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BIOACCUMULATION DYNAMICS AND EXPOSURE ROUTES OF Cd AND Cu AMONG SPECIES OF AQUATIC MAYFLIES

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Abstract—Consumption of periphyton is a potentially important route of metal exposure to benthic invertebrate grazers. The present study examined the bioaccumulation kinetics of dissolved and dietary Cd and Cu in five species of mayflies (class Insecta). Artificial stream water and benthic diatoms were separately labeled with enriched stable metal isotopes to determine physiological rate constants used by a biokinetic bioaccumulation model. The model was employed to simulate the effects of metal partitioning between water and food, expressed as the bioconcentration factor (BCF), as well as ingestion rate (IR) and metal assimilation efficiency of food (AE), on the relative importance of water and food to metal bioaccumulation. For all test species, the contribution of dietary uptake of Cd and Cu increased with BCF. For a given BCF, the contribution of food to the body burden increased with k_{uf}, the metal uptake rate constant from food that combined variation in IR and AE. To explore the relative importance of water and diet exposure routes under field conditions, we used estimated site-specific aqueous free-ion concentrations to model Cd and Cu accumulation from aqueous exposure, exclusively. The predicted concentrations accounted for less than 5% of the observed concentrations, implying that most bioaccumulated metal was acquired from food. At least for the taxa considered in this study, we conclude that consumption of metal-contaminated periphyton can result in elevated metal body burdens and potentially increase the risk of metal toxicity. Environ. Toxicol. Chem. 2011;30:2532–2541. © 2011 SETAC

Keywords—Dietary exposure Benthic grazers Bioaccumulation Cadmium Copper

INTRODUCTION

The global contamination of freshwaters with metals result in bioaccumulation in resident species that may affect species diversity and ecosystem function [1]. Assessment of toxicological risks associated with the metal contamination of these ecosystems begins with accurate assessments of metal exposure. Although dietary exposure is acknowledged as an important exposure route for a growing number of species, difficulties in quantifying dietary and dissolved uptake and relating dietary uptake to toxicity remain sources of uncertainty in risk assessment [2]. Extrapolation of data obtained from laboratory diets to natural settings, where diets for many species are largely undefined, is also problematic. However, if dietary exposure adds to an organism's metal body burden under conditions expected in nature, then the results of bioassays based on dissolved metal concentrations could underestimate toxicological risk.

Biokinetic bioaccumulation models can characterize the fundamental physiological process affecting metal bioaccumulation in aquatic macroinvertebrates [3]. Physiological constants are parameterized experimentally for individual species. After the constants are derived, the model can simulate the relative contribution of each exposure route to metal bioaccumulation at steady state under different geochemical and biological conditions encountered in natural settings [4]. The model can also predict metal bioaccumulation in resident fauna when the constants are combined with well-characterized site-specific metal exposures.

The trophic availability of metals at the base of the food web is of particular concern because of the implications for toxicity to primary consumers and the transfer of metals through the food web. Biokinetic studies of metal accumulation in freshwater herbivores have focused on species that naturally inhabit lakes, ponds, and large rivers [5–7]. In contrast, stream macroinvertebrates, which are largely represented by aquatic insects, have received little attention [8], although they are often used to assess and monitor ecological conditions, including water quality, in these ecosystems [9].

In the present study, biokinetic model parameters were developed for Cd and Cu for five species of grazing mayflies to evaluate the relative contribution of dissolved and dietary uptake to metal bioaccumulation. Metals in periphyton, the principal diet of these animals, can exceed dissolved metal concentrations by several orders of magnitude [10,11], thus making periphyton a potential source of metal exposure. Little information is available on the dietary uptake of Cd for grazing mayflies [12], nor has Cu kinetics from either dissolved or dietary routes been studied, largely because of the absence of a suitable radiotracer. The use of stable Cu isotopes enables kinetic studies of this important element, which is essential but potentially toxic [13]. For the present study, enriched stable isotopes were used to quantify rates of metal uptake from water and food, and elimination rates of absorbed metal after exposure. The effects of varying metal concentrations in food, metal bioavailability of food, and ingestion rate on the relative contribution of dietary exposure to metal body burdens were simulated. To assess the relative importance of aqueous and dietary exposure routes in a natural setting, site-specific estimates of free-ion Cd and Cu concentrations for a river contaminated with mine waste were used to model uptake of dissolved metal alone in three species of mayflies, and these predictions were compared with observed body concentrations.

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METHODS

General

Biokinetic parameters were inferred from a series of separate experiments conducted in artificial soft water (ASW) [14]. The food source for the dietary metal uptake and the depuration studies was the diatom *Nitzschia palea* (UTCC 160, University of Toronto). Batch cultures of *N. palea* were grown axenically in S-diatom media (J. Culp, Environment Canada, personal communication) at room temperature (22°C) under constant light (26 μE/s/m^2). To minimize metal contamination, all glass and plastic used in the experiments, sample preparation, and analysis were soaked in acid (10% hydrochloric acid or 15% nitric acid), rinsed thoroughly with deionized water, and dried under a laminar flow hood equipped with a high-efficiency particulate air filtration system. Double-distilled (Baker Ultrex® II) nitric acid and hydrogen peroxide were used to digest the samples and for procedural blanks. Speciation of Cd and Cu in ASW and in natural water was estimated using WHAM (6.0) [15]. Site-specific predictions were made for sites in the Clark Fork River (MT, USA). All statistical tests were run using Statistica (Ver 7) with a type I error rate of $\alpha = 0.05$.

Experimental organisms

Five mayfly species from two families, Ephemerellidae and Heptageniidae, were tested. Ephemerellids included *Serratella tibialis* and *Drunella flavilinea*, and heptageniid mayflies included *Nixe* sp., *Epeorus albertae*, and *Epeorus longimanus*. These taxa were selected because they have exhibited contrasting bioaccumulation patterns in previous studies [8,16], and they are important prey for fish [17]. The insects were collected from two wadeable streams with no known anthropogenic sources of metals. In 2007, 2008, and 2009, *D. flavilinea*, *Nixe* sp., and *E. longimanus* were collected from Stevens Creek, which is located on the eastern slope of the Santa Cruz Mountains in Santa Clara County, California (N37°16.998′W122°4.485′, elevation ~550 feet). In 2007 and 2010, *S. tibialis* and *E. albertae* were collected from Rock Creek in western Montana, USA (N46°42.224′W113°40.380′, elevation ~3,800 feet). Organisms were collected by hand, then sorted on site into family or genus, and transported in an ice cooler to the laboratory. The survival rate was high (qualitative observation), and animals were active. A voucher sample for each collection was preserved in ethanol for taxonomic identification.

The insects were housed in a constant temperature room, where they were acclimated to the experimental temperature (12 ± 1°C) and test water (ASW) over 3 d. During this time, the insects were held in plastic trays that were initially filled with site water and constantly aerated. Rocks collected from the sample sites were placed into each tray to provide shelter and food. Site water was replaced with ASW by daily replacement of one third of the volume of the water in the holding trays with ASW.

Labeling of water and food

For the determination of dissolved uptake, aliquots of commercially purchased standards (Trace Sciences International) isotopically enriched in ^{106}Cd (96.5%) and in ^{65}Cu (99.4%) were added to 1 L ASW to achieve the desired nominal dissolved concentrations of Cd and Cu. Nominal concentrations were varied from 0.5 to 25 μg/L for Cd and from 5 to 300 μg/L for Cu. Because the standard solutions were acidified, the pH of the ASW was checked and, if necessary, adjusted with NaOH to pH 6 to 7.

Diatoms were labeled simultaneously with ^{106}Cd and ^{65}Cu during a 24-h exposure as described by Croteau and Luoma [18]. Briefly, diatoms were harvested onto filters (1.2 μM Isopore membrane filter), rinsed with ASW, and then resuspended in a 20-ml acid-washed glass scintillation vial filled with ASW spiked simultaneously with enriched ^{106}Cd (1–10 μg/L) and ^{65}Cu (10–100 μg/L). The pH of the exposure media was checked and adjusted with NaOH, if necessary, to pH 6 to 7. Twenty-four hours later, the labeled diatoms were filtered and rinsed with ASW. Five small sections of the filters holding the labeled diatoms were sampled and dried at 40°C to constant weight for the determination of tracer concentrations of the diatom. The remaining filter with the diatom mat was sectioned into pieces, and these were provided to the insects as food.

Dissolved uptake

Uptake rates of tracers from ASW were determined from 4-h exposures. This exposure period was long enough for the insects to acquire detectable levels of tracer and short enough to estimate unidirectional influx (negligible influence of elimination). Exposure containers were 1-L high-density polyethylene containers with lids. Insects ($n = 5$–10) were randomly assigned to different exposure treatments, including control, and provided with constant aeration by means of an air line set through a small hole in the lid of the container. After 4 h, the insects were removed, rinsed for approximately 1 min in deionized water, and then placed individually onto pre-tared acid-washed fluoroethylene sheets. These sheets were then placed into individually labeled snap-cap 1.5-ml tubes and frozen. Water samples (2 ml) were collected in triplicate from each container before the animals were introduced to the solution and after a period of 4 h. The water was acidified with 0.02 ml 16 N HNO_3.

Uptake from diatoms

Labeled *N. palea* was provided to insects ad libitum for 8 h in feeding chambers (150-ml polypropylene vial fitted with 100 μM mesh) suspended into aquaria (7–20 L) equipped with recirculation pumps and charcoal filtration to sorb metal released by the diatoms into solution. Water samples were taken before and after feeding. A previous study estimated the gut residence time of food in the mayfly *Baetis* to be from 4 to 8 h [19]. Thus, an 8-h feeding time was established; we assumed this allowed the insects time to acclimate to the feeding chambers and not exceed the gut residence time. Controls ($n = 10$) were provided unlabeled *N. palea* and housed in a separate aquarium. Insects were periodically monitored for feeding behavior. At the end of the feeding period, each insect was gently rinsed in ASW and then transferred to a 12-ml cup (fitted with 100-μM mesh), and these were moved into another 20-L aquarium filled with ASW. Exposed and control insects were provided with unlabeled *N. palea*. The insects were depurated for 24 h, after which time they were collected following procedures previously described. Feces were harvested from each depuration cup by filtration (1.2-μM membrane filter) and dried (40°C).

Efflux

For 24 h insects were exposed to either ASW labeled with ^{106}Cd and ^{65}Cu or to a combination of ASW and *N. palea* labeled with ^{106}Cd and ^{65}Cu. After the exposure period, the insects were subsampled for metal analysis (initial metal body

burden). The remaining insects were transferred to aquaria filled with ASW and subsampled over a period of 5 to 9 d. During the depuration period, the insects were continuously fed unlabeled *N. palea*, and the ASW was continuously recirculated through activated charcoal to minimize dissolved exposure by metals released by the insects. Water samples were collected before the insects were introduced to the aquaria and after depuration.

Sample preparation and analysis

Insects were removed from the freezer and freeze-dried. Dry weight of individual insects and diatoms was measured on a microbalance (Sartorius, Model M2P) to the nearest hundredth of a milligram. Diatoms, insects, and feces were digested in screw-top fluoroethylene vessels (5–7 ml) with 16 N double-distilled HNO_3 (100 μl acid per milligram sample). Samples were sealed and digested at room temperature for 7 to 10 d, after which time H_2O_2 (40 μl per milligram sample) was added to complete the oxidation of organic material. Deionized water (18 mΩ) was added to produce a 2 to 10% HNO_3 solution. Germanium (Ge) standard was added to each sample (8 μl per milliliter sample) as an internal standard. Insect samples were filtered (0.45 μM) before analysis.

Samples were analyzed for isotopes of Cd (masses 106, 108, 110, 111, 112,113, and 114) and Cu (masses 63 and 65) on a quadrupole inductively coupled plasma mass spectrometer (PerkinElmer, Model Elan 6000). The instrument was calibrated at the beginning of each sample run with serially diluted standards. Analyte concentrations were determined twice for each sample.

Quality assurance

Quality assurance samples comprised procedural blanks and standard reference materials (SLRS-4, riverine water, and Tort-2, lobster hepatopancreas, National Research Council Canada). The SLRS-4 was used as an external standard to confirm the calibration of the inductively coupled plasma mass spectrometer. This standard was analyzed initially after instrument calibration and periodically during the analysis of unknowns (after ~20 samples). Instrument drift and instability were compensated for with the internal Ge standards. Measured Cd and Cu concentrations in the biological reference material (Tort-2) were consistently within ±10% of the certified values.

Calculations of tracer concentration

Tracer concentrations in samples were determined using the equations described in Croteau et al. [20]. Briefly, the natural relative abundance of stable isotopes of Cd and Cu was determined using the signal intensity of each isotope in the calibration standards. These averaged 0.0119 ± 0.0005 (standard deviation) for ^{106}Cd and 0.349 ± 0.015 for ^{65}Cu among batches of samples analyzed on different days. On a given day, the measured relative abundance among the calibration standards typically varied less than ±10% of the mean value for ^{106}Cd and less than ±2% for ^{65}Cu.

For samples analyzed on the same day, the total tracer concentrations were calculated as the product of the tracer's relative abundance and the inferred concentration for the isotope tracer, as shown in Equation 1:

$$[{}^{m}E]_j = {}^{m}p \cdot [T^{m}E]_j \quad (1)$$

where $[{}^{m}E]_j$ is the concentration of metal E of mass m in sample j, ${}^{m}p$ is the calculated mean natural relative abundance of the isotope determined in calibration standards, and $[T^{m}E]_j$ is the inferred total concentration of metal E of mass m in sample j. For samples that are not enriched with the tracer (control samples), the inferred metal concentrations for all isotopes are equivalent. Enrichment of the isotope used for the tracer effectively increases its signal intensity and, thus, its inferred concentration independent of other isotopes. All enriched samples were corrected for the background metal concentration, which yielded the accumulated tracer concentration.

Derivation: rate constants, assimilation efficiency, ingestion rate

The uptake rate constant, k_u, from aqueous exposure was defined as the slope of the linear regression of metal influx rate (μg/g/d) and exposure concentration (μg/L). The influx rate was linear up to 2 μg Cd/L and up to 20 μg Cu/L. Above these concentrations, uptake deviated from linearity (data not shown). These data were not used for estimating the k_u. Speciation modeling for the ASW predicted that 74% of the dissolved Cd and 6% of the dissolved Cu occurred in the free ion form (complexation by organic ligands that the insects might have excreted was ignored). Measured concentrations in the test water were typically ±10% of the nominal concentrations. We estimated the k_u as a function of the nominal total dissolved and the free ion metal concentrations. Multiple linear regression was used to examine for and correct, if necessary, the additional effect of body weight (mg dry wt) on the influx rates. For cases in which body weight was a significant predictor of influx rate in the regression model, the reported value for k_u is the slope for the partial correlation for metal exposure.

Metal elimination was modeled by nonlinear regression using Equation 2, assuming a one-compartment model

$$\frac{C_t}{C_0} = a \cdot \exp(-k_e \cdot t) \quad (2)$$

where C_t is the concentration at time t, C_0 is the initial concentration, a is the intercept (~1), t is days of depuration, and k_e is the estimated efflux rate constant (/d).

Uptake from food was characterized by the metal assimilation efficiency (AE) and ingestion rate (IR in g/g/d). Each was estimated from a mass balance of ^{106}Cd and ^{65}Cu recovered in the bodies of the test animals, M_t (ng), and in their feces, M_{feces} (ng), after depuration of ingested, labeled diatoms. AE was calculated from Equation 3.

$$AE = \frac{M_t}{M_t + M_{feces}} \quad (3)$$

The daily ingestion rate was estimated from Equation 4

$$IR = \frac{(M_t + M_{feces})}{wt \cdot [M]_f} \times 3 \quad (4)$$

where $[M]_f$ (μg/g) is the measured total concentration of ^{106}Cd and ^{65}Cu in *N. palea* used for the feeding experiments, and wt is the individual mayfly dry body weight (mg). The numerator was multiplied by three to convert the 8-h exposure to the daily ingestion rate.

$$\frac{d[M]_t}{dt} = \left(k_u \cdot [M]_w\right) + \left(IR \cdot AE \cdot [M]_f\right) - \left((k_e + g) \cdot [M]_t\right) \quad (5)$$

The kinetic bioaccumulation model is shown in Equation 5. In addition to growth (g in /d), it incorporates the experimen-

tally determined physiological rate constants and metal concentration in water, $[M]_w$, and food, described previously.

Model simulations and predictions

From Equation 5, metal influx from food is dependent on metal concentration of the food source, ingestion rate, and bioavailability of metal in the food. At steady-state, the proportional contribution of dietary uptake, p_f, is estimated from Equation 6.

$$p_f = \frac{[M]_f \cdot IR \cdot AE}{\left(([M]_f \cdot IR \cdot AE) + ([M]_w \cdot k_u)\right)} \quad (6)$$

The product of IR and AE can be expressed as the dietary uptake constant, k_{uf} (g/g/d), a term analogous to the dissolved uptake rate constant [6]. On substitution of this term, Equation 6 simplifies to Equation 7.

$$p_f = \frac{[M]_f \cdot k_{uf}}{\left(([M]_f \cdot k_{uf}) + ([M]_w \cdot k_u)\right)} \quad (7)$$

Metal accumulation in periphyton, and thus its potential contribution to metal bioaccumulation in herbivores will vary with metal speciation and partitioning. A bioconcentration factor (BCF), the ratio of metal accumulation in the periphyton relative to the dissolved metal concentration (L/kg), can be derived as given in Equation 8.

$$BCF = \frac{[M]_f}{[M]_w} \quad (8)$$

To simulate the effect of BCF on dietary metal uptake, we computed values for $[M]_f$ by varying the BCF while keeping $[M]_w$ constant at arbitrary values of 0.05 μg/L for Cd and 5 μg/L for Cu. The BCFs used for these calculations ranged from 1E + 04 to 1E + 06, bracketing published data from field studies [5,10,11]. The resulting values of $[M]_f$ ranged from 0.5 to 50 μg/g for Cd and from 50 to 5,000 μg/g for Cu. Values for $[M]_f$, the values for $[M]_w$, and species-specific values for k_{uf} and k_u were entered into Equation 7 to calculate the proportional contribution from food. The outcome of the simulation is sensitive to BCF (i.e., the relative values of $[M]_w$ and $[M]_f$, not their absolute values). Equation 7 can be re-expressed as functions of BCF and k_{uf} under steady-state conditions.

We modeled Cd and Cu bioaccumulation in mayflies at sites on the Clark Fork River, MT, USA, based on dissolved exposure, only, using the time-integrated form of Equation 5, as shown in Equation 9. For prediction, values for k_u derived from the free-ion Cd and Cu concentrations of the test water were used.

$$[M]_{ss} = \frac{k_u \cdot [M]_w}{k_e + g} \quad (9)$$

Metal concentrations, $[M]_w$, were entered as the free-ion, estimated from water quality data published by the U.S. Geological Survey [21,22]. Because Na, K, Cl, carbonate, and sulfate concentrations were not reported, values were generated based on the recipe for moderately hard artificial stream water [14] adjusted in constant proportions to the site-specific water hardness. Concentrations of those constituents (in mg/L) were set at 13 for Na, 1.0 for K, 0.94 for Cl, 41 for sulfate, and 34 for carbonate. A dissolved organic carbon concentration of 1 mg/L was assumed based on measured dissolved organic carbon in the Clark Fork [23]. A growth rate of 0.02/d [24] was assigned.

RESULTS

Influx of Cd and Cu from water

Influx rates of Cd and Cu in all taxa increased proportionately with dissolved Cd and Cu concentrations (Fig. 1). For each element, influx rates were generally faster in the ephemerellid than in the heptageniid mayflies (analysis of covariance, homogeneous slopes model, $p < 0.001$). Among individual species, the k_u varied 34-fold for Cd and fivefold for Cu (Table 1). Relative to values derived from the nominal dissolved concentrations, the corresponding free-ion specific k_u was 1.3-fold greater for Cd and 17-fold greater for Cu.

Influx rates of Cd and Cu in the ephemerellid mayflies, *S. tibialis* and *D. flavilinea*, decreased with body weight (individual dry wt). For example, dry weight accounted for 3 and 12% of the total variation in observed influx rates of Cd and Cu, respectively, in *S. tibialis*. This effect was accounted for in the k_u, as described previously in the *Methods* section.

Assimilation efficiency and food ingestion rate

Treatment with dissolved ^{106}Cd and ^{65}Cu increased concentrations of the isotopes in the diatoms used for the assimilation efficiency experiments from 3.97 to 42.1 μg/g of ^{106}Cd and 34.9 to 322 μg/g of ^{65}Cu (Table 2). After an 8-h exposure to these labeled diatoms, larval mayflies accumulated from 0.09 to 3.83 μg/g ^{106}Cd and from 0.81 to 19.3 μg/g ^{65}Cu. All taxa assimilated a consistently high percentage of Cd and Cu associated with the diatom (Table 3). Assimilation efficiencies were no lower than 71% and as great as 99%. In general, water samples showed no difference in the dissolved Cd and Cu concentrations before and after feeding (*t* test, $p > 0.05$). When concentrations did increase during feeding (for *Nixe* sp., $[Cd]_w$

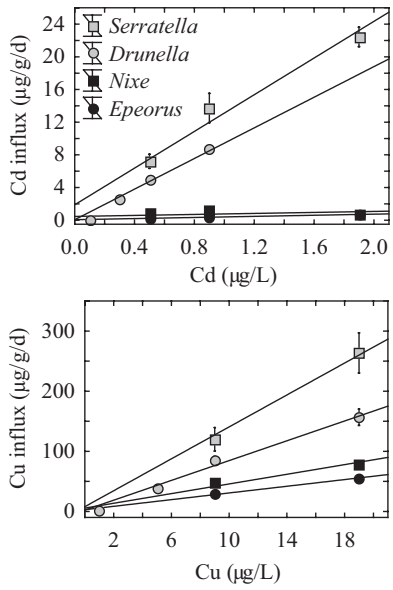

Fig. 1. Influx rates of ^{106}Cd and ^{65}Cu in larval mayflies exposed to the isotopes in artificial stream water. Exposures are shown as the nominal concentrations (μg/L). Data points are the mean ± standard error. Data were modeled with linear regression. *Serratella tibialis* and *Drunella flavilinea* (family Ephemerellidae) are indicated by the symbols with a gray background, and *Nixe* sp. and *Epeorus albertae* (family Heptageniidae) are indicated by symbols with a black background. *Epeorus longimanus* is not shown.

Table 1. Summary of the aqueous uptake rate constant (k_u) for Cd and Cu (mean ± 95% confidence interval) determined in soft artificial stream water for larval mayfly species[a]

Taxon	k_u (L/g/d)			
	$[Cd]_t$	$[Cd^{2+}]$	$[Cu]_t$	$[Cu^{2+}]$
Serratella tibialis	11.3 ± 1.5	15.2 ± 1.9	14.1 ± 2.4	234 ± 39
Drunella flavilinea	8.9 ± 0.7	12.0 ± 0.9	9.9 ± 1.2	164 ± 19
Nixe sp.	1.1 ± 0.5	1.5 ± 0.7	4.1 ± 0.7	67 ± 10
Epeorus albertae	0.33 ± 0.06	0.45 ± 0.09	2.8 ± 0.5	46 ± 7
Epeorus longimanus	0.40 ± 0.13	0.54 ± 0.17	2.6 ± 0.4	44 ± 7

[a] The values of k_u for total dissolved (e.g., $[Cd]_t$) and free-ion (e.g., $[Cd^{2+}]$) metal concentrations are reported on a dry weight basis.

increased from 0.002 to 0.003 μg/L and $[Cu]_w$ increased from 0.047 to 0.074 μg/L), the estimated dissolved uptake ($[M]_w \cdot k_u$) was negligible compared with observed body concentrations after feeding.

Ingestion rates varied from 0.05 g/g/d in *E. albertae* to 0.25 g/g/d in *S. tibialis* (Table 3). The ingestion rate for *E. albertae* should be accepted with reservation, because it was obtained from a small sample ($n = 3$). No correlation was found between ingestion rate and concentration of Cd and Cu in the diatom diets. Visual observations made periodically during the pulse feeding indicated that feeding behavior was erratic and highly individualistic. Although some animals appeared to be grazing on the diatom mats, others were positioned around the mat and not actively feeding. The absence of feeding was supported by the lack of detectable tracer in some animals and their fecal sample. Data from these individuals were not used for the calculation of AE and ingestion rate.

Elimination and estimated k_e

The elimination of Cd and Cu varied greatly among species and metals (Fig. 2). Unlike uptake rates, efflux rates did not differentiate between the two families. Loss of Cd and Cu during the first day (second day for *S. tibialis*) was typically less than 30% of the initial body burden. This loss was assumed to include excretion, egestion of undigested food, or desorption of weakly bound metal from body surfaces in contact with the water, and thus was ignored for the purposes of calculating k_e. In general, dissolved Cd and Cu concentrations did not vary significantly during the depuration period. Equation 2 was used to fit the retention data and estimate the efflux rate constant, k_e (Table 3). The rate of tracer loss was statistically significant ($p < 0.05$) for Cd and Cu in *S. tibialis*, Cd in *D. flavilinea*, Cu in *Nixe* sp., and Cd in *E. albertae*. For these, the k_e ranged from 0.10 to 0.20/d for Cd and from 0.13 to 0.22/d for Cu (Table 3). In contrast, loss was not significant for the remaining metal by species cases.

Model simulations and predictions

Model simulations suggested how the relative contributions of dissolved and dietary uptake to body concentrations of the mayflies could vary as a function of metal partitioning between water and periphyton (BCF). For these simulations, we used species- and metal-specific k_{uf} (the product of AE and IR), and k_e (Tables 1 and 3). As illustrated in Figure 3, model simulations suggested that as metal concentrations in periphyton increased relative to dissolved metal concentrations (increasing BCF), the contribution of the diet to metal concentrations in the mayflies increased rapidly. The effect was more pronounced in the ephemerellid mayflies, *S. tibialis* and *D. flavilinea*, than in the heptageniid mayflies, *Nixe* sp. and *Epeorus* sp., because of the differences in k_u between the species. The higher k_u of the ephemerellids favored dissolved metal uptake at lower BCF, but dietary uptake predominated as BCF increased. In contrast, because of the lower k_u in the heptageniids, uptake from food tended to be the predominant exposure route, even at relatively low BCFs.

The effect of k_{uf} on dietary uptake is exemplified in Figure 4 for Cu in *S. tibialis* over a range of hypothetical rates (g/g/d) of 0.02 (IR = 0.1 g/g/d and AE = 20%), 0.08 (IR = 0.15 g/g/d and AE = 50%), and 0.27 (IR = 0.3 g/g/d and AE = 90%). The values of k_{uf} were plotted against BCF, and as expected, lower values of k_{uf} attributable to lower metal bioavailability in food sources (AE) or lower ingestion rates decreased the dietary contribution of Cu.

Site-specific model predictions of Cd and Cu bioaccumulation limited to dissolved exposure lend additional support to the importance of dietary exposure in metal bioaccumulation by these larval mayflies (Table 4). Cadmium and Cu concentrations predicted from the site-specific free-ion metal concentrations alone accounted for less than 5% of Cd and less than 2% of Cu in *S. tibialis*, *E. albertae*, and *Nixe* sp. (*Drunella* was not present at these sites at the time of collection). These predictions suggested that dietary uptake overwhelmingly accounted for the observed body concentrations of Cd and Cu.

DISCUSSION

Kinetic bioaccumulation models can be used to gain insights into the basic physiological controls on metal bioaccumulation and on the relative importance of aqueous versus dietary exposure routes to metal uptake in aquatic invertebrates (e.g., Wang and Fisher [4]). Larval mayflies are of particular interest because they largely feed on periphyton, tend to accumulate high concentrations of metals relative to insects from other functional feeding groups [25], suffer toxicity when exposed to periphyton contaminated with metals [26], serve as an important food source to higher-level consumers, and are used to indicate where metals adversely affect benthic

Table 2. Concentrations (mean ± standard deviation) of ^{106}Cd and ^{65}Cu in untreated (control) and treated diatoms ($n = 5$), and the accumulation of ^{106}Cd and ^{65}Cu in mayfly larvae exposed for 8 h to treated diatoms for determination of Cd and Cu assimilation efficiency ($n = 3$–19)[a]

Taxon	Untreated diatoms		Treated diatoms		Mayfly larvae	
	^{106}Cd	^{65}Cu	^{106}Cd	^{65}Cu	^{106}Cd	^{65}Cu
Serratella tibialis	0.04 ± 0.01	0.98 ± 0.19	42.1 ± 2.0	322 ± 21	3.83 ± 1.87	19.3 ± 11.5
Drunella flavilinea	0.33 ± 0.18	3.32 ± 1.79	3.97 ± 0.83	34.9 ± 6.8	0.09 ± 0.12	0.81 ± 0.86
Nixe sp.	0.12 ± 0.05	1.10 ± 0.59	24.9 ± 8.6	219 ± 50	1.03 ± 1.07	4.53 ± 3.55
Epeorus albertae	0.09 ± 0.04	2.15 ± 2.7	33.1 ± 2.9	245 ± 22	0.55 ± 0.55	3.69 ± 3.82

[a] Concentrations are reported as μg/g, dry weight.

Table 3. Summary of assimilation efficiency (AE), ingestion rate (IR), and the efflux rate constant (k_e) of Cd and Cu (mean ± 95% confidence interval) for larval mayflies[a]

	AE (%)			k_e (/d)	
Taxon	Cd	Cu	IR (g/g/d)	Cd	Cu
Serratella tibialis	99 ± 1	83 ± 19	0.25 ± 0.09	0.20 ± 0.14*	0.13 ± 0.09*
Drunella flavilinea	71 ± 10	89 ± 4	0.15 ± 0.07	0.10 ± 0.06*	0.04 ± 0.06
Nixe sp.	86 ± 7	86 ± 7	0.19 ± 0.09	0.07 ± 0.10	0.22 ± 0.11*
Epeorus albertae	96	97 ± 4	0.05 ± 0.05	0.12 ± 0.11*	0.05 ± 0.07
Epeorus longimanus	ND[a]	ND	ND	ND	0.04 ± 0.34

ND = Not determined.
[a] Efflux rate constants that were statistically significant ($p < 0.05$) are indicated with an asterisk (*).

macroinvertebrate communities [27]. The present study showed that uptake and efflux kinetics differed widely among species of mayflies (explaining differences in bioaccumulation observed in the field) and suggested that the principal exposure route of Cd and Cu accumulation was ingestion of periphyton.

Dissolved metal uptake

Among the physiological terms used by the biokinetic model, the uptake rate constant from water, k_u, has received the greatest attention in aquatic insects [8,28]. The present study is the first to report data for Cu in larval mayflies, and one of the first to employ stable isotopes as a tracer. The comparability of the stable isotope methodology to previous studies can be assessed by comparing the k_u values of Cd for *D. flavilinea* and *Epeorus* sp. in the present study with those that were derived from data reported by Buchwalter and Luoma [29], who used the radiotracer ^{109}Cd (their data were converted to dry wts assuming a dry wt:wet wt of 0.1). The k_u from the earlier study was estimated to be 9.8 L/g/d for *D. flavilinea* and 0.4 L/g/d for *Epeorus* sp., very close to the values we obtained with the stable isotope methodology. In a comparative study of 21 species, Buchwalter et al. [8] discriminated the k_u for Cd on the basis of phylogenetic divergence at the family level. Results of the present study displayed the same pattern of variation

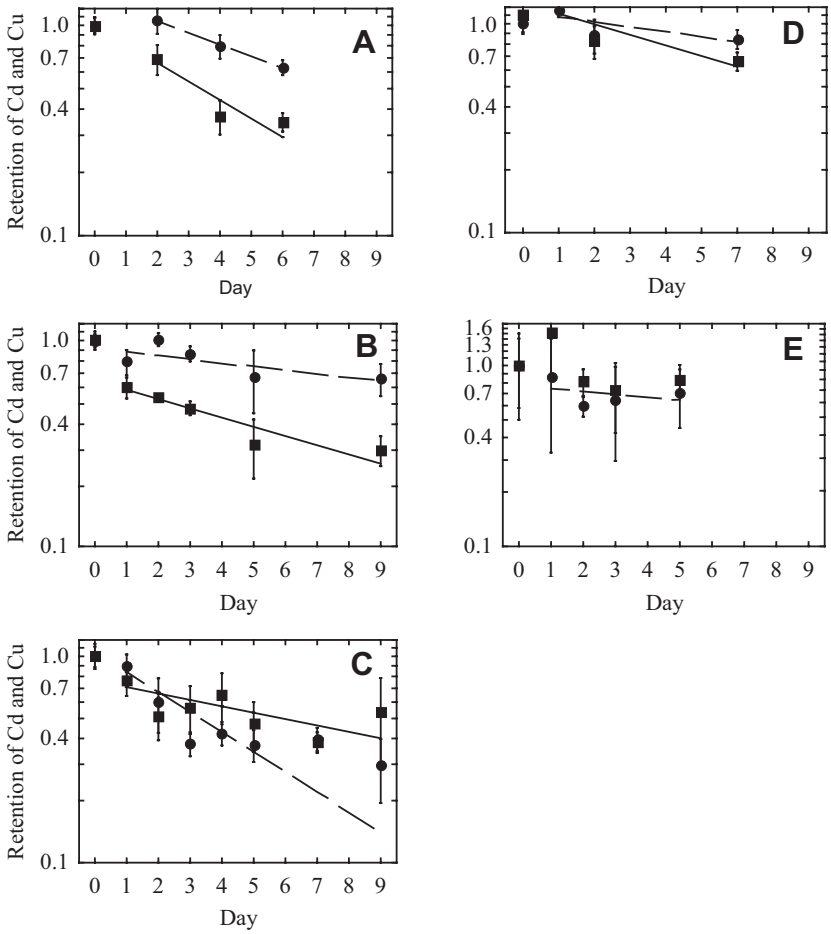

Fig. 2. The retention of accumulated ^{106}Cd (square symbol, solid line) and ^{65}Cu (circle symbol, dashed line) in larval mayflies. The data were modeled with Equation 2. (**A**) *Serratella tibialis*, (**B**) *Drunella flavilinea*, (**C**) *Nixe* sp., (**D**) *Epeorus albertae*, (**E**) *Epeorus longimanus* (Cd was not modeled for *E. longimanus* because the mean retention on day 1 was > 1). Data points are the mean ± standard error.

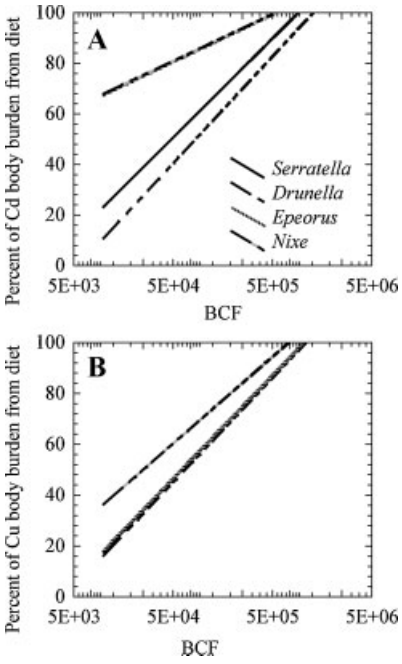

Fig. 3. Model simulations illustrating the influence of metal partitioning between water and periphyton, represented as the bioconcentration factor (BCF), on the relative contribution of food to (**A**) Cd and (**B**) Cu uptake.

between these two familial groups for Cd, as well as for Cu. The values of k_u were less variable among members of the same family than among different families, and the k_u in ephemerellid mayflies was faster than that of heptageniids. The observed variation in k_u among taxa could reflect differences in either the number or the affinity of ion transporters on membrane surfaces [29].

Metal influx rates in aquatic invertebrates are sensitive to animal size [30]. This effect is usually interpreted as a decrease in the effective surface area of metal uptake relative to body mass that occurs with growth of the individual. A previous study noted a negative correlation between Cd influx rates and dry weight of *D. flavilinea* [29]. In the present study, influx rates of Cd and Cu in *S. tibialis* and *D. flavilinea* decreased with dry weight (body size) as well. Failure to normalize for body weight would have biased the estimated k_u for these taxa, although not so much as to obscure the phylogenetic differences described. Body weight had no detectable effect on the influx rates of either *Nixe* sp. or *Epeorus* spp., possibly because a smaller range of body weights affected the statistical resolution, or statistical relationships were less evident because of the species' lesser capacity for uptake of dissolved metals, as reflected in the k_u.

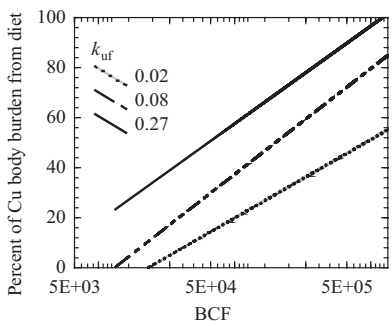

Fig. 4. Model simulation illustrating the combined effects of the bioconcentration factor (BCF) and the dietary uptake rate constant (k_{uf}) on the relative contribution of food to Cu uptake in *Serratella tibialis*.

Metal uptake from food

Assimilation efficiencies of Cd and Cu for the mayflies examined in the present study are among the highest reported for invertebrate herbivores. Measured values (in %) were not less than 70 (Cd in *D. flavilinea*) and were more typically greater than 85. Although AEs for larval mayflies have not previously been reported, comparable AEs for Cd were reported for the gastropod *Lymnaea stagnalis* (85) [6], for *Hyalella azteca* (80) [5], and for the zebra mussel, *Dreissena polymorpha* (71) [7]. In contrast, Cd AE for marine copepods was 35 [31]. Data for Cu AE ranged from 35 to 86 in marine bivalves [32], 32 to 47 in the freshwater bivalve *Corbicula fluminea* [13], 40 in a marine copepod [33], and 86 in the gastropod *L. stagnalis* [6].

Digestive physiology can affect the assimilation of metals by invertebrates [34]. Insects possess a simple tubular gut. Digestion is extracellular, with most digestion and absorption of nutrients and metals occurring in the midgut [35]. The high AEs indicate that Cd and Cu were readily solubilized from the diatom, *N. palea*, and absorbed, presumably, in regions of the midgut.

Dietary assimilation of Cd and Cu from *N. palea* may reflect the form and partitioning of metal to the diatom cell. Metals bound to intracellular ligands such as amino acids and proteins of phytoplankton appear to be highly bioavailable to marine copepods, which have a fairly simple digestive system, like aquatic insects [31]. Our experimental protocol labeled resuspended diatoms for 24 h. Uptake of both Cd and Cu was rapid, and the diatoms were assumed to be uniformly labeled at the end of the 24-h exposure. Preliminary studies of metal partitioning showed that, after metal labeling, little of the bound Cd and Cu were removed from the cells when they were extracted with 5 mM ethylenediaminetetra-acetic acid for 1 min, suggesting that most of the label was either internalized or strongly bound to sites on the cell wall (A. Kleckner, USGS, Menlo Park, CA, USA, unpublished data). Absorption of the label into the cell cytoplasm of *N. palea* would be consistent with the high AEs observed in the present study.

Metal AE determined in the present study could be positively biased if defecation of undigested gut content was not complete after the 24-h depuration period. However, earlier studies using a similar food source have found that assimilation of trace elements in small invertebrate herbivores is mostly complete within 24 h [20,34]; the high AEs derived from *N. palea* were highly unlikely to be experimental artifacts.

Ingestion rates for grazing insects that are directly comparable to the results of the present study do not exist, to our knowledge. The few data on ingestion rates for other freshwater herbivores are highly variable and likely reflect the influence of a number of factors such as species differences, organism size, and the nutritional quality of the food source [36]. Another source of variability is metal contamination of the food source. Aquatic herbivores, including insects, have been observed to reduce their consumption of food that is contaminated with metals, although these effects occurred either at much higher [18] or over much longer exposures [26] than were used in the present study. For example, Irving et al. [26] exposed the grazing mayfly *Baetis tricaudatus* to dietary Cd in short-term (1–1.5 h) and partial life-cycle tests. The insects did not avoid or limit their consumption of diets with Cd concentrations of 10 and 84 μg/g, initially. However, grazing rates were significantly reduced after 10 to 12 d of continuous exposure to 4 and 10 μg/g

Table 4. Observed and site-specific predictions of Cd and Cu[a]

Site	Year	$[Cd^{2+}]$	$[Cu^{2+}]$	Taxon	Observed		Predicted	
					Cd	Cu	Cd	Cu
Turah	2006	1.2E–02	5.1E–04	*Serratella tibialis*	17	380	0.8	1.3
Turah	2006	1.2E–02	5.1E–04	*Nixe* sp.	16	55	0.2	0.1
Turah	2006	1.2E–02	5.1E–04	*Epeorus albertae*	7	170	0.04	0.3
Turah	2008	1.4E–02	2.9E–04	*Nixe* sp.	13	43	0.4	0.1
A. Bearmouth	2008	1.8E–02	2.2E–03	*Nixe* sp	11	39	0.5	0.5
Goldcreek	2008	1.4E–02	1.1E–03	*Nixe* sp.	20	132	0.4	0.2
Galen	2008	1.5E–02	4.4E–04	*Nixe* sp.	30	179	0.4	0.1

[a] Predicted concentrations are the steady-state whole body concentrations based exclusively on the estimated free-ion concentrations of the site water (i.e., dietary exposure was ignored in the calculation). The prediction used the free-ion k_u (Table 1) and k_e (Table 3). Results are for three species collected at four sites on the Clark Fork River (MT, USA) over two years. Observed and predicted concentrations are reported as μg/g, dry weight.

Cd. Our observations indicated that feeding behavior in the insects was highly individualistic and erratic, especially in the early stages of the exposure, as the insects acclimated to the experimental setup. However, the concentrations of Cd and Cu in the diatoms did not correlate with ingestion rates of the larval mayflies, suggesting that the insects did not avoid the food because it was contaminated. The exposure time was established, in part, in recognition of the erratic feeding behavior to reduce the uncertainty around the mean ingestion rate as much as possible. Even so, ingestion rates for the same species (*S. tibialis*) varied by twofold to threefold (0.09–0.25 g/g/d) in replicated experiments (D.J. Cain and M.-N. Croteau, unpublished data).

Metal efflux

The literature shows wide interspecific variation in the rate of metal efflux (parameterized as k_e) (e.g., Wang and Fisher [4], Buchwalter et al. [8]). The rate of metal efflux influences, perhaps greatly, the metal bioaccumulation patterns observed among mayfly species. Low efflux rate constants would favor the accumulation of metals, and high efflux rate constants would limit metal accumulation and the potential for toxicity. In the present study, efflux rate constants estimated from depuration patterns varied from 0.04 to 0.22/d. Although sample variability and slow elimination of acquired metal over the depuration period (e.g., Cu in *Drunella* sp. and *Epeorus* spp.) are sources of uncertainty, the variability in these values is comparable with values of k_e for other species of mayflies and other groups of aquatic insects [8]. Based on a positive correlation between the uptake rate constant, k_u, and k_e for Cd, Buchwalter et al. [8] hypothesized these characteristics may have co-evolved in aquatic insects. Such a relationship was not evident for either Cd or Cu in our more limited dataset, however.

Importance of diet

Model simulations using BCF as a surrogate measure of $[M]_f$ (relative to dissolved metal concentration) suggested that the relative contribution of dietary uptake would increase with increasing BCF. Simulations also illustrated how feeding rates or AE of metal associated with the ingested material could influence the dietary contribution to the metal body burden. For example, reduced feeding rates would reduce the k_{uf} and shift proportionally more uptake to dissolved metals. Physiology will also modify the relative uptake of metal from water and food. Because of their higher k_u, the ephemerellid mayflies *S. tibialis* and *Drunella* spp. are expected to derive more of their accumulated body burden from dissolved metals than the heptageniids, assuming that ingestion rates and AE are comparable.

Site-specific predictions of Cd and Cu bioaccumulation for three of the test species showed that dissolved uptake could account for only a small portion of the observed metal concentrations. In fact, these predictions suggested that the contribution of dissolved metal uptake to the observed body burdens was inconsequential. This outcome was consistent with the predicted low free-ion metal concentrations of the site water and the assumption that the free ion was the only bioavailable species. These predictions would be biased if the k_u were sensitive to water chemistry, such as hardness (e.g., Playle [37]). The uptake rate constant was parameterized in ASW, and applied to the moderately hard to hard water of the Clark Fork River. Although the effect of hardness on k_u in these organisms was not tested, one would expect that the higher concentrations of competing cations and dissolved organic carbon in the Clark Fork River water would result in lower values of k_u than found in the laboratory. This would lower the contribution of dissolved metal uptake and further support the conclusion that consumption of metal-contaminated material was the predominant exposure route.

Prediction of body concentrations in nature is a more difficult problem than understanding basic processes such as exposure routes and interspecies differences in bioaccumulation dynamics because of the differences between laboratory and field conditions that affect both feeding and metal uptake. However, such exercises can be instructive in identifying processes important to improving predictive capabilities. Values for the terms describing dietary uptake must closely represent, in aggregate, the quantity of natural material ingested and the distribution and bioavailability of metals within that material. Thus, IR and AE values derived from pure cultures in the laboratory are a source of uncertainty in predicting metal uptake from a complex natural diet. To account for this, the model can be calibrated for either of these terms, providing that reasonable estimates for the other terms can be assigned. For example, the model can be calibrated to AE by first making assumptions regarding values of IR and $[M]_f$. The particulate organic carbon content in periphyton collected from the Clark Fork River following procedures described in Moulton et al. [38], was 5 to 8% by dry weight, whereas the organic carbon content in cultures of *N. palea* was 32% (D.J. Cain, unpublished data). The lower organic carbon content of the field-collected periphyton reflects substantial quantities of inorganic particles entrained in the periphyton. Presumably, these particles are also ingested by benthic grazers. A reasonable value for IR in nature

might then be higher than the value determined in the laboratory to account for the inferior nutritional quality of natural diets relative to the laboratory diet. Thus, IR was set at 0.98 g/g/d, the value determined for *H. azteca* feeding on natural assemblages of periphyton [5]. The metal concentrations determined in the gut content of *Nixe* sp. from the Clark Fork River were used to represent $[M]_f$ (D.J. Cain, unpublished data). Thus, $[M]_f$ ranged from 38 to 60 μg/g dry weight for Cd and from 165 to 696 μg/g dry weight for Cu. With these assumptions, the model was calibrated to AE. Results for *Nixe* sp. in the Clark Fork River indicated that the aggregate AE from all forms of metals consumed by the insect averaged between 5 and 6% for Cd and Cu, respectively. This result could be reasonable if it is assumed that the bioavailability of Cd and Cu associated with the organic carbon fraction of the periphyton was greater than the bioavailability of inorganic forms of Cd and Cu (e.g., Cd and Cu bound to Fe, Al, and Mn oxides) [39]. The concentration of bioavailable metal from food estimated from the product of the AE and total metal concentration in the gut content was approximately 2 μg/g for Cd and 29 μg/g for Cu. Using these values to represent metal concentrations in ingested periphyton, the BCF*s* relative to the free-ion metal concentrations were on average 2E + 05 and 1E + 07 for Cd and Cu, respectively, consistent with the range of BCFs in which model simulations suggested food would contribute most of the body burden. Because the bioavailable metal from food and water are related and both are reduced in the field compared with the laboratory, the relative contribution of diet to uptake would be expected to follow the model simulations.

In conclusion, our results suggest that under conditions typically encountered in nature, diet is a much more important exposure route of Cd and Cu than uptake from the aqueous phase by algal grazers such as those tested in the present study. Model simulations predicted that uptake of Cd and Cu from diet, relative to water, increased rapidly under environmentally relevant BCF (from 1E + 04 to 1E + 06). However, simulations also suggested that feeding rates and the bioavailability of metal associated with natural diets will affect the actual contribution of metals derived from food, as will the aqueous speciation of the metal. Site-specific predictions in which bioavailable metal was limited to the concentrations of the aqueous free-ions indicated that uptake from the dissolved phase could not account for observed body burdens. These predictions provided evidence supporting the importance of dietary uptake. However, the complexity of natural diets and waters imposes uncertainties on model parameters for prediction of metal uptake in resident populations of larval mayflies, and benthic grazers, in general. Consideration of the bioavailability from both exposure routes is important in using the model for predictive purposes.

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