.63	0.10
	1993	2.45 \pm 0.15	1.10 \pm 0.02	ns	ns
	1995	0.60 \pm 0.05	0.24 \pm 0.02	ns	ns
CF5	1992	2.63	0.37	1.35	0.24
	1993	2.92	1.52	ns	ns
	1995	ns	ns	1.18 \pm 0.07	0.42 \pm 0.07
CF45	1992	0.93	0.24	1.16	0.18
	1993	1.78 \pm 0.07	1.07 \pm 0.08	ns	ns
CF86	1994	1.90 \pm 0.06	0.73 \pm 0.02	1.76	0.72
	1995	ns	ns	2.29 \pm 0.2	1.12 \pm 0.13
CF190	1992	ns	ns	0.33	0.06
	1993	ns	ns	0.98 \pm 0.11	0.51 \pm 0.07
	1994	0.81 \pm 0.08	0.33 \pm 0.02	0.73	0.28
	1995	0.89 \pm 0.09	0.51 \pm 0.06	1.03 \pm 0.22	0.45 \pm 0.01

cytosol (Figure 4) than in the whole body (Figure 3). Lead was not quantified in 1993 (by AAS), but qualitative results (concentrations between the limits of detection and quantitation by ICPAES) indicated Pb concentrations were higher in 1993 than in the other three years. This would be consistent with the results for the three other metals.

Combining species increased the variability in some samples but did not affect the general spatial and temporal patterns. Metal concentrations in *H. cockerelli* and *H. occidentalis* differed by less than two fold in samples collected upstream of CF190 (concentrations were usually – although not always – greater in *H. cockerelli* than in *H. occidentalis*), and bioaccumulation patterns were similar in both species. For example, Cd concentrations in both species were greater in 1993 than in 1992 (Table 1). Differences in whole body concentrations between 1992 and 1995 at SB1 (*H. cockerelli*) and CF5 (*H. occidentalis*) were not consistent with CF190 (*H. occidentalis*), but the cytosolic Cd concentrations were uniformly higher in 1995 than in 1992.

Variation in metal partitioning

Differences in metal accumulation patterns between the cytosol and the whole body were reflected in the

percent of total metal present in the cytosol. The percent of cytosolic metal differed among metals and years. Higher proportions of Cd, Cu and Zn occurred in the cytosol than Pb (Table 2). The latter accounted for an average of 10% among all stations sampled in 1992, and 21% in 1994–95. The average percentages of Cd, Cu and Zn in the same samples ranged between 19% and 36% in 1992 and between 43% and 51% in 1994–95.

The percentage of metals recovered in the cytosol did not vary significantly among years in samples from the Blackfoot River. In contrast, metal partitioning varied significantly in samples from Silver Bow Creek and the Clark Fork (Table 2). The percent of cytosolic metal generally was lower in 1992 than in subsequent years. For example, Cd ranged from 12 to 33% among stations in 1992 and from 36 to 64% in 1993–95. Analysis of individual stations showed that these differences were usually significant (Table 2). Copper, Pb and Zn also tended to be lower in 1992 than in other years, although for Cu this difference was only evident at stations upstream of CF190.

Interannual differences in metal partitioning also are illustrated by differences in the covariance between whole body and cytosolic metal concentrations (Figure 5). Correlations for Cd and Cu were highly significant ($R^2 \geq 0.76$) each year. Differences in the

Table 2. Percent of total metal recovered in the cytosol of *Hydrospyrche* (mean \pm std; $n = 1-5$). Not all stations were sampled every year (ns). Years that differ significantly in the percent of cytosolic metal have different letters (superscript). Individual stations were tested for each element

Station	Cd					Cu					Pb					Zn				
	1992	1993	1994	1995	1995	1992	1993	1994	1995	1995	1992	1993	1994	1995	1995	1992	1993	1994	1995	
SB1	12 \pm 4 ^a	45 \pm 2 ^b	ns	39 \pm 4 ^b	48 \pm 2 ^b	21 \pm 11 ^a	49 \pm 2 ^b	ns	48 \pm 2 ^b	10 \pm 5 ^a	ns	22 \pm 4 ^b	16 \pm 6 ^a	28 \pm 4 ^b	35 \pm 2 ^b	ns	27 \pm 3	ns	ns	
SB2	ns	42 \pm 5	ns	ns	ns	ns	43 \pm 2	ns	ns	ns	ns	ns	ns	27 \pm 3	ns	ns	ns	ns	ns	
CF5	16 \pm 3 ^a	52 \pm 5 ^b	ns	36 \pm 5 ^c	35 \pm 5 ^a	43 \pm 6 ^a	50 \pm 0.2 ^a	ns	35 \pm 5 ^a	3 \pm 1 ^a	ns	2 \pm 2 ^a	26 \pm 3 ^a	35 \pm 2 ^b	34 \pm 1 ^b	ns	ns	ns	ns	
CF45	20 \pm 7 ^a	60 \pm 6 ^b	ns	ns	ns	36 \pm 1 ^a	46 \pm 3 ^b	ns	ns	23	ns	ns	29 \pm 9 ^a	41 \pm 2 ^a	ns	ns	ns	ns	ns	
CF86	ns	ns	39 \pm 7 ^a	49 \pm 11 ^a	42 \pm 8 ^a	ns	ns	34 \pm 0.4 ^a	42 \pm 8 ^a	ns	14 \pm 7 ^a	7 \pm 2 ^a	ns	ns	33 \pm 1 ^a	ns	ns	38 \pm 1 ^b	ns	
CF190	18	52 \pm 8 ^a	40 \pm 5 ^a	52 \pm 9 ^a	46 \pm 2 ^a	44	46 \pm 4 ^a	38 \pm 2 ^b	46 \pm 2 ^a	6	19 \pm 5 ^a	26 \pm 8 ^a	21	37 \pm 4 ^a	41 \pm 1 ^{a,b}	ns	51	44 \pm 2 ^b	ns	
CF273	33 \pm 21 ^a	64 \pm 11 ^a	ns	ns	ns	58 \pm 1 ^a	49 \pm 2 ^b	ns	ns	14 \pm 1	ns	ns	28 \pm 6	51	ns	ns	ns	ns	ns	
CF381	ns	44 \pm 4 ^a	47 \pm 4 ^a	58 \pm 7 ^a	45 \pm 6 ^b	ns	60 \pm 2 ^a	48 \pm 3 ^b	45 \pm 6 ^b	ns	38 \pm 10 ^a	19 \pm 5 ^b	ns	56 \pm 4 ^a	44 \pm 5 ^b	ns	56 \pm 4 ^a	46 \pm 2 ^b	ns	
Blackfoot	ns	61 \pm 23 ^a	78 \pm 5 ^a	48 \pm 11 ^a	43 \pm 22 ^a	ns	57 \pm 1 ^a	51 \pm 2a	43 \pm 22 ^a	ns	31 \pm 11 ^a	28 \pm 16 ^a	ns	57 \pm 5 ^a	48 \pm 26 ^a	ns	63 \pm 2 ^a	63 \pm 2 ^a	ns	
Flint Cr.	15	ns	ns	ns	ns	14	ns	ns	ns	4	ns	ns	33	ns	ns	ns	ns	ns	ns	

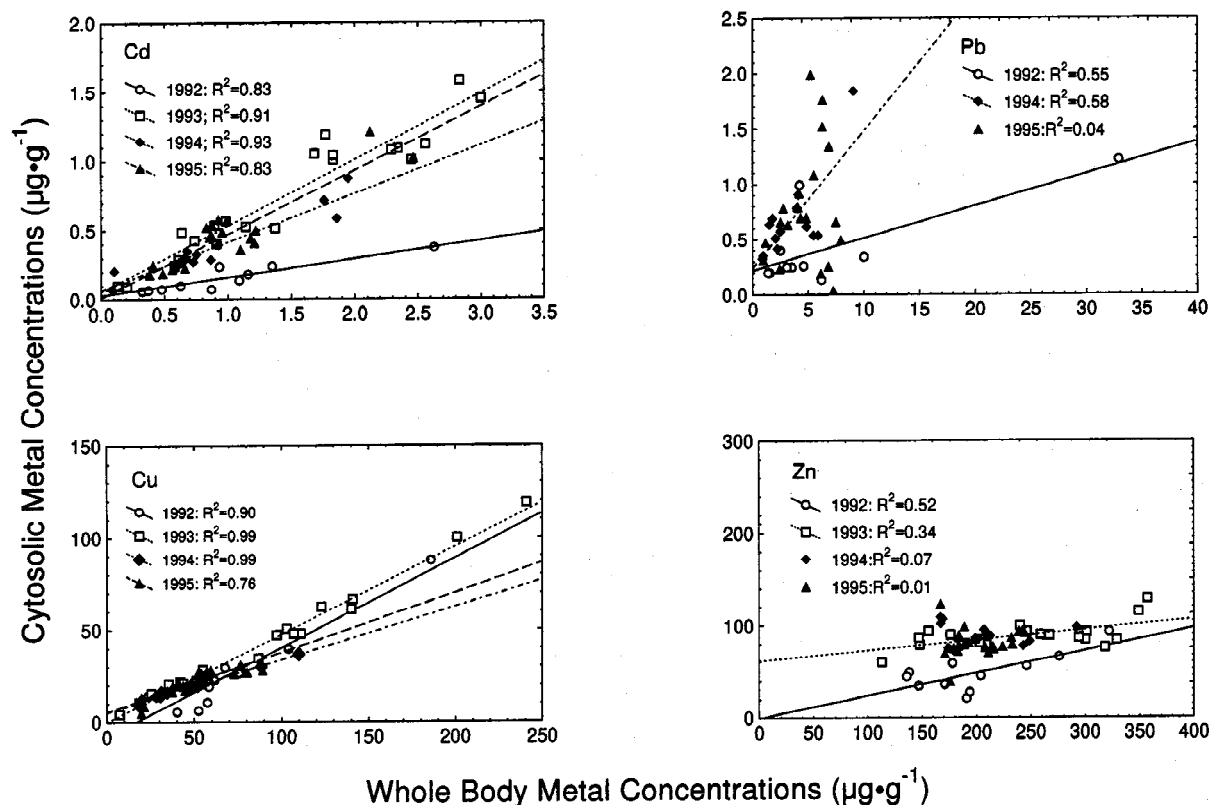


Figure 5. Metal concentrations in the cytosol plotted relative to the whole body of *Hydropsyche* spp. for: 1992 (○); 1993 (□); 1994 (◆); 1995 (▲). Coefficients of determination (R^2) are given for each year. Significant correlations were fitted by linear regression.

correlations indicated that the relationship of whole body to cytosolic metal concentrations varied among years. For example, relative to whole body metal concentrations, cytosolic Cd concentrations were lowest in 1992 (Figure 5), contributing an average of about 19% of the total Cd (Table 2). In 1993, cytosolic Cd accounted for about 52% of the total Cd body burden. Correlations for Pb and Zn were weaker than for Cd and Cu, and in some years, they were insignificant. Nevertheless, the results are consistent with those for Cd: the concentration and the percentage of cytosolic Pb and Zn were lower in 1992 than in later years (Figure 5).

Cytosolic proteins

The concentration of cytosolic proteins varied significantly among stations. However, the effect of metal exposure on these differences was unclear. In 1994, the lowest concentration of total cytosolic protein oc-

curred at CF86, coincident with the highest cytosolic Cd (Figure 6) and Cu (not shown). In 1995, cytosolic metal concentrations at CF86 were greater than in 1994, but protein concentrations were also higher. Thus, cytosolic protein concentrations did not correspond to cytosolic metal concentrations in 1995. However, protein concentrations could be distinguished on the basis of station location in 1995. Proteins were significantly greater at the upstream stations (CF5 and SB1) than at the downstream stations (CF190 and CF381), and intermediate at CF86.

Significant differences in protein between the species (ANOVA) occurred at CF86 in 1994 (mean concentrations were 110 and 124 mg g⁻¹ for *H. occidentalis* and *H. cockerelli*, respectively) and at CF190 in 1995 (165 and 129 mg g⁻¹ for *H. occidentalis* and *H. cockerelli*, respectively). However, these differences were less than the differences among stations or between years.

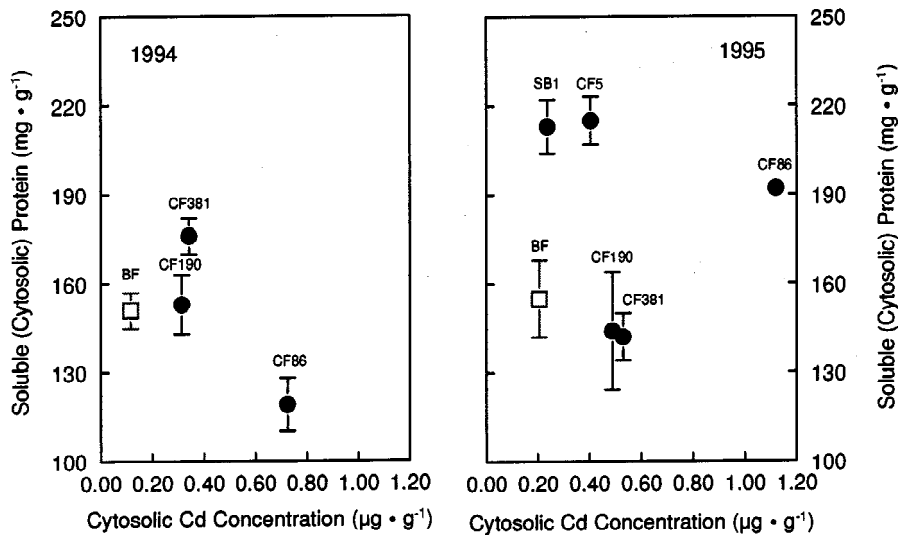


Figure 6. Protein concentrations (mean \pm std) plotted as a function of the cytosolic Cd concentrations in *Hydropsyche* spp. collected in 1994 and 1995 from the Blackfoot River (\square) and the Clark Fork (\bullet). Station locations are indicated above the data.

Discussion

Previous studies on the Clark Fork have demonstrated that immature insects bioaccumulate metals in relation to environmental concentrations (Cain et al., 1992; Axtmann et al., 1997). The present study shows that a portion of the Cd, Cu, Pb and Zn accumulated by *Hydropsyche*, one of the most common insects in the river, is also accumulated intracellularly. Elevated cytosolic metal concentrations in *Hydropsyche* from the Clark Fork strongly indicates that metals released to the river from mining waste are present in biologically available forms.

To our knowledge, only two previous studies have determined cytosolic metal concentrations in natural populations of aquatic insects. Suzuki et al. (1988) examined the cytosolic accumulation of Cd, Cu and Zn in a contaminated population of the mayfly, *Baetis thermicus* from the Mazawa River in Japan, and recently, Cain et al. (unpublished) surveyed metal accumulation in *Hydropsyche californica* in the upper Sacramento River (U.S.A.). The partitioning of metals between the whole body and the cytosol in *Hydropsyche* in the Sacramento River was similar to that observed in the Clark Fork: the cytosolic fraction contained only 12–20% of the whole body Pb, but 49–68% of the Cd, Cu and Zn. Results of Suzuki et al. (1988) indicate that the cytosol contained 94% of the whole body Cd, 36% of the Cu, and only 8% of the Zn in contaminated *Baetis thermicus*. In all

studies, metal concentrations of Cd, Cu and Zn in populations sampled from contaminated stations were elevated compared to uncontaminated sites. Among these metals, Zn exhibited the least enrichment in the cytosol, and the degree of Zn accumulation in the cytosol differed among the studies. There was no substantial difference in Zn accumulation between *Hydropsyche* spp. from contaminated Clark Fork stations and the Blackfoot River. In the Sacramento River, cytosolic Zn concentrations were 1.5–2 times higher than the reference population. Those differences in Zn enrichment reflected the concentrations of the respective reference populations. Zinc concentrations in the Blackfoot River ranged between 80 and 105 $\mu\text{g} \cdot \text{g}^{-1}$. In comparison, the cytosolic Zn concentration of the Sacramento River reference site population was 59 $\mu\text{g} \cdot \text{g}^{-1}$. Among contaminated sites in the Clark Fork and the Sacramento River, Zn concentrations in *Hydropsyche* were similar; ranging between 30 and 120 $\mu\text{g} \cdot \text{g}^{-1}$ with the majority of samples falling between 80 and 100 $\mu\text{g} \cdot \text{g}^{-1}$. Concentrations reported for *B. thermicus* by Suzuki et al. (1988) are given as $\mu\text{g} \cdot \text{ml}^{-1}$, so direct comparisons of concentrations between that study and the Clark Fork and Sacramento River studies are not useful. However, the reported values indicate that the Zn concentration of the contaminated population was about 7 times greater than concentrations in the uncontaminated population. Causes for the difference in Zn accumulation between *B. thermicus* and *Hydropsyche* spp. could in-

clude species-specific differences in Zn accumulation or different Zn exposures in the Mazawa River.

In *Hydropsyche* spp. from the Clark Fork, the relationship between whole body and cytosolic concentrations was metal-specific and changed among years. A strong, positive, linear relationship was consistently found between whole body and cytosolic concentrations of Cd and Cu within years. However, differences in metal partitioning between the whole body and cytosol changed the relationship among years, especially for Cd. Relationships between whole body and cytosolic Zn and Pb concentrations were typically weak within years, but changed among years.

The complexity in metal partitioning observed in *Hydropsyche* spp. has also been reported in bivalves. These studies have generally shown a correspondence between cytosolic and whole soft tissue concentrations. However, the details of the relationship appear variable among taxa, metals, season, and level of contamination. In a highly contaminated population of *Macoma balthica* in south San Francisco Bay (the monthly mean tissue concentrations of Ag and Cu were 42–182 and 139–474 $\mu\text{g g}^{-1}$, respectively), temporal changes in cytosolic metal concentrations generally reflected whole soft tissue concentrations (Johansson et al., 1986). However, the relationship was weaker at the lowest tissue metal concentrations. Other studies of *M. balthica* in less contaminated environments (tissue concentrations of Cd and Cu were 0.1–1.3 and 14–37 $\mu\text{g g}^{-1}$, respectively) also showed that cytosolic metal concentrations were not strongly correlated with whole tissue (Bordin et al., 1996). Correlations were improved when animals were experimentally exposed to Cd and Cu (Johansson et al., 1986; Bordin et al., 1996). Roesjadi (1994) also demonstrated that cytosolic metal concentrations in native populations of the mussel *Mytilus edulis* exhibited spatial and seasonal complexity that was not represented by whole tissues.

Differences in metal accumulation between the cytosol and the whole body could reflect differences in exposure-dependent intracellular uptake and affinities for cytosolic metal-binding proteins. Experimental exposures with other insects have demonstrated that Cd and Cu are efficiently accumulated into the cytosol. In *Chironomus* larvae, 60–75% of the total accumulated Cd was recovered in the cytosol (Yamamura et al., 1983; Seidman et al., 1986). In *Drosophila melanogaster* larvae, 98% of the total Cd and 84% of the Cu was taken up into the cytosol (Maroni & Watson, 1985). As in other organisms, a portion of the cytosolic

Cd and Cu in insects binds to inducible ligands. These ligands are functionally similar to metallothionein, although their structure is generally distinct from mammalian and molluscan metallothioneins and varies among different insect taxa (Clubb et al., 1975; Everard & Swain, 1983; Yamamura et al., 1983; Maroni & Watson, 1985; Seidman et al., 1986; Aoki et al., 1989; Suzuki et al., 1989). Zinc does not appear to either induce or strongly bind to Cd/Cu-metal binding proteins, but instead, is largely bound to constitutive proteins (Maroni & Watson, 1985; Suzuki et al., 1989). A variety of invertebrates appear to be able to physiologically regulate intracellular concentrations of essential elements, including Cu and Zn, when challenged with elevated environmental concentrations (Devineau & Amiard-Triquet, 1985; Amiard et al., 1987; Krantzberg & Stokes, 1989; Kaland et al., 1993; Couillard et al., 1994; Bordin et al., 1996). We saw no conclusive evidence supporting regulation of either Cu or Zn in *Hydropsyche*. Concentrations of cytosolic Cu varied widely and generally corresponded to environmental (bed sediment) contamination levels. Within years, concentrations of cytosolic Zn were fairly uniform among most stations in Silver Bow Creek and the Clark Fork, and only increased substantially at the most contaminated station (CF5). It seems unlikely that exposures to bioavailable Zn were constant over a large segment of the river given the magnitude of the contamination gradient (Zn concentrations in bed sediments of the Clark Fork are 8–33 times greater than regional reference concentrations (Axtmann & Luoma, 1991)). Thus, the spatial pattern is suggestive of Zn regulation over a limited range of exposure. However, between 1992 and 1993, cytosolic Zn concentrations increased at stations all along the river, indicating that the animal did not regulate the increased Zn exposures. From a practical standpoint, cytosolic Zn concentrations could not be predicted precisely from either concentrations in the whole animal or its environment. The relationship between whole body and cytosolic Pb also was not highly predictive, even though cytosolic Pb tended to increase with higher whole body Pb concentrations.

Non-cytosolic metal is an important fraction of the whole body metal burden in insects such as *Hydropsyche*. Non-cytosolic metal can occur in a variety of forms, and its presence can complicate relationships between cytosolic and whole body concentrations. The gut content contributes variable amounts of metal depending on the level of contamination, taxon and metal (Smock, 1983; Gower & Darling-

ton, 1990; Hare et al., 1989, 1991b; Cain et al., 1995). For example, the gut content of *Hydropsyche*, in 1992, accounted for approximately 32–46% of the total Cd, Cu and Pb (Zn was not quantified) (Cain et al. 1995). A residual fraction (unaccounted for by the gut content and cytosol) made up 21–51% of the Cd, 4–25% of the Cu and 46–63% of the Pb. This fraction could be comprised of metals sorbed to external body parts or insoluble, intracellular metal. External coatings of potentially metal-rich iron and manganese oxides have been observed on insects from metal-contaminated rivers (Cain et al., 1992), including the Clark Fork (Boggs, 1994). Lead, in particular, can occur principally as an external contaminant (Krantzberg & Stokes, 1988; Hare et al., 1989, 1991b). For Cd, Cu and Zn, intracellular accumulation and storage occurs in insoluble inclusions (e.g. granules), membranes, or other organelles (Timmermans & Walker, 1989; Gower & Darlington, 1990; Wallace & Lopez, 1997). The midgut epithelium, malpighian tubules, fat body and anal papillae have been reported to be major accumulation sites for Cd, Cu and Zn in a variety of insects (Sohal et al., 1976; Marshall, 1983; Suzuki et al., 1984; Seidman et al., 1986; Krantzberg & Stokes, 1990; Hare et al., 1991a). The 2–3 fold changes in whole body Zn concentrations observed in our study might have been influenced by Zn associated with some of these intracellular components. Darlington and Gower (1990) reported that Cu is concentrated in sulphur-containing granules in cells of the malpighian tubules and in the subcuticular area of larvae of *Plectrocnemia*. The densities of those granules were greater in specimens from Cu-contaminated sites than from uncontaminated sites.

Interannual variability in metal exposure has not been well studied in lotic environments, although large variability in weather patterns, runoff and other factors could affect metal inputs and metal bioaccumulation. The consistency of change in cytosolic metals in 1993, relative to 1992, suggests that a common set of conditions affected exposures over a broad reach of the river. At some stations in the upper Clark Fork, variation in whole body Cd and Cu concentrations in benthic insects collected annually since 1986 correlate positively with yearly total discharge and with yearly metal loads (Hornberger et al., 1997). Statistically significant correlations also occur between annual discharge and the proportion of cytosolic Cd in *Hydropsyche* spp. Although these correlations do not identify causative mechanisms, large yearly differences in hydrologic conditions, linked to weather patterns, might affect

inputs from contaminated floodplain sediment, contaminated bed sediment or contaminated groundwater. In any case, the processes attendant to higher stream discharge that increase whole body metal exposures also increase intracellular metal concentrations.

Metals have been shown to retard growth and development in insects (Hatakeyama, 1989; Timmermans et al., 1992; Simkiss et al., 1993), and to have inhibitory effects on total cytosolic proteins in other invertebrates (Hylland et al., 1994). However, our results did not indicate a simple, linear relationship between cytosolic metal bioaccumulation and cytosolic protein concentrations in *Hydropsyche* spp. Differences in the relative proportion of different species and instars among samples could add variability to relationships. The relative abundance of different instars in samples was not quantified in this study, however, species-specific differences in protein (and metals) concentrations were too small to explain spatial or temporal differences. It is possible that metal effects were not highly expressed in *Hydropsyche*, a genus that is relatively metal-tolerant.

Other environmental factors may have affected protein concentrations in *Hydropsyche* spp. For example, water temperatures are slightly higher during spring to fall in Silver Bow Creek and the upper Clark Fork compared to the lower Clark Fork (Lambing et al., 1996). Differences in river discharge between 1994 and 1995 could have affected detrital inputs and food availability in the Clark Fork, while food availability to *Hydropsyche* in Silver Bow Creek may be regulated by outflow from the Warm Spring Ponds. These factors could be principally responsible for protein levels or they could interact with metal exposures to produce complex spatial and temporal responses. Luoma (1996) suggested that large, comprehensive data sets are needed to differentiate the influence of multiple environmental stressors on animals living in contaminated ecosystems. Such a database does not yet exist for cytosolic proteins.

Measurement of intracellular metals could provide a better understanding of linkages between environmental exposure to metals and toxic effects. In this study, analysis of cytosolic metals in *Hydropsyche* spp. demonstrated that macroinvertebrates in the upper Clark Fork river were persistently exposed to relatively high concentrations of biologically available Cd, Cu and Pb. The implications of this exposure could be difficult to resolve. Although high cytosolic metal concentrations were not directly related to differences in cytosolic protein concentrations in *Hydropsyche* spp.,

a metal-tolerant taxon (Clements et al., 1992), effects of metals on other taxa have been indicated in surveys of the macroinvertebrate community. McGuire (1995) reported that species richness was lower in the upper Clark Fork compared to the lower Clark Fork, partly due to the loss of taxa believed to be sensitive to metals. Fish also have exhibited signs of metal stress. Compared to trout from uncontaminated sites, trout in the upper Clark Fork had elevated metal concentrations in tissues, higher levels of lipid peroxidation products, higher metallothionein levels, and a greater incidence of Cu-containing granules in liver tissue (Farag et al., 1995). Consumption of insects is one source of metals to Clark Fork trout. Experimental studies have reported that fish accumulate metals and experience adverse effects when fed metal-contaminated insects from the Clark Fork river (Woodward et al., 1995). The cytosolic fraction of metal found in macroinvertebrates, including *Hydropsyche*, could constitute a highly available dietary source of metals to these predators (Reinfelder & Fisher, 1991; 1995; Wallace & Lopez, 1997).

Conclusions

Whole body concentrations represent the sum of a variety of metal forms accumulated in and on the body. Although the whole body concentration is useful in assessing the relative level of metal concentration in an organism's environment, it may not accurately reflect intracellular concentrations. In this study, we have examined the conformity between metal concentrations of the whole body and an intracellular fraction (cytosol), assuming the latter is more indicative of an element's biological availability and that it would provide a basis for assessing toxic risk. Comparison of cytosolic and whole body metal concentrations enhanced assessments of metal bioavailability to *Hydropsyche* spp. along an extensive contamination gradient by demonstrating that the cytosolic metal concentration was not always predictable from the whole body metal concentration. Inconsistencies resulted from metal-specific and interannual differences in metal partitioning between the cytosol and the whole body. Sixty-two to ninety-eight percent of the whole body Pb occurred in particulate forms that probably were not available for intracellular uptake. Thus, intracellular accumulation of Pb was much lower and more variable than indicated by the whole body concentrations. The cytosol was an important accumulation site for Cd,

Cu and Zn (containing 12–64%) of the body burden. Cytosolic concentrations of Cd and Cu were directly proportional to whole body concentrations within a given year. Thus, whole body concentrations were indicative of spatial patterns in exposure to bioavailable Cd and Cu. Cytosolic Zn concentrations were fairly uniform along the contamination gradient, and were not indicative of changes in whole body concentrations. Interannual differences in metal concentrations were generally greater in the cytosol than the whole body. Therefore, cytosolic metal concentration was the more sensitive indicator of interannual differences in exposure to bioavailable metal. Cytosolic metal concentration did not correlate with cytosolic protein, a general biomarker of protein metabolism. Additional studies are required to examine dose-response relationships in organisms from contaminated ecosystems like the Clark Fork.

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