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LINKING METAL BIOACCUMULATION OF AQUATIC INSECTS TO THEIR DISTRIBUTION PATTERNS IN A MINING-IMPACTED RIVER

DANIEL J. CAIN,* SAMUEL N. LUOMA, and WILLIAM G. WALLACE
U.S. Geological Survey, Menlo Park, California 94025

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Abstract—Although the differential responses of stream taxa to metal exposure have been exploited for bioassessment and monitoring, the mechanisms affecting these responses are not well understood. In this study, the subcellular partitioning of metals in operationally defined metal-sensitive and detoxified fractions were analyzed in five insect taxa. Samples were collected in two separate years along an extensive metal contamination gradient in the Clark Fork River (MT, USA) to determine if interspecific differences in the metal concentrations of metal-sensitive fractions and detoxified fractions were linked to the differences in distributions of taxa relative to the gradient. Most of the Cd, Cu, and Zn body burdens were internalized and potentially biologically active in all taxa, although all taxa appeared to detoxify metals (e.g., metal bound to cytosolic metal-binding proteins). Metal concentrations associated with metal-sensitive fractions were highest in the mayflies *Epeorus albertae* and *Serratella tibialis*, which were rare or absent from the most contaminated sites but occurred at less contaminated sites. Relatively low concentrations of Cu were common to the tolerant taxa *Hydropsyche* spp. and *Baetis* spp., which were widely distributed and dominant in the most contaminated sections of the river. This suggested that distributions of taxa along the contamination gradient were more closely related to the bioaccumulation of Cu than of other metals. Metal bioaccumulation did not appear to explain the spatial distribution of the caddisfly *Arctopsyche grandis*, considered to be a bioindicator of metal effects in the river. Thus, in this system the presence/absence of most of these taxa from sites where metal exposure was elevated could be differentiated on the basis of differences in metal bioaccumulation.

Keywords—Metals Bioaccumulation Aquatic insects Tolerance Bioassessment

INTRODUCTION

The response of stream macroinvertebrates to metal contamination has received considerable attention because of its utility as an indicator of ecological damage [1]. Changes in community attributes stems from species-specific differences in sensitivity to metals that result in reduced abundances and ultimately local extinctions of some species. A body of literature is developing showing that oligochaetes, chironomids, and hydropsychid caddisflies are relatively metal tolerant, whereas many mayflies, particularly within the family Heptageniidae, are highly sensitive to metals [2–4]. Metal tolerance mechanisms are generally understood, but their expression in stream insects and significance to population- and community-level effects have not been established.

It is generally accepted that physiological and biochemical processes that affect the bioaccumulation of and toxicity to metals differ among species [5,6]. Some species may limit bioaccumulation of some metals by uptake and loss, thereby maintaining low intracellular metal concentrations and effectively preventing metal toxicity. However, most species cannot regulate bioaccumulation of many metals, particularly nonessential metals such as Cd. In these instances, species may bind metals to inducible cytosolic proteins such as metallothionein or store metal in intracellular structures. These differences may fundamentally affect species' sensitivities to metals. For example, differences in Cd tolerance among species of the mayfly *Baetis* were related to differences in the species' capacities to induce a Cd-binding protein, but not total body burden, following Cd exposure [7]. Recently, Wallace et al. [8] proposed that species-specific metal tolerance might be expressed as differences in the metal partitioning among operationally defined subcellular fractions representing detoxified and nondetoxified compartments. Interspecific expression of metal tolerance has largely been studied experimentally where detoxification mechanisms were activated and promoted by controlled metal exposures.

Wild populations of lotic insects have not been directly sampled to determine if detoxification mechanisms are differentially expressed under exposures experienced in the field and if they provide a foundation for explaining interspecific effects of metals, as reflected in ecological endpoints (e.g., abundance, tolerance indices, and richness). In this paper, we analyzed and compared the subcellular partitioning among several insect taxa inhabiting a river chronically contaminated by mining. The taxa were selected on the basis of previously established site-specific differences in metal tolerance inferred from their abundance along a metal contamination gradient [9]. Our premise was that differences in metal tolerance would be expressed in differences in the concentration and subcellular partitioning of metals.

MATERIALS AND METHODS

Study site

Samples were collected from the Clark Fork, the Blackfoot River, and Rock Creek (MT, USA) (Fig. 1). The mine at Butte and the ore smelter at Anaconda, Montana, both located in the headwaters of the Clark Fork, were active from the late 1860s to the mid-1980s and once constituted the largest copper-producing complex in the United States. Mine wastes have created an extensive longitudinal gradient in metal concentrations in bed sediments extending from Silver Bow Creek to the Clark Fork near the confluence of the Flathead River [10]. The Black-

* To whom correspondence may be addressed (djcain@usgs.gov).

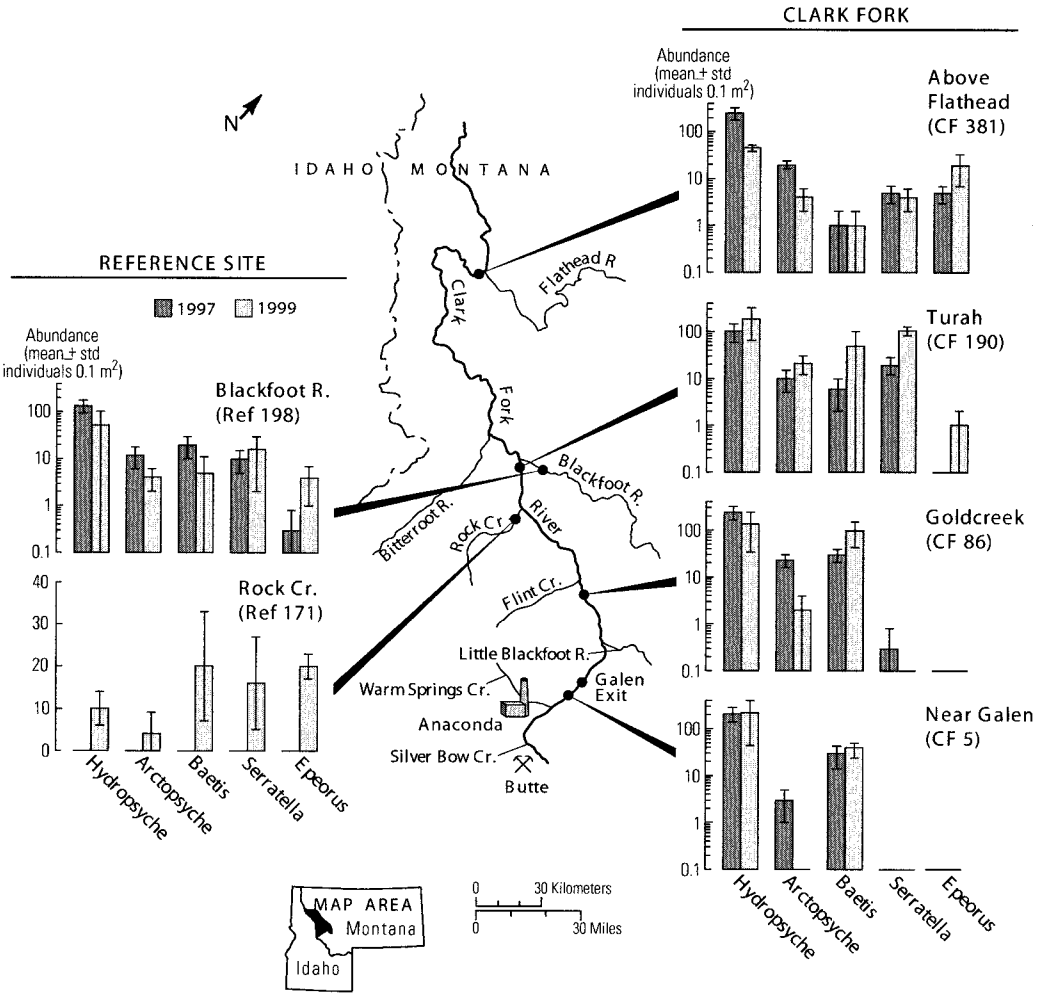

Fig. 1. Map showing the sampling locations on the Clark Fork, Blackfoot River, and Rock Creek (MT, USA) and the abundances (individuals/0.1 m²; mean ± 1 standard deviation) of taxa targeted for this study. Abundance data were reproduced from McGuire [9,12].

foot River and Rock Creek are tributaries to the Clark Fork and have no significant source of metal contamination at their confluences with the Clark Fork.

Sample collection

Samples for this study were collected from five stations encompassing 380 km of the Clark Fork (Fig. 1) in August 1997 and 1999 during annual base flow. These stations, which form part of a larger network of stations that have been sampled annually since 1990 for metals analysis of tissues and sediments [11], were chosen to represent the widest possible range of metal exposures and to match the scale of ecological effects [9]. Samples were not collected from Silver Bow Creek because either all the target species were absent or their densities were too low to obtain enough biomass for analytical purposes. Samples collected from uncontaminated sites in Rock Creek and the Blackfoot River (Fig. 1) served as regional references.

McGuire [9] derived metal tolerance values for Clark Fork macroinvertebrates from distribution and abundance data relative to the historical, longitudinal contamination gradient and from the relevant literature. Based on these assigned rankings, several taxa were targeted for collection in this study. The net-spinning caddisfly *Hydropsyche* spp. (Trichoptera) and the mayfly *Baetis* spp. (Ephemeroptera), both abundant throughout the Clark Fork drainage (Fig. 1), were considered to be metal tolerant by McGuire. Another caddisfly, *Arctopsyche grandis* (Trichoptera), was considered moderately tolerant, while the mayflies *Epeorus albertae* and *Serratella tibialis* (Ephemeroptera) were deemed highly sensitive. Typically, *A. grandis*, *E. albertae*, and *S. tibialis* do not occur upstream of Goldcreek (Fig. 1), where contamination has been greatest, although rare collections of *A. grandis* have been reported in recent years [12,13].

Larvae were collected with large kick nets and by hand from a single, wadable (<0.5-m deep) riffle at each station. The insects were sorted to order, family or genus on site. Handling and preservation of samples in the field followed procedures described by Cain and Luoma [14]. The samples were moved to the laboratory and stored at −80°C until analysis.

Sample preparation

Larvae were partially thawed; rinsed with refrigerated, deionized water to remove adhering particles; and then transferred to a sorting dish that was placed on a bed of ice. Species were identified to genus and species using descriptions from Alstad [15], Merritt and Cummins [16], Schefter and Wiggins [17], and voucher specimens prepared by the U.S. Geological Survey National Water Quality Laboratory (Denver, CO, USA). Identifications also were checked against identifications in McGuire [9,12]. Once identified, larvae were immediately

transferred to an ice cooler. Younger instars that could not be identified and individuals that appeared to have recently molted were discarded. Because of their small size, individual larvae were pooled into one to six replicates weighing between 0.1 and 6 g wet weight. Differences in the weight and numbers of replicates reflected differences in the body size and relative abundance of taxa collected at each station.

Larvae were then homogenized in refrigerated, N_2-saturated, 0.05-M Tris-HCl buffer (pH 7.4). The homogenate was subsampled to obtain a whole-body concentration and subsequently separated by differential centrifugation and chemical and heat treatments into five operationally defined subcellular fractions following procedures modified from Cain and Luoma [14] and Wallace et al. [8]. These fractions included cell debris containing metals solubilized from the exoskeleton, tissue fragments, mitochondria, nuclei, and gut content by digestion in 0.5 N NaOH for 1 h at 70°C, a residual fraction containing metals not solubilized by the NaOH treatment (e.g., intracellular granules), the microsomal fraction, cytosolic proteins denatured by heat treatment, and heat-stable cytosolic proteins. Although we did not directly assay the heat-stable protein fraction for metallothionein, others have found that nearly all the Cd and Cu in this fraction is bound to metallothionein [18,19]. Zinc-binding ligands would include metallothionein and possibly other heat-stable ligands [20]. The heat-denatured protein fraction represents a variety of larger cytosolic macromolecules. For convenience, hereafter we refer to the heat-stable and heat-denatured protein fractions as metal-binding protein (MBP) and non-MBP, respectively.

Data from the subcellular fractions were summed into operationally defined metal-sensitive and detoxified compartments [8]. The metal-sensitive compartment comprised the non-MBP and microsomal fractions. The detoxified metal compartment included the MBP and residual fractions. Although the procedure used for the residual fraction was designed to isolate intracellular granules, other metal forms could be present that do not represent the product of metal detoxification pathways (e.g., surface contamination, undigested inorganic particles from the gut). Visual inspection of the residual fraction obtained from *Hydropsyche* spp. and *Baetis* spp. (×200–400 magnification) revealed chitinous body parts in both taxa. Spherical structures forming aggregates of various sizes that resembled intracellular granules also were observed in *Hydropsyche* spp. but not in *Baetis* spp. The residual fraction might be better characterized as those metals that were detoxified (metal-rich granules) and biologically inactive. However, for the sake of consistency [8], we reserve the term ''detoxified''. The cell debris was not considered in the compartmental approach because of questions about its toxicological significance. The fraction likely contains metals bound to sensitive sites (e.g., nonspecific binding to membrane-bound proteins) and nonsensitive sites (e.g., exoskeleton, undigested gut contents).

All fractions were frozen at −40°C, freeze-dried, weighed, digested in a subboiling 16 N HNO_3 reflux, and evaporated to dryness. Fractions were reconstituted in 2% HNO_3, filtered (0.45 μm), and analyzed by either inductively coupled plasma optical emission spectrophotometry or graphite furnace atomic spectrophotometry. Metal body burdens in each fraction were calculated on a weight-specific basis (nmol/g).

Quality assurance

Procedural blanks, metal-spiked blanks and samples, and standard reference materials (SRM 2976 and NRC Tort-2) were analyzed for quality control. In addition, metal concentrations in subcellular fractions were summed and compared to concentrations in the whole homogenate. Observed concentrations (mean ± 95% CI) of Cd, Cu, and Zn in National Institute of Standards and Technology 2967 were within the reported uncertainties. Observed concentrations of Cu in Tort-2 also were within the reported uncertainty. Observed concentrations of Cd and Zn in Tort-2 were typically less than the reported uncertainties and averaged 91 and 89%, respectively, of the reported means. The mean and standard deviation of predigestion spike recoveries of Cd, Cu, and Zn were 100 ± 5%, 104 ± 4%, and 105 ± 11%, respectively. Recoveries of Cd, Cu, and Zn in subcellular fractions were 95 ± 13%, 91 ± 10%, and 93 ± 11%, respectively.

Statistical analysis

Samples of *Hydropsyche* and *Baetis* were represented by one or two species. We suggest that pooling species within these genera had a negligible effect on our results. Samples of *Hydropsyche* were represented by *H. occidentalis* and *Ceratopsyche* (*Hydropsyche*) *cockerelli*. Metals were analyzed for each species separately. Concentrations between species were not consistently different, as observed previously [14], and therefore data were pooled for statistical description and analysis, and the results were reported as *Hydropsyche* spp. Identifications from voucher specimens and field data [9,12] showed that *Baetis* was represented by a single species, *B. tricaudatus*, at most sites. *Pladitus* (*Baetis*) *puntiventris* was not present in voucher samples but was identified in field samples with *B. tricaudatus* at Goldcreek and Near Galen in 1999, although in relatively low numbers (<15% of the total abundance of *Baetis* species) [12]. Therefore, it is possible that our samples at those sites included both species. However, metal partitioning patterns in these samples and in samples comprised of *B. tricaudatus* were not significantly different (analysis of covariance).

Differences in whole-body metal concentrations among taxa and intraspecific differences in metal partitioning (between populations from reference sites and from the Clark Fork) were analyzed by analysis of variance (ANOVA) and the Kruskal–Wallis test. Statistical comparisons among taxa were made within collections (collection at a station within a given year) for those samples that comprised $n > 1$. Untransformed and, where necessary, log-transformed data were analyzed by single-factor ANOVA. Post hoc multiple comparisons of taxa were made using Tukey's test for unequal *n*. Data that could not meet assumptions of ANOVA after log transformation were analyzed by the Kruskal–Wallis test. Although the replicates representing the mean concentrations were derived by randomly subsampling the entire collection and therefore were not true replicates, we felt this approach provided a reasonable analysis of interspecific differences for site-specific exposures.

Correlations are generally described with the Pearson product-moment coefficient. However, metal concentrations in the subcellular fractions were first correlated against whole-body or cytosolic metal concentrations using the general linear model (Statsoft®) [21] to determine if metal partitioning among subcellular fractions varied significantly between years. Because of differences in sample sizes among taxa, stations, and years, these analyses were run with the sample means rather than all replicates. We believed this better represented the metal partitioning behavior of each taxon. The equality of slopes

of the relationships (metal concentration of subcellular fraction vs whole body) between years was initially tested with the homogeneous slopes model, a form of the general linear model. Depending on whether a significant interaction was detected, the data were reanalyzed with either a separate slopes model or analysis of covariance. Data for *E. albertae* were not analyzed for yearly differences because samples were collected at only two stations in 1997. For the purposes of presentation, data for both years were combined, and relevant changes in partitioning between years are discussed.

RESULTS

Metal exposure

Concentrations of Cd, Cu, and Zn in sediments from the Clark Fork declined from near the head of the river to Above Flathead (Table 1). Between years, Cu and Cd concentrations appeared more variable than Zn within the upper 190 km of the river. Dissolved Cu and Zn (dissolved Cd concentrations were typically less than method reporting limits) generally corresponded with the spatial gradient in bed sediments in 1997 but were more uniform across stations in 1999 (Table 1). Annual differences in dissolved metals and stream flow corresponded (Table 1), but spatial gradients in water chemistry were consistent between years.

Spatial and temporal variation in metal concentrations of insects generally corresponded to contamination levels in sediments and water. Maximum whole-body concentrations occurred in the upper section of the Clark Fork (Near Galen to Goldcreek) (Table 2). Correlations between metal concentrations in *Hydropsyche* spp., the most widely distributed taxa, and sediments (including reference streams) were significant for Cu and Zn ($r = 0.67$ and $r = 0.85$ for Cu and Zn, respectively) but not significant for Cd. Stronger coefficients resulted when Cu and Zn concentrations in *Hydropsyche* spp. were correlated with the average monthly dissolved concentration for the May through August period (coinciding with spring runoff and hatching and rapid growth of insects) ($r = 0.94$ and $r = 0.96$ for Cu and Zn, respectively). Metal concentrations in insects were typically higher in 1997 than in 1999 (Table 2). These differences corresponded with interannual differences in dissolved and sediment metal concentrations (Table 1).

Interspecific differences in bioaccumulation

Interspecific differences in whole-body concentrations consistently ranked the same regardless of the station or year of the collection, although all species were not present in all collections (Table 2). Metal concentrations were typically lowest in the caddisflies *Hydropsyche* spp. and *A. grandis*, intermediate in *Baetis* spp. and *E. albertae*, and highest in *S. tibialis* (Fig. 2). Differences in metal concentrations between years affected the magnitude of differences among some taxa at some stations (e.g., Turah; Table 2) but not the relative differences among them. Interspecific differences for Zn were less evident at reference sites, where concentrations in all taxa except *S. tibialis* ranged between 1,170 and 2,400 nmol/g, than under the higher exposures of the Clark Fork (Fig. 2).

Metal accumulation in subcellular fractions was related primarily to whole-body concentrations. Metal concentrations in each subcellular fraction were usually positively correlated with whole-body concentrations ($r \geq 0.73$) (Table 3). The exception was Zn in MBPs and the microsomal fraction of

Table 1. Water quality data and metal concentrations of fine sediments (<63 μM) for the Clark Fork, Rock Creek, and Blackfoot River (MT, USA). Yearly water quality data are for the period October to August. Data are the range in values within each year. Concentrations of dissolved Cd were usually below method reporting limits. Metal concentrations of sediments are the means of replicate, composite samples collected once annually in August. Data reproduced from Dodge et al. [38,39]. Water quality data were not collected (designated NC) at Above Flathead and Galen Exit. Sediment data at these stations provided by M. Hornberger, U.S. Geological Survey

Station name (km)	Year	Stream flow (ft^3/s)	Conductance (μs/s)	pH	Temp. (°C)	Total hardness (mg/L $CaCO_3$)	Dissolved metal (μg/L)		Sediment metal (μg/g)		
							Cu	Zn	Cd	Cu	Zn
Reference sites											
Blackfoot River (198)	1996–1997	701–13,400	147–258	8.3–8.5	1.0–14.5	72–130	<1–2	<3	<0.8	29	68
	1998–1999	634–8,990	139–276	8.2–8.7	4.5–18.0	70–130	<1–2	<1–<20	<0.2	27	67
Rock Creek (171)	1996–1997	284–5,060	55–133	7.7–8.3	1.5–15.5	22–59	<1–1	<3	<0.8	14	45
	1998–1999	252–3,040	53–142	7.8–8.8	4.0–15.5	23–63	<1–2	2–<20	<0.2	16	47
Clark Fork											
Above Flathead (381)	1996–1997	NC	NC	NC	NC	NC	NC	NC	1.5	134	357
	1998–1999	NC	NC	NC	NC	NC	NC	NC	1.0	94	246
Turah (190)	1996–1997	845–9,650	136–392	7.8–8.5	2.0–17.0	58–180	3–20	<3–12	4.4	635	1,050
	1998–1999	596–6,410	138–380	7.7–8.8	4.0–19.5	59–170	3–9	2–<20	3.9	479	1,080
Goldcreek (85)	1996–1997	356–3,120	207–462	8.1–8.7	2.0–13.5	86–210	4–18	<3–19	5.5	1,080	1,070
	1998–1999	248–1,720	237–458	8.2–8.7	1.5–18.5	100–200	3–8	2–<20	3.5	780	1,080
Galen Exit (12)	1998–1999	NC	NC	NC	NC	NC	NC	NC	6.5	1,442	1,489
Near Galen (5)	1996–1997	109–1,050	237–511	8.0–9.0	1.5–19.0	100–230	5–21	<3–31	7.6	1,540	1,160
	1998–1999	91–522	197–517	8.4–8.8	4.0–15.0	81–230	3–7	2–<20	4.0	991	1,120

Table 2. Whole-body metal concentrations (nmol/g; mean ± standard deviation, n = 1–6) in taxa collected from the references sites (Blackfoot River and Rock Creek, MT, USA) and the Clark Fork (MT, USA). Letters signify results of statistical tests (among taxa, within sites and years). Samples with different letters were significantly different ($p < 0.05$). NA means ''not available'' and refers to station/year where abundance was too low to meet analytical requirements

Station name (km)	Year	Taxon	Cd	Cu	Zn
Reference sites					
Blackfoot River (198)	1997	*Hydropsyche* spp.	1.3 ± 0.10A	346 ± 11A	2,240 ± 29A
		Arctopsyche grandis	1.3 ± 0.2A	272 ± 44A	2,180 ± 123A
		Baetis spp.	4.7	267	1,170
		Epeorus albertae	7.1	950	1,630
		Serratella tibialis	19	1,640	4,130
	1999	*Hydropsyche* spp.	1.0 ± 0.01A	387 ± 9A	2,410 ± 12A
		A. grandis	1.92	426	2,480
		Baetis spp.	10.7	965	2,030
		E. albertae	8.6 ± 1.3A	1,160 ± 149A	1,930 ± 233A
		S. tibialis	12.3	1,860	3,350
Rock Creek (171)	1999	*Hydropsyche* spp.	NA	NA	NA
		A. grandis	1.8 ± 0.7A	265 ± 69A	2,140 ± 199A
		Baetis spp.	10.4	588	1,890
		E. albertae	14.1 ± 1.2B	658 ± 37B	1,620 ± 95B
		S. tibialis	37.3	1,300	4,920
Clark Fork					
Above Flathead (381)	1997	*Hydropsyche* spp.	10.7 ± 0.5A	696 ± 36A	2,530 ± 353A
		A. grandis	12.1 ± 0.6B	780 ±14B	2,760 ± 88A
		Baetis spp.	NA	NA	NA
		E. albertae	165	4,690	11,800
		S. tibialis	220	5,640	28,200
	1999	*Hydropsyche* spp.	11.6 ± 1.6A	696 ± 120A	2,740 ± 177A
		A. grandis	13.3 ± 1.0A	510 ± 97A	3,140 ± 343A
		Baetis spp.	NA	NA	NA
		E. albertae	66.6 ± 1.4B	2,710 ± 153B	7,680 ± 250B
		S. tibialis	190 ± 38C	5,330 ± 1,380C	25,600 ± 4,820C
Turah (190)	1997	*Hydropsyche* spp.	16.3 ± 0.2A	1,960 ± 57A	3,670 ± 147A
		A. grandis	24.6 ± 1.4B	1,740 ± 347A	4,130 ± 406A
		Baetis spp.	NA	NA	NA
		E. albertae	NA	NA	NA
		S. tibialis	353 ± 11C	6,900 ± 189B	45,800 ± 700B
	1999	*Hydropsyche* spp.	8.7 ± 0.4A	966 ± 29A	3,140 ± 317A
		A. grandis	5.2 ± 1.2A	867 ± 175A	3,080 ± 565A
		Baetis spp.	38.1	1,720	19,400
		E. albertae	66.3	2,690	13,600
		S. tibialis	148 ± 2B	5,100 ± 198B	40,500 ± 251B
Goldcreek (85)	1997	*Hydropsyche* spp.	15.4 ± 2.1A	3,100 ± 372A	3,880 ± 159A
		A. grandis	17.4 ± 2.1A	2,210 ± 313B	3,670 ± 369A
		Baetis spp.	239 ± 0.2B	2,210 ± 125B	21,200 ± 752B
	1999	*Hydropsyche* spp.	16.7 ± 3.4A	1,120 ± 223A	3,900 ± 741A
		A. grandis	32.8 ± 4.3B	712 ± 66B	4,480 ± 495A
		Baetis spp.	71.5	1,500	14,600
Galen Exit (12)	1999	*Hydropsyche* spp.	8.9 ± 0.8	1,360 ± 49	3,840 ± 102
		Baetis spp.	134	2,210	16,200
Near Galen (5)	1997	*Hydropsyche* spp.	9.0 ± 0.4	1,770 ± 66	3,270 ± 41
		Baetis spp.	102	1,630	12,100
	1999	*Hydropsyche* spp.	8.1 ± 0.3	1,100 ± 36	2,910 ± 108
		Baetis spp.	NA	NA	NA

both caddisflies. Zinc concentrations in those fractions were not significantly different between reference and contaminated populations (ANOVA; $p > 0.05$). Metal partitioning (the proportional contribution to the body burden described by the slope of the regression between the whole body and a subcellular fraction) generally did not vary significantly between samples from reference sites and the Clark Fork or between years. *Serratella* accumulated a greater proportion of Cd in the residual fraction in the Clark Fork (ANOVA; $p < 0.05$ between reference sites and the Clark Fork). Minor differences occurred in some fractions in *A. grandis* between years. Zinc bound to non-MBPs correlated significantly with whole-body concentrations in 1999 but not 1997 (Table 3); also, proportionally more Cu was associated with cytosolic proteins and the microsomal fraction in 1997 than in 1999. These differences appeared to be influenced by disparities in the range of concentrations represented in the samples from each year. For example, the correlation between whole-body and non-MBP Zn in 1999 was influenced by a single sample at the upper end of the concentration range (4,480 nmol/g Zn in the whole body and 1,100 nmol/g Zn in non-MBP). At comparable whole-body concentrations, metal concentrations among subcellular fractions were similar between years.

Metals were concentrated in cytosolic proteins, cell debris, and the residual fraction (Table 3). The microsomal fraction usually represented a relatively small percentage of the body burden. Cumulatively, cytosolic proteins and cell debris represented about 70 to 81% of the Cd body burden in all taxa

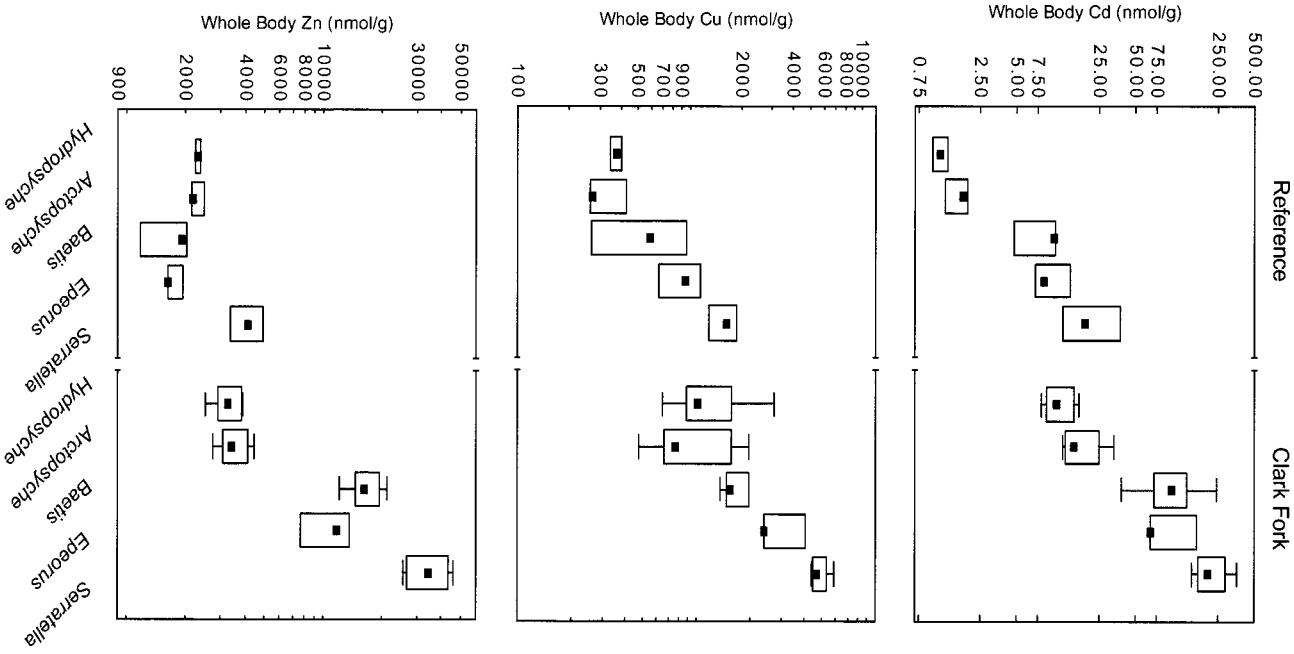

Fig. 2. Statistical summary of whole-body metal concentrations (Cd, Cu, and Zn) (nmol/g) for each taxon. Data for all samples collected from the reference sites (Blackfoot River and Rock Creek, MT, USA) (Table 1) were combined, as were all data from the Clark Fork (MT, USA) (symbol: median; box: 25th and 75th percentiles; whiskers: minimum and maximum).

except *S. tibialis* (Table 3). Accumulation of Cd in non-MBPs of the mayflies *Baetis* spp. and *E. albertae* was exceptional, with this fraction accounting for 43% of the body burden. Copper was largely distributed among cytosolic proteins, cell debris, and the residual fractions in all taxa (Table 3). More Zn was contained in the cell debris than any other fraction, and mayflies tended to accumulate more Zn in this fraction than caddisflies. Cytosolic proteins represented the secondary pool of Zn.

Metal partitioning to and the resulting metal concentrations of cytosolic MBP and non-MBPs varied considerably among taxa (Fig. 3). Metal-specific patterns also were evident in some

Table 3. The range in metal concentrations of the whole-body and subcellular fractions (nmol/g). Correlations between metal concentrations in the whole-body and subcellular fractions were usually significant ($r \geq 0.73$), except for those fractions designated NS ($p > 0.05$). Metal partitioning to each subcellular faction, expressed as the mean percentage (%) of the whole-body metal burden, is given as well

Element	Whole-body fraction	*Hydropsyche* spp. (n = 11)		*Arctopsyche grandis* (n = 9)		*Baetis* spp. (n = 8)		*Epeorus albertae* (n = 6)		*Serratella tibialis* (n = 7)	
		Range	%	Range	%	Range	%	Range	%	Range	%
Cd	Whole body	1.0–16.7		1.3–32.8		4.7–239		7.1–165		12.2–353	
	Residual	<0.2–3.8	16	<0.04–4.6	10	<0.05–8	1	<0.3–22.0	9	1.6–144	28
	MBP[a]	0.2–4.7	22	0.40–4.6	23	1.25–37	20	1.4–31.3	21	1.4–40	11
	Non-MBP	0.2–3.1	18	0.37–5.3	24	1.78–83	43	2.4–61.6	43	1.8–31	15
	Microsomal	0.02–1.4	6	0.06–2.0	9	0.09–33	9	0.6–17.3	8	3.1–36	18
	Cell debris	0.4–6.6	30	0.20–18.6	43	0.53–37	18	0.8–19.6	13	2.7–77	19
Cu	Whole body	346–3,100		265–2,210		267–2,210		658–4,690		1,300–6,900	
	Residual	64–953	27	56.5–599	27	60.5–1,029	35	170–674	20	212–2,540	26
	MBP	70–503	18	39.5–231	17	45.9–327	14	70–795	18	356–1,580	23
	Non-MBP	40–209	11	33.9–259	14	32.1–270	11	89–1,220	21	93–1,080	12
	Microsomal	28–229	7	17.5–143	8	31.1–154	6	66–464	10	144–629	11
	Cell debris	97–878	27	59.0–581	26	84.1–535	25	145–1,040	21	216–1,390	20
Zn	Whole body	2,240–3,900		2,140–4,480		1,170–21,200		1,615–13,600		3,350–45,800	
	Residual	292–1,192	19	204–1,050	16	37–3,850	9	225–1,650	15	247–14,300	17
	MBP	205–490 NS	14	347–601 NS	15	1,226–1,200	9	131–1,290	10	211–4,300	8
	Non-MBP	172–957 NS	21	571–1,100	24	200–1,630	13	206–1,690	16	309–2,170	8
	Microsomal	180–324 NS	8	184–364 NS	10	232–1,690	11	196–1,120	11	571–2,180	10
	Cell debris	518–1,647	32	441–2,150	33	405–11,000	53	584–7,640	45	1,301–19,500	41

[a] MBP = metal-binding protein.

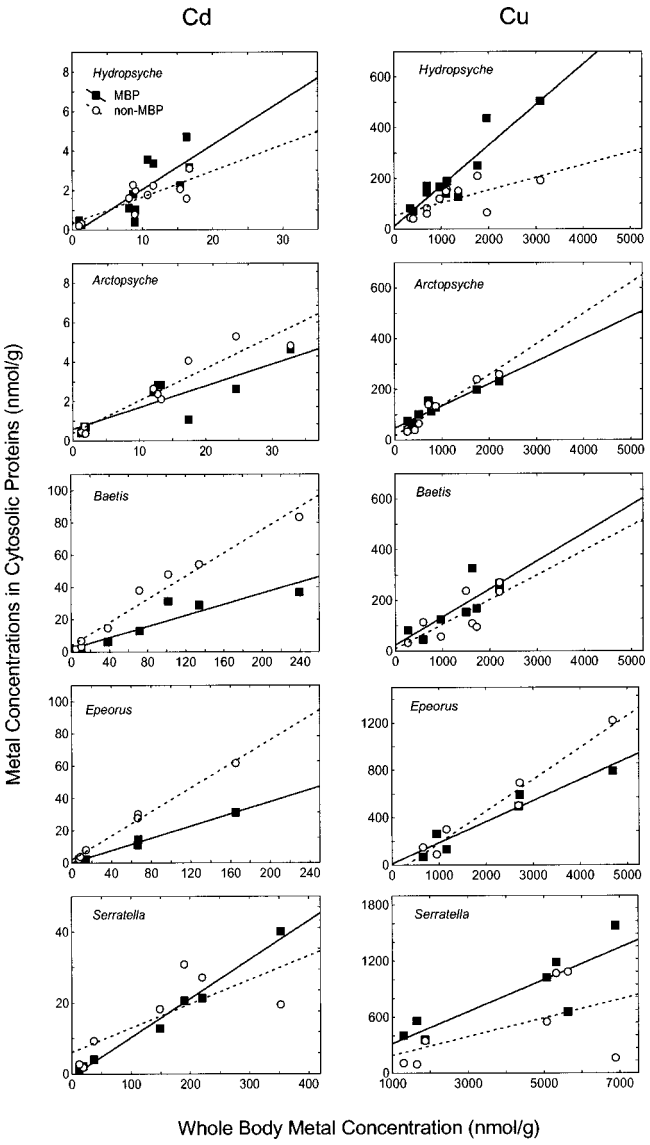

Fig. 3. Cadmium and Cu concentrations (nmol/g) of cytosolic metal-binding proteins (MBPs) (solid square) and non-MBPs (open circle) compared among taxa. Metal concentrations in cytosolic protein fractions are plotted relative to whole-body concentration. Data are the means of the samples.

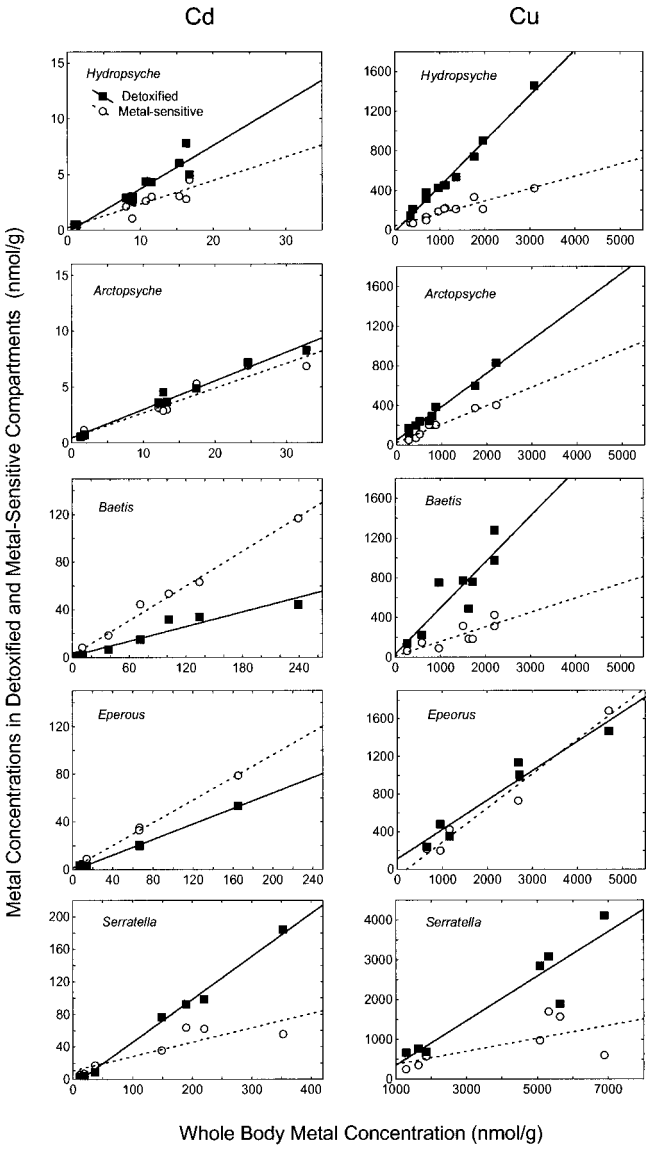

Fig. 4. Cadmium and Cu concentrations (nmol/g) of detoxified (solid square) and metal-sensitive (open circle) compartments compared among taxa. Metal concentrations of the compartments are plotted relative to whole-body concentrations. Data are the means of the samples.

taxa. However, no evidence was observed of a disproportionate shift in binding from one to the other protein fraction relative to increasing whole-body or cytosolic metal concentrations. In *Hydropsyche* spp., more Cu was bound to MBP than to non-MBP as whole-body concentrations increased (homogeneous slopes model, general linear model). This binding pattern was suggested for Cd also, although the slopes describing the binding of Cd to MBP and non-MBP were not significantly different. *Arctopsyche* partitioned Cd and Cu equally between MBP and non-MBPs. Metal concentrations of cytosolic proteins were generally less in caddisflies than mayflies. In *Baetis* spp. and *E. albertae*, Cd was bound largely to non-MBPs. Higher concentrations of Cu also were associated with non-MBPs than MBPs in *E. albertae*. In *Baetis* spp., however, the binding pattern and concentrations of Cu in the protein fractions were similar to those of *A. grandis*. Partitioning of Cd and Cu to cytosolic proteins in *S. tibialis* was variable among samples; however, the slopes and the mean concentrations of Cd and Cu for the two fractions were not significantly different. The majority of cytosolic Zn in caddisflies was bound to non-MBPs (Table 3). In mayflies, Zn was partitioned more equally between both cytosolic fractions, and the concentrations in each fraction were greater than those in caddisflies.

Metal concentrations in metal-sensitive (non-MBP plus microsomal) and detoxified compartments (MBP plus residual) were closely related to whole-body concentrations, but partitioning patterns differed somewhat to those observed in the cytosolic protein fractions (Fig. 4). The contribution of the residual fraction to the detoxified compartment generally increased metals in that compartment relative to those in the metal-sensitive compartment. This was not the case for Cd in *Baetis* spp. and *E. albertae* (Table 3), and consequently approximately 50% of their Cd body burdens were associated with the metal-sensitive compartment (Fig. 5). *Epeorus* also accumulated a higher proportion of its Cu body burden in the metal-sensitive compartment than other taxa (Fig. 5). Zinc in

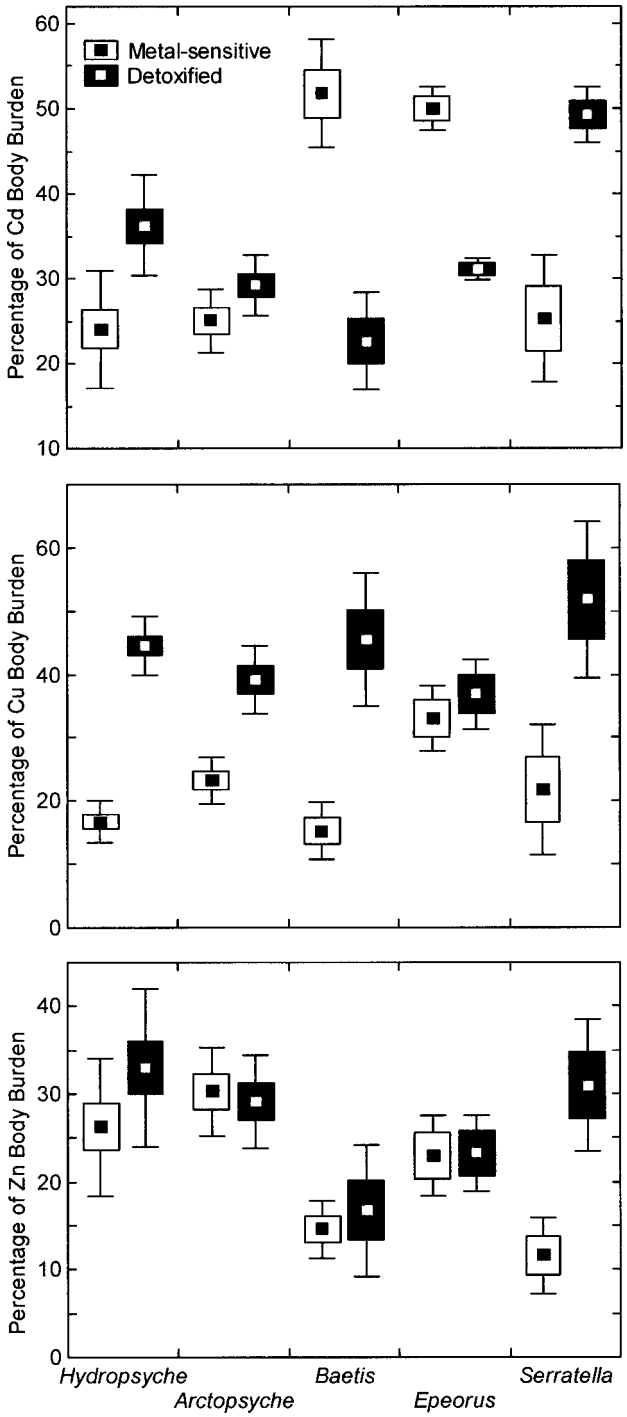

Fig. 5. Metal partitioning in Cd, Cu, and Zn between the detoxified (solid box with open symbol) and metal-sensitive (open box with solid symbol) compartments of taxa collected from the Clark Fork (MT, USA), exclusively (data from reference sites excluded). Partitioning is expressed as the percentage of the total body burden (symbol: mean; box: ±1 standard error; whiskers: ±1 standard deviation). Data represented are the means of the samples.

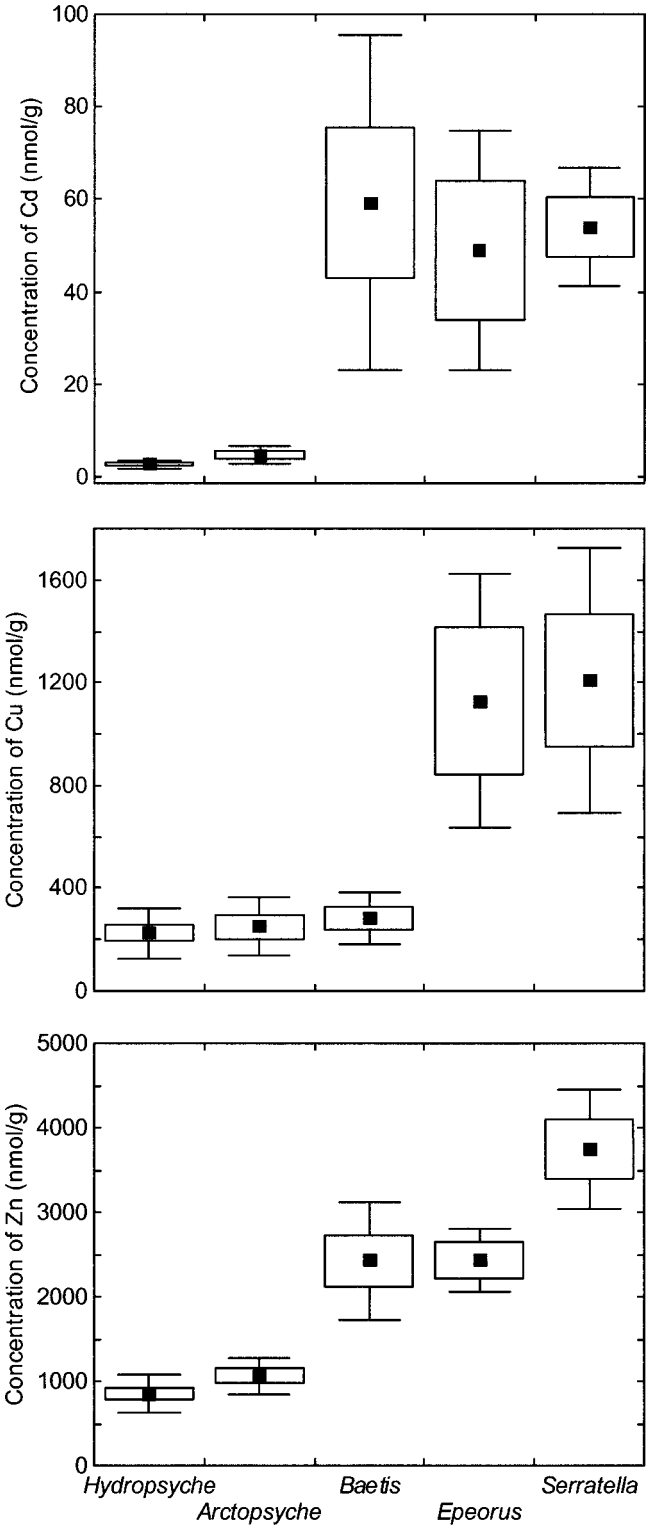

Fig. 6. Metal concentrations in Cd, Cu, and Zn (nmol/g) (symbol: mean; box: ±1 standard error; whiskers: ±1 standard deviation) of the metal-sensitive compartment in taxa from the Clark Fork (MT, USA) exclusively (data from reference sites excluded). Data represented are the means of the samples.

both compartments correlated with whole-body Zn in mayflies (data not shown). In caddisflies, however, Zn in the detoxified compartment but not the metal-sensitive compartment correlated with whole-body concentrations, as expected from results in Table 3. Zinc was equally partitioned between the metal-sensitive and detoxified compartments in all taxa, except *S. tibialis* (Fig. 5).

Metal concentrations in the metal-sensitive compartment of each taxon are summarized in Figure 6. Metal concentrations were consistently low in *Hydropsyche* spp. and *A. grandis* and consistently high in *E. albertae* and *S. tibialis* (despite the high proportion of detoxified metal in *S. tibialis*). Cadmium

and Zn concentrations in *Baetis* spp. were relatively high, like other mayflies. However, Cu concentrations in *Baetis* spp. and caddisflies were comparable. Recalling that *Baetis* spp. and the caddisflies were metal tolerant while *E. albertae* and *S. tibialis* were sensitive, interspecific differences in metal tolerance would appear to be most strongly associated with interspecific differences in Cu concentrations than either Cd or Zn.

DISCUSSION

The objective of this study was to determine if interspecific differences in metal bioaccumulation could be mechanistically linked to observed population-level effects (abundance) in the Clark Fork. The relatively low whole-body concentrations of metals in caddisflies suggested that they would be less vulnerable to metal contamination than mayflies. Analysis of subcellular partitioning provided further evidence of differences in vulnerability that related to the spatial distribution of most of the taxa we examined.

Metal exposure

Metal exposures to benthic macroinvertebrates in the Clark Fork reflect physical and chemical processes affecting the dispersion of contaminated sediments and the influence of remediation [11,14,22]. Site-specific exposures generally corresponded to the longitudinal gradient of metal concentrations in sediments that is predicted from physical mixing and dilution of contaminated sediments from upstream with uncontaminated sediments delivered by tributaries [22]. Interannual differences in tissue concentration appear related to hydrologic processes controlling the mobilization of sediment-bound metals stored in the streambed and floodplain [11]. Metal concentrations in water, in invertebrates, and to a lesser extent in sediments have declined in the upper 40 km of the river. These changes, first noticeable in 1998, appear to be in response to remediation of streambanks and the riparian zone, initiated in 1991. Remediation also includes treatment of water from Silver Bow Creek and release of that water to the Clark Fork. This might mitigate exposures to organisms in the upper Clark Fork even though sediments remain contaminated.

Interspecific differences in bioaccumulation

Some studies have related the onset of toxicity to an increase in the concentration of metal associated with non-MBP cytosolic ligands [23,24], manifested as a shift in the distribution of metal between MBPs and non-MBPs. The inflection is thought to signal the saturation of MBP, allowing metals to bind nonspecifically to other macromolecules where they exert toxic effects. This accumulation pattern, termed spillover, was not observed in any of the taxa we examined from the Clark Fork. The proportional metal accumulation into the non-MBP and MBP fractions was fairly constant. In all species, whole-body and total cytosolic Cu and Cd concentrations correlated positively with Cu and Cd bound to MBPs, suggesting the induction of MBP and increased metal tolerance in response to elevated metal exposures [25]. Metal accumulation in non-MBPs was typically observed also, which is consistent with both laboratory [26,27] and field studies [28,29].

Nonetheless, binding patterns between non-MBPs and MBP exhibited by these taxa may be an indication of their vulnerability to metal exposure. Slopes describing the accumulation of Cu and Cd into the respective protein fractions, relative to whole-body concentrations, indicated that more Cd was accumulated into non-MBPs than MBPs in both *Baetis* spp. and *E. albertae*. *Epeorus* also accumulated more Cu into non-MBPs than MBPs. But in *Baetis* spp., Cu accumulation into non-MBPs and MBP was equivalent. The patterns in both taxa suggest partial compensation of the cytosolic dose by MBP, but compensation appeared less effective at the higher cytosolic concentrations experienced by *E. albertae*. *Epeorus* accumulated higher concentrations of Cu (about fourfold) into non-MBPs than *Baetis* spp. at contaminated sites, and thus it seems that it would be more vulnerable to Cu toxicity. In *S. tibialis*, the absolute concentrations of Cd and Cu bound to non-MBPs were comparable to those in *E. albertae* and *Baetis* spp. (Cd only), although they represented a lower proportion of the body burden. Saturation of the binding capacity of MBP (where metal uptake exceeds the rate of MBP synthesis) accompanied by a disproportionate accumulation of metals into non-MBPs might occur at higher exposures, but it is possible that a constant, higher rate of binding to non-MBPs might lead to the same result in chronically contaminated populations as the more traditional concept of spillover. *Hydropsyche* spp., in contrast to mayflies, accumulated a greater proportion of cytosolic Cu and Cd into MBPs than into non-MBPs at the highest cytosolic concentrations. The resultant low cytosolic concentrations, coupled with more efficient detoxification, should reduce *Hydropsyche*'s vulnerability to these metals. In *A. grandis*, concentrations of cytosolic Cd and Cu were relatively low, and these metals were distributed more or less equally between MBP and non-MBPs.

The binding patterns of cytosolic Zn were consistent with chromatographic data that have shown that Zn is bound to a variety of low- and high-molecular-weight (non-MBP) molecules [18]. Interspecific differences were evident in the concentrations and cytosolic partitioning of Zn between caddisflies and mayflies. Cytosolic Zn concentrations in caddisflies did not increase with whole-body concentrations and were lower than in mayflies. This partitioning pattern might be expected if cytosolic Zn was effectively regulated in these caddisflies. In mayflies, Zn in both MBP and non-MBP fractions increased proportionately with whole-body Zn. The higher cytosolic Zn in mayflies may promote the induction of MBP, which binds excess Zn. Alternatively, Zn recovered with heat-stable proteins may actually be bound to ligands that are chromatographically distinct from metallothionein [20]. Nonspecific binding of Zn to heat-denatured molecules, represented by Zn in the non-MBP fraction, was suggested as well.

The microsomal fraction typically accounted for less than 10% of the total body burden and evidently has a relatively low number of metal-binding sites. This is consistent with results that have been reported for other invertebrates [26,30].

The residual and cell debris fractions were less well characterized than the microsomal and cytosolic protein fractions. Both fractions contained relatively high proportions of the metal body burden in most taxa, indicating that they comprise a large number and variety of metal-binding sites. As stated earlier, the residual fraction probably represented biologically inactive and detoxified forms of metal, and for that reason it was included in the detoxified compartment.

Inclusion of residual metal substantially increased the concentration of detoxified metals to the point where concentrations frequently equaled or exceeded those in the metal-sensitive compartment. However, MBPs accounted for the majority of detoxified Cd in *Baetis* spp. and *E. albertae*. Metals associated with non-MBPs usually contributed more metal

than the microsomal fraction to the metal-sensitive compartment. Therefore, metal partitioning to non-MBPs was particularly important in determining interspecific differences in the metal concentrations of the metal-sensitive compartment.

Ecological implications

Results of an ecological risk assessment performed in the Clark Fork basin indicated that metal exposure stemming from the chronically contaminated sediments stored in the stream channel and floodplain has impaired biological communities in the upper Clark Fork [31]. Changes in structural features of the macroinvertebrate community associated with the historical metal contamination gradient [9,12] were typical of rivers of the western United States affected by metals [2].

The longitudinal distributions—and inferred metal tolerances—of most taxa considered in this study corresponded to the subcellular partitioning and the resulting metal concentrations associated with metal-sensitive fractions. The metal sensitivity of *E. albertae* and *S. tibialis* may generally result from their high concentrations of Cd, Cu, and Zn and accumulation of these metals into non-MBPs and other metal-sensitive fractions. In contrast, metal tolerance of *Hydropsyche* spp. may be explained by its ability to limit metal accumulation, sequester cytosolic metals into MBPs, and thereby reduce the interaction of metals with metal-sensitive sites. However, interspecific differences in Cu concentrations seemed the most significant aspect of bioaccumulation relative to differential metal tolerance expressed among these five taxa. In particular, metal tolerance in *Baetis* spp. appeared to be related to the bioaccumulation of Cu. Concentrations of Cu in the metal-sensitive compartment of *Hydropsyche* spp. and *Baetis* spp. were similar to one another and one-third or less than those in *E. albertae* and *S. tibialis*. Limiting Cu accumulation provides a mechanism by which *Baetis* spp. could tolerate the high Cu exposures in the Clark Fork and conceivably reduce its overall risk.

However, the high concentrations of Cd and Zn, which were associated primarily with non-MBP, indicate that *Baetis* spp. could be vulnerable to Cd and Zn toxicity. *Baetis* spp. collected from the Arkansas River (CO, USA) exhibited the same metal-specific accumulation of Cd, Cu, and Zn that we observed in the Clark Fork [32]. The authors of that study attributed high Zn exposures in the Arkansas River to lower abundances of the taxon.

In the Clark Fork, *Arctopsyche grandis* has been considered moderately tolerant to metals because of its longitudinal distribution patterns. Surveys, conducted annually since 1986, have shown that *A. grandis* has rarely occupied sites upstream of Goldcreek, where contamination levels have been greatest historically [9], inferring that metals exposure had prevented colonization of these sites. We suggest that other environmental factors may be as important as metals in determining its distribution in the Clark Fork. Our results showed that this species limited metal accumulation to relatively low concentrations (comparable to *Hydropsyche* spp.). Therefore, we would expect *A. grandis* to be relatively tolerant. In fact, *A. grandis* inhabits other, highly contaminated streams in the western United States, consistent with the notion that it is metal tolerant [32]. Thus, it is likely that certain ecological preferences of *A. grandis* are not met in the upper Clark Fork.

High metal concentrations in herbivores and detritivores (e.g., *E. albertae* and *S. tibialis*) may reflect ingestion of highly contaminated periphyton, as suggested by others [33]. However, the relatively low Cu concentrations in the herbivore *Baetis* spp. exemplifies the difficulty in generalizing metal bioaccumulation patterns based on functional feeding category. A better understanding of uptake from solute and dietary pathways could help resolve such ambiguities [34,35].

An understanding of the relative vulnerabilities of taxa suggests possible recovery scenarios where metal exposures are being mitigated by remediation. For example, if metal toxicity were the primary limiting factor for metal-sensitive taxa, one would expect populations in the upper Clark Fork to recover in response to remediation. Recovery within the upper 10 to 15 km of the river is in fact indicated by an increase in taxa richness and Ephemeroptera, Plecoptera, and Trichoptera richness in recent years [13]. Abundance data show little evidence that either heptageniid mayflies, including *E. albertae*, or *S. tibialis* have colonized the upper Clark Fork, although populations occur in nearby tributaries (Warm Springs Creek and the Little Blackfoot River). It is possible that these taxa will become established over time if exposures continue to decline and other factors (e.g., availability of suitable substrate) are favorable. The influence of other environmental factors on species assemblages has not been as thoroughly characterized as metals, however. These factors could confound impacts attributed to metal toxicity and would be expected to have greater influence on the structure of species assemblages as metal exposures are mitigated.

CONCLUSION

Others have pointed out that studies at lower levels of biological organization are needed to provide mechanistic support for population- and community-level effects [36,37]. Changes in species assemblages associated with increasing metal exposure appear to operate through the progressive selection against the least tolerant to the most tolerant taxa within the community. Local extinction of sensitive species is the most obvious way that metal exposure is manifested in community-level attributes. However, even when metal concentrations in the environment are known, it is often difficult to isolate metals from other factors that may be affecting ecological change. Metal-specific sensitivities of resident taxa are rarely known [3]. Typically, sensitivities are based on nonspecific tolerance indices and/or surveys of the site. Therefore, demonstration of a plausible mechanism relating metal exposure to species vulnerability would improve the interpretation of causation. In this study, comparative analysis of subcellular metal partitioning into metal-sensitive and detoxified compartments was used as a basis to interpret vulnerabilities of different taxa to metal exposure. In accordance with conceptual views of metal toxicity, accumulation of high concentrations of metal in fractions containing metal-sensitive sites appeared symptomatic of species deemed sensitive in the Clark Fork. We suggest that the absence of those species from sites where metal exposures were highest was an indication of where metal-specific effects were most likely occurring. Recovery of sensitive taxa might be expected as metal exposure is mitigated. We suggest that diagnostic evidence based on an understanding of mechanisms underlying interspecific sensitivities to metals could help resolve metal-specific effects on resident organisms.

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