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Differences in Dissolved Cadmium and Zinc Uptake among Stream Insects: Mechanistic Explanations

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This study examined the extent to which dissolved Cd and Zn uptake rates vary in several aquatic insect taxa commonly used as indicators of ecological health. We further attempted to explain the mechanisms underlying observed differences. By comparing dissolved Cd and Zn uptake rates in several aquatic insect species, we demonstrated that species vary widely in these processes. Dissolved uptake rates were not related to gross morphological features such as body size or gill size—features that influence water permeability and therefore have ionoregulatory importance. However, finer morphological features, specifically, the relative numbers of ionoregulatory cells (chloride cells), appeared to be related to dissolved metal uptake rates. This observation was supported by Michaelis−Menten type kinetics experiments, which showed that dissolved Cd uptake rates were driven by the numbers of Cd transporters and not by the affinities of those transporters to Cd. Calcium concentrations in exposure media similarly affected Cd and Zn uptake rates in the caddisfly *Hydropsyche californica*. Dissolved Cd and Zn uptake rates strongly co-varied among species, suggesting that these metals are transported by similar mechanisms.

Introduction

Aquatic insects are widely utilized for freshwater bioassessment and ecological risk assessment. They are useful ecological indicators for several reasons, including their taxonomic richness and their differential susceptibilities to environmental stressors (*1*) such as trace metal pollution (*2*, *3*). Differences in metal sensitivity might ultimately result in the loss of some species but not others from metal-contaminated areas. Determining the underlying mechanisms responsible for species-specific sensitivity differences could reduce ambiguities in separating metal effects from other stressors and help to define which suites of aquatic insects might be the most effective indicators of metal pollution. The overall metal sensitivity of a given taxon results from the interplay among parameters that govern exposure (bioaccumulation via aqueous and dietary routes and loss dynamics) as well as detoxification processes (*4*), reflected in internal distribution patterns (*5*, *6*). However, the degree to which these processes differ across stream insect species is largely unknown. This paper explores physiological and morphological characteristics involved in dissolved cadmium and zinc uptake rate differences in a variety of insect species that are thought to differ in their sensitivities to trace metal pollution.

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Wide variation in physiological and morphological characteristics accompanies the broad taxonomic diversity of aquatic insects. Much of this variation is associated with the insects' evolutionary history as secondarily aquatic organisms. Though the earliest insects are believed to have been aquatic, current aquatic insect lineages are thought to have arisen from numerous invasions of aquatic habitats by several different terrestrial ancestors over evolutionary history (*7*). Primary challenges associated with the transition from land to freshwater include exchanging respiratory gases and maintaining salt/water balance in a hypo-osmotic environment (*8*). The terrestrial ancestors of aquatic insects performed their osmoregulatory functions in the gut, malpighian tubules, and the rectum (*9*). Aquatic insects also perform osmoregulatory functions in these locations. However, aquatic insect taxa (Megalopterans excluded) can have specialized chloride cells or chloride epithelia on the body surface for the sequestration of ions from the water column (*8*). Chloride cells help to counteract diffusive ion loss via paracellular pathways as well as the urinary loss of ions associated with the removal of excess body water (*9*−*12*). As a result, osmoregulation in aquatic insects occurs both on the body surface as well as in the digestive tract.

Insects have developed both morphological and physiological solutions to the respiratory and osmoregulatory challenges associated with aquatic life. Some of these solutions are evasive in nature while others are compensatory (*13*). For example, evasive strategies include breathing atmospheric gases and maintaining a relatively impermeable barrier to help control the inflow of water and the dissipative loss of ions. Compensatory strategies include utilizing dissolved oxygen through gills and performing significant osmoregulatory functions on the body surface using chloride cells (*8*).

Where species fall on the continuum of evasive to compensatory in terms of gas, water, and ion exchange has potential consequences for contaminant bioaccumulation. For example, large gill surfaces lead to high water permeability and fast accumulation rates of the organophosphate pesticide chlorpyrifos (*14*, *15*). Water permeability might secondarily affect dissolved metal bioaccumulation if animals have accelerated ion uptake mechanisms to compensate for ion loss through leaky epithelia or urinary ion loss associated with excreting excess body water. Alternatively, the number of chloride cells could affect metal uptake if they affect the number of ion channels associated with the uptake of ions such as calcium.

Morphological characteristics such as body size could possibly influence the fluxes of water and ions. The typically small body size of insects results in large surface area-to-volume relationships, which, in turn, place unique demands on the integument and transporting epithelia to maintain salt/water balance (*16*). Body size can affect metal uptake rates within species (*17*), demonstrating the potential importance of surface area-to-volume ratios. Another potentially important morphological characteristic is gill size for reasons described above.

Here we ask: Do dissolved Cd and Zn uptake rates vary across species, and if so, why do they differ? Can morphological characteristics such as body size/gill size explain differences? Do physiological characters associated with respiration, water permeability, or osmoregulation explain or predict differences? Are the numbers of metal transporters, as defined by kinetics experiments, associated with differences in Cd uptake dynamics between species?

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TABLE 1. Test Species

order	family	genus species	habitat & feeding
Ephemeroptera	Ephemerellidae	*Drunella grandis*	riffles, scrapers
Ephemeroptera	Ephemerellidae	*Drunella flavilinea*	riffles, scrapers
Ephemeroptera	Heptageniidae	*Rhythrogena morrisoni*	riffles, scrapers
Ephemeroptera	Heptageniidae	*Epeorus* sp.	riffles, scrapers
Ephemeroptera	Ameletidae	*Ameletus* sp.	riffles, glides, collectors
Ephemeroptera	Siphlonuridae	*Siphlonuris* sp.	backwaters, collectors
Trichoptera	Hydropsychidae	*Hydropsyche californica*	riffles, omnivorous
Trichoptera	Hydropsychidae	*Hydropsyche oslari*	riffles, omnivorous
Plecoptera	Perlidae	*Calineuria californica*	riffles, predatory

Materials and Methods

Insect Collecting and Handling. All of the animals used in these studies were field collected, wild populations belonging to the orders Ephemeroptera, Plecoptera, or Trichoptera (EPT). These taxa are commonly focused upon in bioassessments. The majority of taxa chosen for this study were mayflies, on the basis of numerous reports of their apparent sensitivity to metals relative to other taxa (*3*, *6*, *18*, *19*). In addition, the range of gill sizes among mayflies allowed us to explore this character as a determinant of metal uptake.

Two ephemeropteran species each were tested from the families Heptageniidae and Ephemerelidae. Both are described as being particularly sensitive to metal pollution (*3*, *6*, *19*). Conversely, two trichpteran species, both Hydropsychidae, were chosen on the basis of descriptions of their tolerance to metal pollution (*6*). Although diet was not a part of this study, the species used in this study represent the major insect functional feeding groups, summarized in Table 1.

The insects were collected from streams in California with a D-frame kick net. The mayflies *Drunella grandis, Ameletus* sp., *Rhythrogena morrisoni,* and the stonefly *Calineuria californica* were collected from the Trinity River near Douglas City, CA (40°39′30″ N, 122°55′30″ W). The mayfly *Epeorus* sp. and caddisfly *H. oslari* were collected from Stevens Creek above the Stevens Creek reservoir, Santa Clara County, CA, while *H. californica* was collected from Stevens Creek below the reservoir (37°17′20″ N, 122°04′20″ W). The mayfly *Siphlonuris* sp. was collected from San Francisquito Creek in Menlo Park, CA (37°27′15″ N, 122°09′37″ W). The mayfly *D. flavilinea* was collected from Pescadero Creek, upstream from Pescadero, CA (37°16′30″ N, 122°17′45″ W).

Insects were transported on ice in Whirl Pac bags containing streamwater from the insects' respective locations. They were held in an environmentally controlled room with a light:dark photoperiod of 16:8 h and a constant temperature of 15 °C. Soft artificial river water (ATSM) (48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4 \cdot 2H_2O$, 30 mg/L $MgSO_4$, and 2 mg/L KCl), pH 6.85, was used for acclimation to laboratory conditions and experiments. Insects were acclimated to lab conditions for a minimum of 4 days prior to experimentation and fed ad libitum a diet consisting of alfalfa and Spirulina aquarium flake food (O. S. I. Marine Lab, Inc., Hayward, CA). Insects were not fed for 1 day prior to experiments. This reduced fecal material output and metal sorption onto fecal material during exposures. Only apparently healthy, active animals were used in these experiments.

Water samples were collected to ensure that none of the habitats were heavily contaminated with metals. Samples were collected into 500-mL polypropylene screw-cap bottles that had been previously acid-washed, rinsed in deionized water (>18 MΩ), dried, and sealed. At the site, the bottles were submerged, opened, and then rinsed three times. Water was collected by slowly filling the bottles while moving them vertically from the near subsurface to about 0.1 m above the stream bed. The bottles were resealed underwater and then placed in an ice cooler. Replicate subsamples (i.e., sample splits) of whole-water samples were prepared for ICP-MS analysis by the method described by Garbarino and Hoffman (*20*), except samples were digested in 0.3 N HNO_3. Samples were filtered (0.4 μm Millipore) under a laminar flow hood. Quality control for elemental analysis by ICP-MS included reanalysis of a calibration standard (∼every 10 analysis) and analysis of process blanks, sample splits, and certified references for river water (SLRS-4, National Research Council Canada). Cadmium concentrations at all of the sites excluding San Francisquito Creek were <0.01 and 0.02 μg/L in dissolved and whole water samples, respectively. Zinc concentrations at all of the sites excluding San Francisquito Creek were ≤1.9 and 3.3 μg/L in dissolved and whole water samples, respectively. San Francisquito Creek is an urban stream and had Cd concentrations of 0.01 and 1.2 μg/L and Zn concentrations of 5.4 and 20.1 μg/L in dissolved and whole water samples, respectively. These values are below the Environmental Protection Agency's chronic water quality criteria for both metals (*21*), but nonetheless can be considered moderately contaminated.

Metal Accumulation Studies. ^{109}Cd and ^{65}Zn (1 mCi each) were obtained from U.S. Department of Energy, Los Alamos, NM, in 0.1 N HNO_3. Both metal solutions contained a 1:1 stable and isotopic metal ratio upon receipt. Primary stock solutions were prepared in 0.1 N HNO_3 to obtain activities of 1.428 μCi/mL Cd and 0.625 μCi/mL Zn. Exposure chambers were 100 mL Nalgene polypropylene beakers containing 50 mL artificial soft water, metal stock solution, and NaOH to adjust pH to 6.85, yielding final activities of 4.9 and 4.5 μCi/L for Cd and Zn, respectively. The combined radioactive and stable metal concentrations in the exposure media were 32 ng/L for both Cd and Zn (0.293 nM Cd and 0.492 nM Zn). The Windermere humic aqueous model (WHAM) was used to calculate free-ion concentrations of 0.269 nM Cd^{+2} and 0.372 nM Zn^{2+} under these experimental conditions.

Water from each exposure chamber was assayed for radioactivity using a Wallac 1480 gamma counter (Gaithersburg, MD) at both the beginning and end of exposures. Loss of dissolved metals to the containers and organisms was <10% in all experiments excluding those using *Drunella*. All gamma counting was accompanied by the use of counting standards and blanks, with the appropriate adjustments made for counting efficiencies and radioactive decay.

Uptake rates of Cd and Zn were measured separately for each taxon. Very short exposure periods (4 h) were used in these studies for several reasons. We wanted to minimize the potentially confounding influences of metal efflux, changes in water chemistry resulting from excretion of metabolic wastes, and feeding during exposure (*22*). A single larva was placed in each exposure chamber, with a minimum of five individuals used per taxon for each experiment. Exposure chambers were placed on a tray, which was affixed to an orbital shaker, providing a gentle swirling motion to decrease boundary layer effects while not interfering with the animal's ability to maintain its position in the chamber.

Animals were held in exposure chambers for a total of 4 h. After each hour of exposure, individuals were removed from their respective chambers, rinsed with artificial river water, transferred to 20-mL vials containing artificial river water, assayed in vivo for radioactivity, and then returned to their respective exposure chambers. Metal uptake was linear for all species over the 4-h time course, and uptake rates were calculated as the average of the slopes of each individual (from 1 to 4 h) in a given taxon. Slopes were not fit through the origin because we assumed that sorption of Cd and Zn onto the integument of the larvae would occur instantaneously and be completed within 1 h of exposure. *Y*-intercepts were estimated from the slopes of the uptake curves to provide the basis for comparing the relative affinities of the integument of different species to metals.

Water Permeability Studies. The water permeabilities of *C. californica*, *Ameletus* sp., *D. grandis*, and *R. morrisoni* were compared by holding five individual larvae in 48 mL artificial river water spiked with ^3H_2O to obtain specific activities of 15.0 μCi/L. Exposure water activities were verified by taking two 1-mL aliquots from each exposure chamber prior to initiating exposures. These water samples were counted in 18 mL of Scintisafe liquid scintillation cocktail (LSC) for 5 min. Larvae were exposed to ^3H_2O solutions for 45 min. This time point was chosen on the basis of previous studies of water permeability in aquatic insects (*15*). After exposure, insects were rinsed, weighed, and digested with Scintigest in 20-mL liquid scintillation vials at 50 °C. The digests were neutralized with HCl, and 18 mL of Scintisafe was added to each vial for liquid scintillation counting for 5 min. Five individuals of each taxon were blotted dry, weighed, and then dried at 50 °C for 36 h to determine differences in percent water content among taxa.

Microscopy. Whole larvae and isolated gills were fixed in 100% ethanol, freeze-dried, and mounted for scanning electron microscopy. The mounted specimens were coated with approximately 50 Angstroms Au/Pd, 60/40 with a Hummer X (*10*) sputter coater. A LEO 982 FESEM field emission scanning electron microscope was used to qualitatively examine chloride cell densities on the surfaces of gills and other structures. In other studies, live larvae were immersed in 4% w/v $AgNO_3$ for 20 min. Following Ag exposure, the larvae were fixed in 75% ethanol and exposed to light for 30 min. Chloride cells were readily identified as darkly stained structures, generally concentrated on the gill surfaces. Gill sizes were not measured in these studies but varied widely among species.

Michaelis−Menten Kinetics. A kinetic approach was taken to assess the relative importance of the numbers of transporters versus the transporter affinity in determining differences in dissolved Cd uptake. Experiments were not conducted with Zn. We chose the fastest Cd accumulator (*D. flavilinea*) and a slow Cd accumulator (*Siphlonuris* sp.) from previous experiments for this study. The effect of ambient Cd concentration on Cd uptake rate was assessed by exposing these two mayfly species to six nominal Cd concentrations (total dissolved) ranging from 32 to 5090 ng/L (0.30−47.0 nM). At each Cd concentration, 4-h time course experiments were conducted as described above. V_{max} and K_d values were calculated in two ways for each species using Prism 4.0 software. The first used rate data (the slopes of the uptake lines from 1 to 4 h, to minimize the effects of Cd bound to the integument) as a function of Cd concentration. The second used total accumulation divided by exposure time (4 h), which included Cd accumulated internally and on the body surface. The equation

$$Y = V_{max} \times [Cd]/(K_d + [Cd]) \quad (1)$$

was used for both analyses, where V_{max} is maximal binding of Cd to transporters, [Cd] is expressed in nanomoles, and K_d is the concentration of Cd required to reach half-maximal saturation. *Y* is the accumulation of Cd in terms of rate or total accumulation/exposure duration.

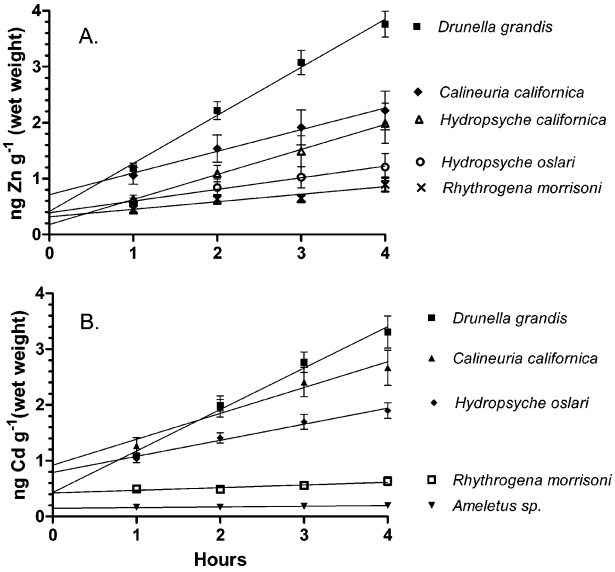

FIGURE 1. Dissolved zinc (panel A) and cadmium (panel B) uptake rates in several species of aquatic insects. Error bars represent the standard errors of the means.

Calcium Competition and Calcium Channel Blocker Effects on Dissolved Cd Uptake. We tested if calcium channels might be a route of uptake and whether the number of channels affected uptake rates by evaluating the effects of ambient calcium concentrations and verapamil, a calcium channel blocker, on metal uptake. Calcium concentration effects on metal accumulation were examined for both Cd and Zn in the caddisfly *H. californica* ($n = 12$ per treatment group) and for Cd only in the mayfly *D. flavilinea* ($n = 15$ per treatment group), with exposures lasting 7 h. These taxa were chosen solely on the basis of their local availability. Exposure media was artificial water described above (178 μM Ca), with additional $CaCO_3$ added to the high Ca groups (712 μM Ca for experiments with *D. flavilinea* and 1.424 mM Ca for experiments with *H. californica*). The effect of water chemistry on metal speciation was examined using WHAM, and metal uptake was normalized for free-ion concentration for each metal. In studies with *H. californica*, the calcium channel blocker verapamil (100 μM) was tested in artificial soft water (178 μM Ca) with both Cd and Zn present in the exposure media. In studies with *D. flavilinea*, verapamil at 10 and 300 μM was tested with Cd only.

Statistical Analysis. Because metal uptake was linear for each individual of every taxon tested, uptake rates for a given species were calculated as the average of the uptake slopes for all individuals. The comparison of dissolved Zn and Cd uptake rates across species was assessed via a Pearson correlation coefficient. Michealis−Menten constants (V_{max} and K_d) were calculated from both rate data and total accumulation data to highlight the importance of metal binding to the integument. T-tests were used to compare the effects of calcium concentration on Cd and Zn uptake (*23*).

Results and Discussion

Metal Bioconcentration by Dissolved Uptake. Dissolved Cd and Zn uptake rates were remarkably variable across the seven species tested for Zn and nine species tested for Cd (Table 2 and Figure 1a−b). The ephemerellid mayflies *D. grandis* (0.74 ± 0.06 ng g^{-1} h^{-1}) and *D. flavilinea* (1.33 ± 0.85 ng g^{-1} h^{-1}) accumulated Cd and Zn more rapidly than other

TABLE 2. Cd and Zn Accumulation Rates in Several Aquatic Insect Species

taxon	Zn ($ng\ g^{-1}\ h^{-1}$)	*y*-intercept ($ng\ g^{-1}$)	wet wt (mg)	*n*	r^2	*p*	Cd ($ng\ g^{-1}\ h^{-1}$)	*y*-intercept ($ng\ g^{-1}$)	wet wt (mg)	*n*	r^2	*p*
Drunella grandis	0.86 ± 0.06	0.42 ± 0.15	101 ± 4	5	0.99	<0.01	0.74 ± 0.06	0.43 ± 0.16	96.1 ± 4.4	5	0.99	<0.05
Drunella flavilinea	nd						1.30 ± 0.09	0.86 ± 0.23	18.1 ± 3.3	5	0.99	<0.01
Rhythroge na morrisoni	0.14 ± 0.03	0.32 ± 0.08	25 ± 4	9	0.92	0.04	0.05 ± 0.01	0.43 ± 0.04	45.5 ± 5.4	5	0.85	0.07
Epeorus sp.	0.07 ± 0.01	0.90 ± 0.04	10 ± 1	5	0.93	0.03	0.04 ± 0.01	0.55 ± 0.04	20.5 ± 3.9	5	0.82	0.09
Ameletus sp.	0.02 ± 0.01	0.48 ± 0.02	17 ± 2	5	0.77	0.12	0.01 ± 0.00	0.15 ± 0.01	23.6 ± 3.2	5	0.88	0.06
Siphlonuris sp.	nd						0.00 ± 0.00	0.13 ± 0.01	38.3	5	0.74	0.13
Hydropsyche californica	0.45 ± 0.01	0.18 ± 0.04	26 ± 5	5	0.99	0.03	0.45 ± 0.05	1.02 ± 0.15	33.2	5	0.95	0.02
Hydropsyche oslari	0.21 ± 0.02	0.39 ± 0.04	22 ± 2	10	0.99	<0.01	0.29 ± 0.02	0.79 ± 0.08	20.2	10	0.98	<0.01
Calineuria californica	0.39 ± 0.03	0.71 ± 0.08	78 ± 6	5	0.99	<0.01	0.46 ± 0.07	0.92 ± 0.19	118. 3	5	0.95	0.02

TABLE 3. Water (^3H_2O) Permeability in Selected Species

taxon	wet wt (mg)	water content (μL)	^3H_2O uptake (μL)	^3H_2O uptake (μL)/mg (wet wt)
Calineuria californica	7.9 ± 2.0	6.3 ± 1.5	4.8 ± 0.9	0.61
Ameletus sp.	18.7 ± 2.4	15.6 ± 2.0	7.4 ± 0.7	0.39
Rhythrogena morrisoni	12.6 ± 2.3	10.7 ± 2.0	7.8 ± 1.3	0.62
Drunella grandis	96.5 ± 16.9	68.3 ± 12.0	13.1 ± 1.0	0.14

species. However, other mayfly taxa accumulated metals at much slower rates. Cd uptake rates varied approximately 250-fold between *Siphlonuris* sp. and *D. flavilinea*, both mayflies. Similarly, Zn uptake rates varied approximately 35-fold between *Ameletus* sp. and *D. grandis*. Cd uptake was also similar and moderately rapid for congeneric hydropsychid caddisflies *H. californica* ($0.45 \pm 0.05\ ng\ g^{-1}\ h^{-1}$) and *Hydropsyche oslari* ($0.29 \pm 0.03\ ng\ g^{-1}\ h^{-1}$). Zn uptake rates in *Hydropsyche* were similar to Cd uptake rates and were 0.45 ± 0.03 and 0.21 ± 0.02 ng/g/h for *H. californica* and *H. oslari*, respectively.

Sources of Variability in Dissolved Cd and Zn Uptake Rates. The most common explanation as to why species varied in their metal uptake rates is gross morphology. We examined the two characteristics most likely to affect water permeability differences among species: body size (weight) and gill size. Linear regression showed no significant relationship between metal uptake rates and body weights among all species tested for Cd ($p = 0.71$), and Zn ($p = 0.18$) excluding *D. grandis*. These results agree with the finding that in the Clark Fork River, Montana, size did not explain large differences in Cd and Cu bioaccumulation among species (*6*).

Within species, effects of body size on bioaccumulation are well known. In a few of our test species, size appeared to be negatively correlated with metal uptake rates. The trend was only statistically significant for Cd in *D. flavilinea* ($p < 0.05$) and for Zn in *R. morrisoni* ($p < 0.05$). For most species, this was not the case; however, we cannot discount that small sample sizes may have hindered detection of significant size effects. For this suite of insects, therefore, surface area per unit mass (gill and body surface) may be important within a given species, but basic biological differences among species appeared to be more important than surface area in determining differences in uptake rates.

Gill size was also not related to Cd or Zn uptake rates. For example, taxa with relatively large gills could have relatively slow (*Siphlonuris* and *Rhythrogena)* or fast (*Calineuria* and *Hydropsyche)* metal uptake rates. To examine this relationship quantitatively, water permeability was tested directly in selected taxa (Table 3). There was no relationship between ^3H_2O uptake rates and metal uptake rates, demonstrated by Pearson correlation coefficients of 0.36 ($p = 0.64$) for Cd and 0.50 ($p = 0.49$) for Zn.

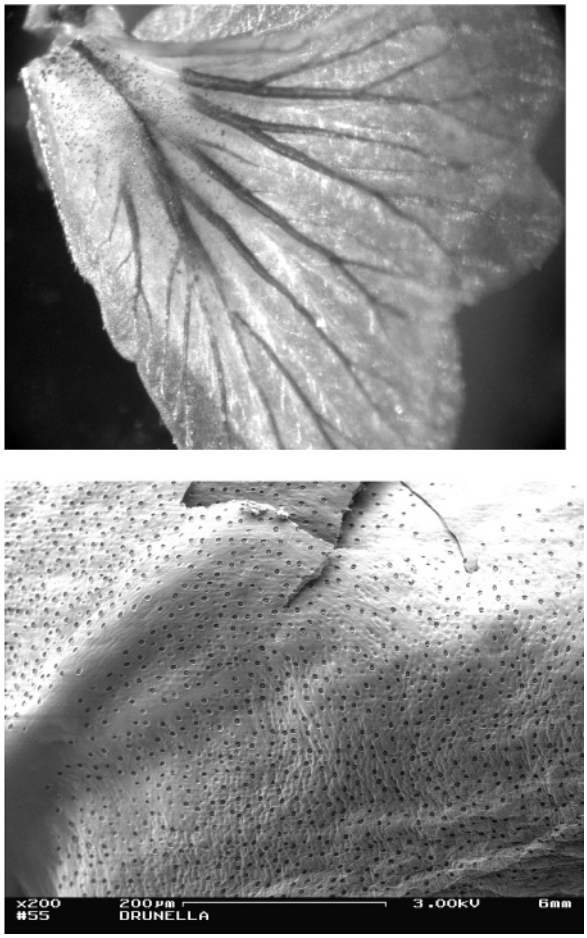

FIGURE 2. Chloride cell densities vary on the gills mayfly species *Drunella flavilinea* (a rapid Cd accumulator) (panel a) and *Siphlonuris* sp. (a slow Cd accumulator) (panel b). Chloride cells on *D. flavilinea* are visible by SEM as dark, disklike structures, densely populating all apical surfaces of the complex in this species. Chloride cells on the gills of *Siphlonuris* sp. are stained with silver chloride precipitates and are limited to the medial portion of the gill surface in this species.

Finer scale morphological features related to ionoregulation appeared to be more closely associated with interspecific differences in metal uptake. Light and scanning electron microscopic techniques allowed for qualitative comparisons of chloride cell densities on the gill surfaces of mayflies and stoneflies. The relative densities of chloride cells varied widely among species but were unrelated to gill size. The small, bafflelike gills of *Drunella* were densely populated by chloride cells on every observed surface (Figure 2a). In contrast, the large gills of *Siphlonuris* were sparsely populated by chloride cells (Figure 2b). Although the numbers of chloride cells were difficult to quantify, there appeared to be

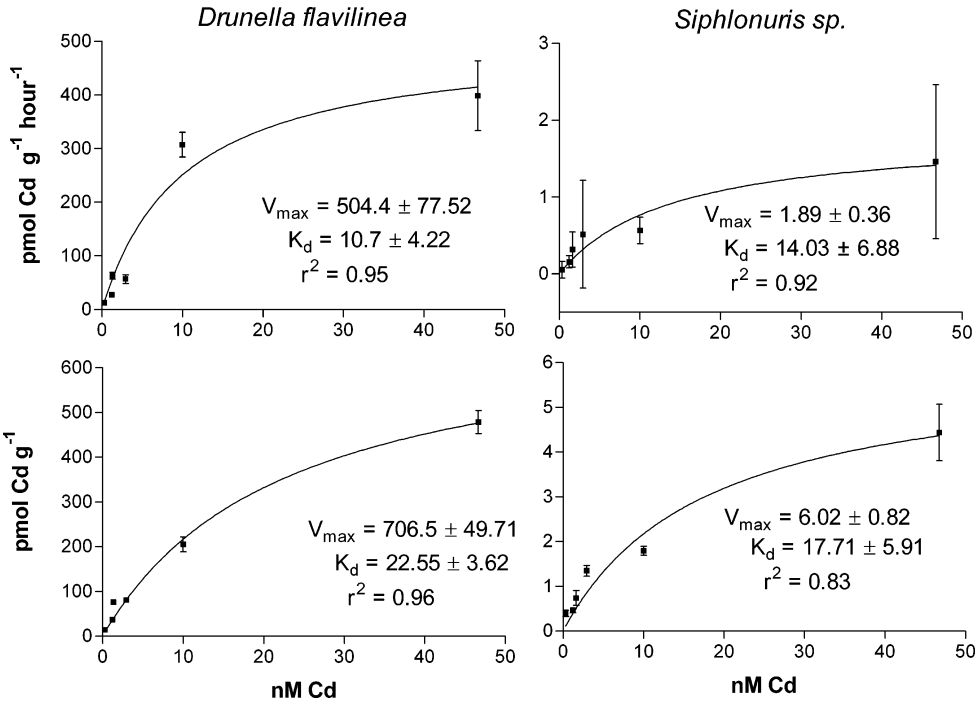

FIGURE 3. Michaelis−Menten kinetics based on uptake rates (panel a) and total accumulation (panel b) in the mayflies *Drunella flavilinea* and *Siphlonuris* sp. Error bars represent the standard errors of the means.

a relationship between chloride cell densities and metal uptake rates. Both *Drunella* species rapidly accumulated Cd and Zn, whereas uptake rates were much slower in *Siphlonuris*. In other cases, such as *C. californica*, visual evidence of larger numbers of chloride cells was accompanied by faster metal uptake rates. Other taxa such as *Ameletus* sp. had relatively few chloride cells and slow metal uptake rates.

Michaelis−Menten kinetics. We further explored if a larger number or higher affinity of transport sites was the mechanistic reason that chloride cell density seemed related to metal uptake rates, using Michaelis−Menten type kinetic experiments with *Siphlonuris* and *Drunella* (Figure 3). When kinetics were based on uptake rates (the slopes of uptake curves as in Figure 1), the mayfly *D. flavilinea* was determined to have 266-fold more transporters ($V_{max} = 504.4 \pm 77.52$) for cadmium than the mayfly *Siphlonuris* sp. ($V_{max} = 1.89 \pm 0.36$). K_d values of 10.7 ± 4.22 for *D. flavilinea* and 14.03 ± 6.88 for *Siphlonuris* sp. were not significantly different (two-tailed $p = 0.68$), suggesting that there is little difference in affinity of Cd for the transporters in these two species. Observed differences in uptake rates are thus determined by the number of channels rather than by the affinity of the channels to Cd.

When kinetics were based on total uptake after 4 h of exposure, rather than the rates of uptake, the mayfly *D. flavilinea* was determined to have 117-fold more transporters ($V_{max} = 2826 \pm 198.8$) for cadmium than the mayfly *Siphlonuris* sp. ($V_{max} = 24.07 \pm 3.28$). K_d values of 22.55 ± 3.62 for *D. flavilinea* and 17.70 ± 5.91 for *Siphlonuris* sp. were not significantly different (two-tailed $p = 0.50$).

Uptake Pathways. To test if Ca channels were among the type of sites that played a role in determining metal uptake rates (particularly Cd, given its similar ionic diameter to Ca), we examined the effects of ambient calcium concentrations and verapamil-a calcium channel blocker on metal uptake rates (Figure 4). In experiments with the mayfly *D. flavilinea*, only Cd was used. Increasing calcium concentrations from 178 to 712 μM decreased Cd uptake in *D. flavilinea* by 13% (unpaired t-test, two-tailed $p < 0.05$). Increasing calcium concentrations from 178 μM to 1.42 mM decreased Cd and Zn uptake by 34 and 36%, respectively, in the caddisfly *H. californica*. Competition between Ca and Cd ions has also been observed in other invertebrate species (e.g., the midge *Chironomous staegeri* (*24*).

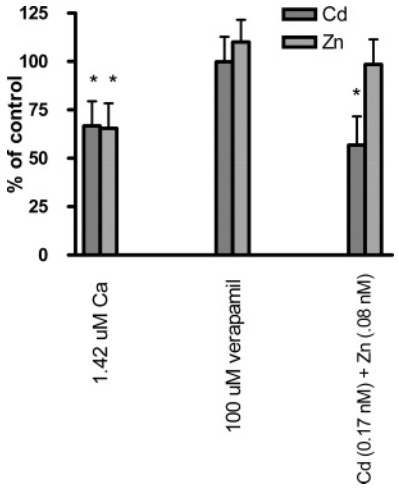

FIGURE 4. Calcium concentration affected Cd and Zn uptake in the caddisfly *Hydropsyche californica*. Verapamil did not affect Cd or Zn uptake in this species. Simultaneous exposure to Cd and Zn did not affect Zn accumulation rate but markedly decreased Cd uptake.

Verapamil (300 μM) inhibited Cd uptake in *D. flavilinea* by 19.6% compared to controls (unpaired t-test, two-tailed $p < 0.05$). However, verapamil (300 μM) had no effect on Cd or Zn uptake relative to controls in *H. californica* ($p > 0.05$). The lack of a verapamil effect in *Hydropsyche* demonstrates that verapamil inhibition of the site was not confounded by complexation of Cd under these experimental conditions.

Mixed results with verapamil inhibition were observed in other studies as well. Craig et al. (*24*) found verapamil to effectively inhibit Cd uptake in *Chironomous staegeri*. Wang and Fisher (*25*) found that verapamil significantly inhibited Cd and Zn influx in the marine bivalves *Macoma balthica* and *Mytilus edulus*. In other taxa, such as the Rainbow Trout, verapamil is not an effective Ca channel blocker (*26*). Several

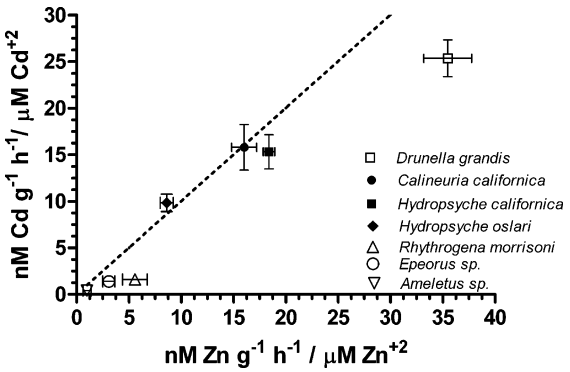

FIGURE 5. Zinc and cadmium uptake rates normalized to free-ion concentrations co-varied among taxa. Open symbols are ephemeropteran species, while closed symbols are trichopterans or plecopterans. Error bars represent the standard errors of the means.

different types of calcium channels have been described in animals (*27*), some of which are not inhibited by verapamil. It is likely that there are at least a few different types of Ca channels in aquatic insects.

In experiments with *H. californica* and verapamil, both Cd and Zn were present in control and verapamil treatments. Although verapamil had no effect on metal uptake, there was an interactive affect of the metals with each other. Zn uptake was unaffected by the presence of Cd, but Cd uptake was reduced by the presence of Zn by 45.5 and 43% in control and verapamil groups, respectively.

Co-Variation of Cd and Zn Uptake Rates. If osmoregulatory mechanisms (e.g., number of ion channels) are of general importance in governing differences in metal uptake among species, then interspecific differences in the rates of Zn and Cd uptake should be related. As shown in Figure 5, Cd and Zn uptake rates strongly co-varied among all taxa tested (Pearson coefficient of co-variation = 0.96, $p < 0.01$) consistent with similar dependency on chloride cells and the associated number of ion channels. The similar effect of calcium on Cd and Zn uptake in *D. flavilinea* coupled with the apparent competition between the two metals in the verapamil experiments also suggested similar transport mechanisms for the two metals.

To our knowledge, this is the first demonstration of the coupling of Cd and Zn uptake rates in aquatic insects. However, co-variation of Cd and Zn uptake rates has been observed in other species, including marine bivalves (*28*) and crustaceans (*29*).

The use of aquatic insects as ecological indicators in trace metal contaminated ecosystems would benefit from an increased understanding of how and why such species differ in their uptake of trace metals (*4*). In North America alone, there are well over 6000 aquatic insect species described to date (*30*). If differential biodynamics is among the key determinants of sensitivity differences among these species (*6*), then understanding the fundamental biological attributes responsible for controlling metal accumulation patterns could point toward generalizations about which species are most likely to be adversely affected as contamination increases.

However, few studies compare interspecific differences in biological attributes such as osmoregulatory biology to processes that might affect internal exposure (e.g., bioaccumulation) and effects of contaminants. For example, we know of no previous comparisons of aquatic insect osmoregulatory biology in relation to metal uptake (*31*−*33*). Here, we show that Cd and Zn uptake rates from solution vary by orders of magnitude among species of aquatic insects. That variation is directly tied to morphological characteristics ultimately associated with osmoregulatory function. Osmoregulatory structures provide first-order control of dissolved metal uptake. Additionally, it appears that dissolved metal uptake varies among species to the extent that species perform osmoregulatory processes on their body surface relative to their digestive tracts. This may also help explain why many taxa such as the dipterans *Chaoborus* spp. (*34*−*35*) and megalopteran *Sialis velata* (*36*) accumulate the majority of their body burdens from dietary sources.

In the organisms we studied, metal uptake was similar among species of the same genus, but not within orders. Some ecological indices for detecting stressed communities are based upon an implicit assumption about broad differences in sensitivity among orders. Yet our results show that no generalizations could be made about metal uptake rates at the level of order; variability within orders is as large or larger than variability among orders. Understanding the mechanistic basis for bioaccumulation and sensitivity differences among species might strengthen the argument for choosing particular taxonomic groups as sentinel species (*19*) for detecting metal effects. Furthering our understanding of the basic biology of different aquatic insect groups might point toward important generalizations in that regard.

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Literature Cited

(1) Rosenberg, D. M.; Resh, V. H. *Freshwater Biomonitoring and Benthic Macroinvertebrates*; Chapman and Hall: New York, 1993.
(2) Winner, R. W.; Boesel, M. W.; Farrell, M. P. Insect community structure as an index of heavy-metal pollution in lotic ecosystems. *Can. J. Fish. Aquat. Sci.* **1980**, *37*, 647−655.
(3) Clements, W. H.; Carlisle D. M.; Lazorchak, J. M.; Johnson, P. C. Heavy metals structure benthic communities in Colorado mountain streams. *Ecol. Appl.* **2000**, *10*, 626−638.
(4) Rainbow, P. S. Trace metal concentrations in aquatic invertebrates: why and so what? *Environ. Pollut.* **2002**, *120*, 497−507.
(5) Wallace, W. G.; Lee, B.-G.; Luoma, S. N. Subcellular compartmentalization of Cd and Zn in two bivalves. 1. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Mar. Ecol. Prog. Ser.* **2003**, *249*, 183−197.
(6) Cain, D. J.; Luoma S. N.; Wallace, W. G. Linking metal accumulation of aquatic insects to their distribution patterns in a mining-impacted river. *Environ. Toxicol. Chem.* **2004**, *23*, 1463−1473.
(7) Kristensen, N. P. Phylogeny of insect orders. *Annu. Rev. Entomol.* **1981**, *26*, 135−157.
(8) Komnick, H. Chloride cells and chloride epithelia of aquatic insects. *Int. Rev. Cytol.* **1977**, *49*, 285−329.
(9) Stobbart, R. H.; Shaw, J. Salt and water balance: excretion. In *The Physiology of Insecta*; Rothstein, M., Ed.; Academic Press: New York, 1974.
(10) Gonzalez, R. J.; McDonald, D. G. The relationship between oxygen consumption and ion loss in a freshwater fish. *J. Exp. Biol.* **1992**, *163*, 317−332.
(11) Patrick, M. L.; Gonzalez, R. J.; Bradley, T. J. Sodium and chloride regulation in freshwater and osmoconforming larvae of *Culex* mosquitoes. *J. Exp. Biol.* **2001**, *204*, 3345−3354.
(12) Patrick, M. L.; Ferreira, R. L.; Gonzalez, R. J.; Wood, C. M.; Wilson, R. W.; Bradley, T. J.; Val, A. L. The characterization of ion regulation in Amazonian mosquito larvae: Evidence of phenotypic plasticity, population based disparity, and novel mechanisms of ion uptake. *Physiol. Biochem. Zool.* **2002**, *75*, 215−222.
(13) Kirschner, L. B. Water and Ions. In *Environmental and Metabolic Animal Physiology;* Prosser, C. L., Ed.; Wiley-Liss: New York, 1991; pp 13−108.
(14) Buchwalter, D. B.; Jenkins, J. J.; Curtis, L. R. Temperature influences on water permeability and chlorpyrifos uptake in aquatic insects with differing respiratory strategies. *Environ. Toxicol. Chem.* **2003**, *22*, 11, 2806−2812.

(15) Buchwalter, D. B.; Jenkins, J. J.; Curtis, L. R. Respiratory strategy is a major determinant of [^{3}H]water and [^{14}C]chlorpyrifos uptake in aquatic insects. *Can. J. Fish. Aquat. Sci.* **2002**, *59*, 1315−1322.
(16) Dow, J. A. T. V-ATPases in insects. In *Organellar Proton-ATPases*; Nelson, N., Ed.; R. G. Landes Co.: Georgetown, TX, 1994.
(17) Langton, W. J.; Spence, S. K. Biological factors involved in metal concentrations observed in aquatic organisms In *Metal Speciation and Bioavailability in Aquatic Systems*; Tessier, A., Turner, D. R., Eds.; JohnWiley & Sons: New York, 1995; pp 407−478.
(18) Gower, A. M.; Myers, G.; Kent, M.; Foulkes, M. E. Relationship between macroinvertebrate communities and environmental variables in metal-contaminated streams of southwest England. *Freshwater Biol.* **1994**, *32*, 199−221.
(19) Kiffney, P. M.; Clements, W. H. Effects of heavy metals on a macroinvertebrate assemblage from a Rocky Mountain stream in experimental mesocosms. *J. N. Am. Benthol. Soc.* **1994**, *13*, 511−523.
(20) Garbarino, J. R.; Hoffman, G. L. *Methods of Analysis of the U.S. Geological Survey National Water Quality Laboratory—Comparison of a nitric acid in-bottle digestion procedure with other whole-water digestion procedures*; U.S. Geological Survey Open-file Report 99-094; U.S. Geological Survey: Denver, CO, 1999.
(21) U.S. EPA 2001 Update on Ambient Water Quality Criteria for Cadmium. http:// www.epa.gov/waterscience/criteria/aqualife/cadmium/cad2001upd.pdf (accessed 8/2004).
(22) Luoma, S. N.; Fisher, N. In *Ecological Risk Assessment for Contaminated Sediments*; Ingersoll, C., Biddinger G., Dillon, T., Eds.; SETAC Press: Pensacola, FL, 1997; pp 211−232.
(23) Sokal, R. R.; Rohlf, F. J. *Biometry*; W. H. Freeman and Company: San Francisco, CA, 1981.
(24) Craig, A.; Hare, L.; Tessier, A. Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomous staegeri. Aquat. Toxicol.* **1999**, *44*, 255−262.
(25) Wang W.-X.; Fisher, N. S. Effects of calcium and metabolic inhibitors on trace element uptake in two marine bivalves. *J. Exp. Mar. Biol. Ecol.* **1999**, *236*, 149−164.
(26) Rodgers, J. T.; Wood, C. M. Characterization of lead-calcium interaction in the freshwater rainbow trout *(Oncorhynchus mykiss). J. Exp. Biol.* **2004**, *207*, 813−825.
(27) Anderson, P. A. V.; Greenberg, R. M. Phylogeny of ion channels: clues to structure and function. *Comp. Biochem. Physiol., B* **2001**, *129*, 17−28.
(28) Wang, W.-X.; Dei, R. C. H. Factors affecting trace element uptake in the black mussel *Septifer virgatus. Mar. Ecol. Prog. Ser.* **1999**, *186*, 161−172.
(29) Rainbow, P. S. Physiology, physiochemistry and metal uptake-a Crustacean perspective. *Mar. Pollut. Bull.* **1995**, *31*, 55−59.
(30) Merritt, R. W.; Cummins, K. W. *An Introduction to the Aquatic Insects of North America;* Kendall-Hunt: Dubuque, IA, 1996.
(31) Vuori, K.-M. Rapid behavioural and morphological responses of Hydropsychid larvae (Trichoptera, Hydropsychidae) to sublethal cadmium exposure. *Environ. Pollut.* **1994**, *84*, 291−299.
(32) Guerold, F.; Giamberini, L.; Tourmann, J.-L.; Pihan J.-C.; Kaufmann, R. Occurrence of aluminium in chloride cells of *Perla marginata* after exposure to low pH and elevated aluminum concentration. *Bull. Environ. Contam. Toxicol.* **1995**, *54*, 620−625.
(33) Vuori, K.-M.; Kukkonen, Metal concentrations in *Hydropsyche pellucidula* larvae (Trichoptera, Hydropsychidae) in relation to the anal papillae abnormalities and age of exocuticle. *Water Res.* **1996**, *30*, 2265−2272.
(34) Munger, C.; Hare, L. Relative importance of water and food as cadmium sources to an aquatic insect (*Chaoborus punctipennis*): implications for predicting Cd bioaccumulation in nature. *Environ. Sci. Technol.* **1997**, *31*, 891−895.
(35) Croteau, M.-N.; Hare, L.; Tessier, A. Differences in Cd accumulation among species of the lake-dwelling biomonitor *Chaoborus. Can. J. Fish. Aquat. Sci.* **2001**, *58*, 1737−1746.
(36) Roy, I.; Hare, L. Relative importance of water and food as Cd sources to the predatory insect *Sialis velata* (Megaloptera). *Can. J. Fish. Aquat. Sci.* **1999**, *56*, 1143−1149.

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