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Using Biodynamic Models to Reconcile Differences Between Laboratory Toxicity Tests and Field Biomonitoring with Aquatic Insects

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Aquatic insects often dominate lotic ecosystems, yet these organisms are under-represented in trace metal toxicity databases. Furthermore, toxicity data for aquatic insects do not appear to reflect their actual sensitivities to metals in nature, because the concentrations required to elicit toxicity in the laboratory are considerably higher than those found to impact insect communities in the field. New approaches are therefore needed to better understand how and why insects are differentially susceptible to metal exposures. Biodynamic modeling is a powerful tool for understanding interspecific differences in trace metal bioaccumulation. Because bioaccumulation alone does not necessarily correlate with toxicity, we combined biokinetic parameters associated with dissolved cadmium exposures with studies of the subcellular compartmentalization of accumulated Cd. This combination of physiological traits allowed us to make predictions of susceptibility differences to dissolved Cd in three aquatic insect taxa: *Ephemerella excrucians*, *Rhithrogena morrisoni*, and *Rhyacophila* sp. We compared these predictions with long-term field monitoring data and toxicity tests with closely related taxa: *Ephemerella infrequens*, *Rhithrogena hageni*, and *Rhyacophila brunea.* Kinetic parameters allowed us to estimate steady-state concentrations, the time required to reach steady state, and the concentrations of Cd projected to be in potentially toxic compartments for different species. Species-specific physiological traits identified using biodynamic models provided a means for better understanding why toxicity assays with insects have failed to provide meaningful estimates for metal concentrations that would be expected to be protective in nature.

Introduction

Trace metal contamination is a problem in freshwater ecosystems throughout the world (*1*). Aquatic insects are often the dominant invertebrate fauna in such systems, particularly in lotic ecosystems where they can comprise 70−90% of the macrofaunal species pool (*2*). Because insects play critical ecological roles in stream ecosystems (*3*, *4*) and show considerable variation in sensitivity to anthropogenic stressors, they are commonly used in bioassessment and biomonitoring (*5*−*7*).

Clearly, some insect species are highly sensitive to trace metals in nature. The most compelling evidence for metal effects on stream fauna has come from field-based observational studies (*8*−*10*). In field studies, the loss of certain insect taxa but not others correlates with metal exposure. Differences between locations are typically characterized by indices reflecting changes in community composition, although these indices are generally not stressor-specific. Because observational studies cannot by nature establish causal relationships, experimental approaches are needed to better understand how and why species differ in their susceptibilities to trace metal exposure.

Toxicity testing has historically been the most straightforward approach to understanding the hazards associated with contaminant exposure, and it remains the cornerstone for establishing water quality criteria. Existing toxicity data show that relative to many other faunal groups, insects tend to be more tolerant to cadmium, copper, lead, nickel, and zinc in acute tests, with LC_{50} values almost 4 orders of magnitude higher than concentrations found in nature (*11*). LC_{50} values for several species of aquatic insects reported in the updated ambient water quality criteria for Cd (*12*) are also several orders of magnitude greater than concentrations known to affect benthic communities in the field (*13*, *14*). This inconsistency (*9*) has important ramifications for the inclusion of insect data in species sensitivity distributions and ultimately the development of water quality criteria. If laboratory toxicity data with aquatic insects are included, they would likely skew species sensitivity distributions toward less protective water quality standards. However, if insect data are excluded, a dominant faunal group would not be represented. Ideally, species sensitivity distributions should be somewhat reflective of the communities that water quality guidelines are meant to protect (*11*, *15*). Alternative methodologies are, therefore, needed to better understand the sensitivities of aquatic insects to metal contamination and to resolve the apparent discrepancy between field data and toxicity testing.

Here, we ask if insect community changes in response to trace metal contamination might eventually be explained by understanding physiological traits that make some species more sensitive than others. Our principal purpose is to test whether biokinetic and compartmentalization studies can be merged to better predict Cd susceptibility differences among taxa. Rankings derived from physiological studies with the mayflies *Rhithrogena morrisoni* (Heptageniidae), *Ephemerella excrucians* (Ephemerellidae), and the caddisfly *Rhyacophila* sp. (Rhyacophilidae) are compared to rankings derived from both long-term field monitoring and mesocosm experiments with closely related taxa. We further explore how physiological traits might help to explain apparent discrepancies between field biomonitoring and toxicity testing with insects.

Materials and Methods

Selection of Taxa. Taxa were chosen for field and toxicity studies based on their relative abundance and importance in North American streams. The mayflies *Ephemerella* and *Rhithrogena* and the caddisfly *Rhyacophila* are each well represented in cold water streams in many ecoregions. Because of their importance in Rocky Mountain streams,

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they are among the few taxa for which both field-based observational data (long-term monitoring of population densities in relation to measured metal concentrations) and toxicity data are available. Ongoing physiologically based studies of Cd accumulation and compartmentalization have generated data for several aquatic insect species (Buchwalter, unpublished data). Three of these species (*Ephemerella excrucians*, *Rhithrogena morrisoni*, and *Rhyacophila* sp.) are closely related to taxa (*Ephemerella infrequens*, *Rhithrogena hageni*, and *Rhyacophila brunea*) for which field and experimental data were available.

Long-Term Monitoring and Stream Microcosm Experiments. Data from previously published field monitoring studies (*14*) and stream microcosm experiments (*16*) were used to establish concentration−response relationships between dissolved trace metal concentrations and abundance of aquatic insects. Water quality and aquatic insect samples were collected biannually (spring and fall) from the Arkansas River (Colorado) as part of a long-term (1989−2004) investigation of the effects of trace metals on benthic macroinvertebrate communities. Here we report data collected from station AR1, located downstream from the Leadville Mine Drainage Tunnel. Metal concentrations were elevated at the start of the study, but have declined significantly as a result of remediation activities initiated in 1992 (*17*). We selected data from station AR1 to facilitate comparisons with results of microcosm experiments which were conducted using benthic communities collected from this same site (see below). Details of the study site and collection procedures have been published previously (*14*, *17*). Briefly, aquatic insects were collected using a 0.1 m Hess sampler ($n = 5$) fitted with a 350 μm mesh net. Samples were washed through a 350 μm sieve in the field and organisms were preserved in 80% ethanol. In the laboratory, organisms were identified and enumerated. Water samples for analysis of total and dissolved trace metals (Cd, Cu, and Zn) were collected in 14 mL acid washed containers and measured using flame and/or furnace atomic absorption spectroscopy.

Stream microcosm experiments were conducted in July 2002 and May 2003 to establish concentration−response relationships between trace metals and abundance of *R. hageni*, *E. infrequens*, and *R. brunea*. Details of these experimental procedures were reported in Clark and Clements (*16*). Benthic macroinvertebrate communities established over 30 days on substrate-filled trays were transported to a series of 18 experimental stream microcosms located at the Stream Research Laboratory at the Colorado State University Foothills campus (Fort Collins, CO). For each experiment, three trays were placed into each of 18 experimental streams, which were randomly assigned to six treatments (three replicates per treatment). Benthic communities were exposed to an equitoxic mixture of Cd, Cu, and Zn at target concentrations bracketing levels previously measured at polluted stations in the Arkansas River (*17*). Target concentrations ranged from 0.0 (control) to 10.0 μg Cd/L, 36 μg Cu/L, and 1200 μg Zn/L in the highest treated streams. We used 50% dilutions of these highest target concentrations (e.g., 600, 300, 150, 75 μg Zn/L) to achieve the remaining four treatments. After 7 days, the three trays from each stream microcosm were combined, surviving organisms were preserved in 80% ethanol, and samples were processed as described above. Metal concentrations and water hardness were measured on days 2, 4, and 7 of both experiments and analyzed as described above.

Because field monitoring studies and stream microcosm experiments involved a mixture of metals, we used an additive measure of toxicity to express metal concentrations relative to the U.S. Environmental Protection Agency's chronic water quality criteria (*18*). The cumulative criterion unit (CCU) is defined as the ratio of the measured dissolved metal concentration (m_i) to the hardness-adjusted chronic criterion value (c_i), summed for all metals:

$$\mathrm{CCU} = \sum m_i/c_i \qquad (1)$$

Because water hardness affects toxicity and bioavailability of some trace metals, criterion values are adjusted for water hardness using equations derived from laboratory toxicity tests. Assuming no synergistic or antagonistic interactions, a CCU value ≤ 1.0 represents the concentration of a mixture of metals that should be protective of aquatic organisms. The CCU has been previously employed to quantify metal contamination in streams receiving mixtures of metals (*9*, *19*).

All statistical analyses were conducted using SAS/STAT v8.2. Monitoring and stream microcosm results were analyzed using linear regression to examine the relationship between abundance of aquatic insects and CCU. If necessary, data were log-transformed and plots of residuals were examined to verify that data met assumptions of parametric statistics.

Physiological Studies. *Insect Collecting and Handling.* All insect larvae used in these studies were field collected from streams using a D-frame kick net. The mayflies *Ephemerella excrucians* and *Rhithrogena morrisoni* were collected from the Trinity River in Northern California (40°40′11″N, 122°59′04”W). The caddisfly *Rhyacophila* sp. was collected from Pescardeo Creek in Northern California (37°16′07″N, 122°18′51″W). Taxa were selected based on their local availability from sites with no known sources of metal contamination. Water samples were taken from these streams when insects were collected and analyzed by ICP−MS (*20*). These samples showed no evidence of metal contamination (data not shown). Insects were transported on ice in Whirl Pac bags containing streamwater. Insects were maintained in a light:dark photoperiod of 16:8 h and a constant temperature of 15 °C. Artificial soft water (ASW) (48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4{\cdot}2H_2O$, 30 mg/L $MgSO_4$, and 2 mg/L KCl, adjusted to pH 6.85 by additions of 0.1 N NaOH) was used for acclimation to laboratory conditions. Insects were acclimated to laboratory conditions for a minimum of 4 days prior to experimentation and fed a diet consisting of conditioned leaves, *Spirulina* aquarium flake food (O.S.I. Marine Lab, Inc., Hayward, CA), and TetraMin fish food ad libitum. Food was withheld for 1 day prior to initiating experiments to reduce fecal production (and associated metal sorption) during aqueous exposures. Only apparently healthy, intact, active animals were used in these experiments.

Experimental animals comprised a mix of different instars based on available individuals at the times of collecting. This is important because variability associated with developmental state was incorporated into the rate constants derived to predict bioconcentration factors. We have observed that earlier instars tend to have faster uptake and efflux rates than mature larvae (Buchwalter, unpublished data). Sample sizes and average masses are reported in Table 1.

Cadmium Accumulation Experiments. Cadmium was used in these experiments, because Cd is a nonessential metal in insects, and has been shown to affect aquatic life in many areas (*21*). Organisms were exposed in 1 L Nalgene beakers containing an 11 × 16.5 cm piece of polyethylene mesh that the insects used to maintain their position in the chamber. Two replicate chambers were used for each taxon. A steady flow of air was bubbled into the containers, which maintained dissolved oxygen concentrations and provided constant water movement. ASW (997.5 mL) was spiked with 0.5 mL $CdCl_2$ stock solution (1 μg/mL in 0.1N HNO_3), 2.0 mL $^{109}CdCl_2$ stock solution (9 ng/mL in 0.1N HNO_3) to provide a cadmium concentration of 518 ng/L (4.6 nM) with an initial corresponding activity of 2.14 μCi/L. The final pH of both

TABLE 1. Taxa, Source Location, Mass, and Numbers of Individuals Used in Physiological Experiments

order	family	genus species	sampling location	weight (g) ± std. err.	*n*
Ephemeroptera	Heptageniidae	*Rhithrogena morrisoni*	40°39′20″N, 122°57′34″W	27.4 ± 1.3	22
Ephemeroptera	Ephemerellidae	*Ephemerella excrucians*	40°39′20″N, 122°57′34″W	6.6 ± 1.1	30
Trichoptera	Rhyacophilidae	*Rhyacophila* sp.	37°16′07″N, 122°18′51″W	34.2 ± 1.9	30

acclimation and exposure solutions was adjusted to pH 6.85 with 0.1 N NaOH.

Cadmium accumulation was measured over 5 days in mayflies, and 6 days in the caddisfly. After each day of exposure, 5−10 individuals were removed from each beaker, rinsed with ASW, transferred to 20 mL vials containing ASW, assayed in vivo for radioactivity and then returned to the exposure chambers. The radioactivity of the exposure water was monitored daily, and was renewed after days 1 and 3 for the mayflies, and after days 2 and 4 for the caddisfly. All gamma counting was performed using a Wallac 1480 gamma counter (Gaithersburg, MD) for 3 min with the appropriate corrections for decay and counting efficiency. At the end of the accumulation phase of the experiment, half of the individuals were rinsed with ASW, and transferred to a −80 °C freezer for subsequent subcellular fractionation.

Cadmium Efflux Experiments. The remainder of the exposed populations was rinsed and depurated for 9−10 days in cadmium-free ASW. Depuration chambers were maintained free of effluxed Cd by monitoring water for radioactivity and changing the water as needed. Clean gravel, cobble, and nylon mesh were placed in the aquaria for substrate. Food was provided to effluxing animals. Individuals were subsampled daily and assayed in vivo for 5 days, and then assayed every 2 days thereafter until they had effluxed Cd for 9−10 days.

Derivation of Rate Constants. The rate constants for efflux (k_e) were estimated by fitting data from the depuration phase of the experiment to a nonlinear least-squares regression model. The rate constants of loss presented here are those from the slowest component of loss because this component most likely represents physiological loss in organisms chronically exposed in nature (*22*, *23*). Loss of Cd during depuration was generally biphasic, characterized by rapid loss during the first day, followed by slower and essentially constant loss for the remaining days. Therefore, loss constants (k_e) were estimated by fitting efflux data after the first day of loss to eq 2:

$$C_t = C_i\, e^{-(ke(t))} \quad (2)$$

Where C_t = Cd concentration in the larva (ng Cd g^{-1} wet weight) at time t; C_i = Cd concentration in the larva (ng Cd g^{-1} wet weight) at the beginning of the efflux phase of the experiment; k_e = efflux rate constant (day^{-1}).

Uptake rate constants (k_u) values were calculated from the following equation:

$$k_u = C_t\, k_e / ([1 - e^{-ket}]C_w) \quad (3)$$

Where C_w = concentration of Cd in the exposure water; C_t = concentration of Cd observed in the larvae after 1 day of exposure; k_u = uptake rate constant (L g^{-1} day^{-1}).

The inherent tendency for a taxon to accumulate Cd by the dissolved route of exposure is defined as the ratio of the estimated steady-state concentration of Cd in tissue resulting from dissolved exposure relative to the dissolved Cd concentration in water. This term can be derived experimentally as the ratio of k_u/k_e (*24*), as shown in eq 4:

$$\text{dissolved accumulation tendency} = (k_u/k_e) \times 10^3 \quad (4)$$

The term bioconcentration factor (BCF) is avoided here to avoid the widespread confusion associated with that term.

Subcellular Fractionation. On the final day of exposure, insects were assayed for ^{109}Cd, then immediately homogenized in refrigerated, N_2-saturated, 0.05 M Tris-HCl buffer (pH 7.4). The homogenate was subsequently separated into five operationally defined subcellular fractions by differential centrifugation and chemical and heat treatments following procedures previously described (*25*) and modified by Cain et al. (2006). These fractions included cell debris, cellular organelles, microsomes, cytosolic proteins denatured by heat treatment, and heat-stable cytosolic proteins. We assumed that Cd in the heat-stable protein fraction was largely bound to metallothionein-like proteins (*26*−*28*) and glutathione, while the heat-denatured fraction represented a variety of larger cytosolic proteins (*25*). We refer to the heat-stable and heat-denatured protein fractions as metallothionein-like protein (MTLP) and non-MTLP, respectively. Data from the subcellular fractions were summed into operationally defined metal-sensitive and detoxified compartments. The metal-sensitive compartment comprised the non-MTLP, microsomal and organellar fractions, each containing sites potentially vulnerable to Cd binding. The detoxified metal compartment was Cd associated with MTLP. Cd in the cell debris fraction was interpreted as being biologically inert tissue such as chitin.

Predictions of sensitivity differences among species were derived by merging rate constants with subcellular fractionation data. Rate constants provided estimates of accumulated Cd concentrations after 1 year of exposure. This estimate was subsequently modified to only consider Cd concentrations accumulated in potentially sensitive sites (organelles, microsomes, and non-MTLP cytosolic proteins). We term this calculation the dissolved cadmium sensitivity index (DSCI).

We performed these calculations with the following caveats in mind with respect to exposures experienced in the field: (1) only aqueous Cd accumulation was measured; (2) kinetic parameters, k_u and k_e, are only representative of the life stages tested; and (3) the subcellular fractionation patterns of metals after short-term exposures may not be identical to patterns after longer, chronic exposures. Nevertheless, these calculations provide insights into how interspecific variation in physiological processes influences Cd susceptibility.

Results

Long-Term Monitoring and Microcosm Experiments. To investigate the response of aquatic insects to metal contamination in the Arkansas River, long-term monitoring of insect population densities was conducted with corresponding water chemistry data. Abundance of the mayflies *Rhithrogena hageni* (Heptageniidae) and *Ephemerella infrequens* (Ephemerellidae) at station AR1 in the Arkansas River was significantly ($p < 0.0001$) correlated to dissolved metal concentrations over the 15 year sampling period (Figure 1). Dissolved metal concentrations, expressed as CCU, explained 51 and 62% of the variation in abundance of *Rhithrogena* and *Ephemerella*, respectively. EC_{50} values, operationally defined as the CCU level associated with a 50% reduction in field abundance, were estimated from these regression equations. EC_{50} values derived from these analyses

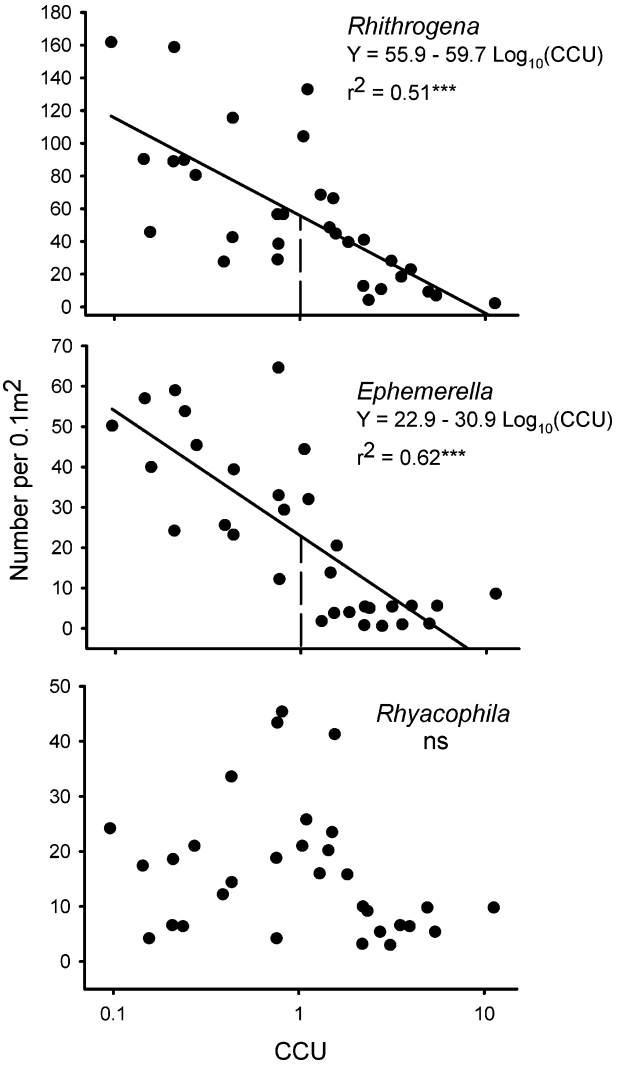

FIGURE 1. Relationship between trace metal concentration and abundance of *Ephemerella infrequens, Rhithrogena hageni,* and *Rhyacophila brunea* at Arkansas River station AR1 near Leadville, Colorado. Each point represents the mean number of organisms per five Hess samples collected in spring and fall from 1989 to 2004. CCU is the cumulative criterion unit, defined as the ratio of the measured metal concentration to the U.S. EPA hardness-adjusted criterion concentration, summed for all metals measured (see text for details). The vertical line represents a CCU value of 1.0, considered to be a level of metals that would be protective of aquatic organisms. The figure also shows regression equations and r^2 values for all significant relationships. ***$p < 0.0001$; ns = not significant.

were 0.93 CCU for *Rhithrogena* and 0.74 CCU for *Ephemerella.* In contrast to these results, there was no relationship between dissolved metal concentration and abundance of the caddisfly *Rhyacophila brunnea* (Rhyacophilidae).

To establish concentration−response relationships between dissolved metal concentrations and insect population densities in the laboratory, we conducted microcosm experiments that exposed these three species to a range of metal concentrations. Highly significant concentration−response relationships between abundance of *R. hageni* and metal concentration were observed in stream microcosm experiments conducted in July and May (Figure 2). Although metal concentration explained a significant amount of variation in both experiments (78−86%), effects were much greater in July. Abundance of *E. infrequens* was also significantly correlated to metal concentration, but these effects were only observed in July. Abundance of *R. brunnea* was not affected by metal concentration during either seasonal period.

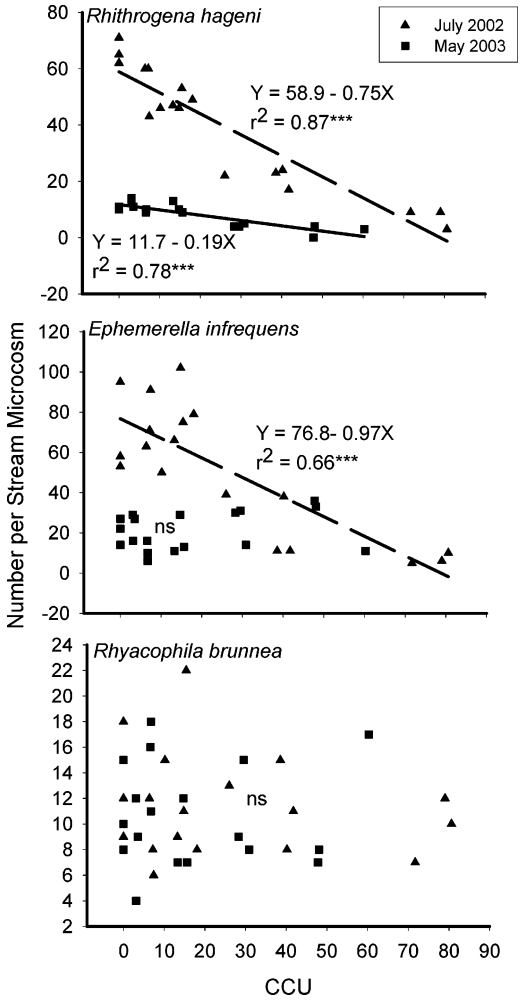

FIGURE 2. Concentration−response relationships between metals (expressed as CCU; see details in Figure 1) and abundance of *Ephemerella infrequens, Rhithrogena hageni* and *Rhyacophila brunea* in stream microcosms. Microcosm experiments were conducted in July 2002 and May 2003 using macroinvertebrate communities collected from Arkansas River station AR1. The figure also shows regression equations and r^2 values for all significant relationships. ***$p < 0.0001$; ns = not significant.

EC_{50} values, defined as the CCU value that correlated with a reduction of population size by 50% relative to control streams, were estimated for the mayflies *R. hageni* and *E. infrequens* using regression equations from the July microcosm experiment. EC_{50} values for these species were indistinguishable: 39.3 CCU for *R. hageni* to 39.6 CCU for *E. infrequens*. These values are 42 and 53 fold higher than the EC_{50} values estimated from the field monitoring data for *Rhithrogena* and *Ephemerella*, respectively. In the equitoxic mixture of metals used in the microcosm experiments, a CCU value of 39.5 corresponded to approximately 450 μg Zn /L + 50 μg Cu/L + 5 μg Cd/L. These metal levels were significantly greater than those measured on any sampling occasion at station AR1 in the Arkansas River, where the highest concentration recorded was approximately 11.2 CCU in spring 1990. Thus, water quality criteria derived from these microcosm experiments would not be protective of *Ephemerella* and *Rhithrogena* based on results of long-term monitoring of these taxa.

Physiological Studies. Cd accumulation from solution varied over 27 fold among the three taxa tested (Figure 3). *Ephemerella inermis* had the fastest accumulation rate at 172.3 ng Cd per gram per day, whereas the caddisfly *Rhyacophila* sp. had a very slow accumulation rate of 6.3 ng

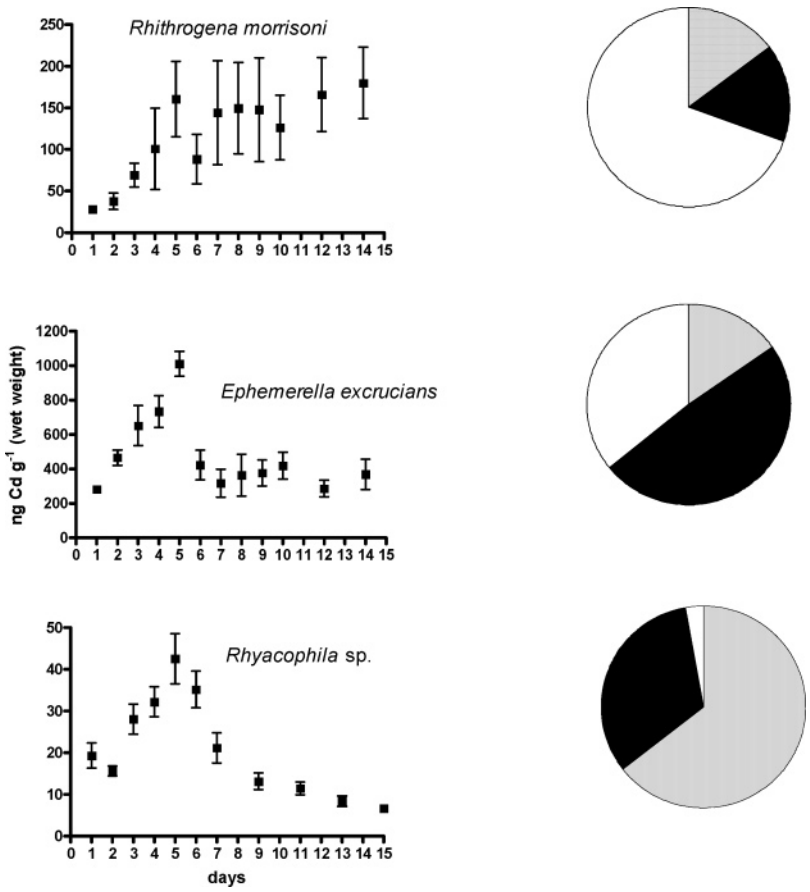

FIGURE 3. Radiotracer experiments show large differences among species in Cd uptake and efflux rates. Pie charts represent the subcellular compartmentalization of Cd into cell debris (hatched), biologically active or potentially toxic sites (black), and detoxified or associated with metallothionein like proteins (white). The combination of these parameters provides estimates of steady-state concentrations in biologically active compartments in addition to providing estimates of times required to reach steady-state concentrations (see Table 2).

TABLE 2. Derivation of a Dissolved Cd Sensitivity Index[a]

taxon	k_u ($L \cdot g^{-1} \cdot d^{-1}$)	k_e (d^{-1})	k_u/k_e	[Cd]ss ($\mu g \cdot g^{-1}$)	Tss (days)	[Cd]year ($\mu g \cdot g^{-1}$)	DCSI
Rhithrogena morrisoni	0.054 ± 0.007	0.001 ± 0.001[b]	54 000	27.9	5588	1.9	0.302
Ephemerella excrucians	0.554 ± 0.040	0.014 ± 0.021	39 593	20.5	399	18.8	9.156
Rhyacophila sp.	0.042 ± 0.007	0.137 ± 0.014	309	0.2	41	0.2	0.065

[a] The ratio of the uptake rate constant (k_u) to the efflux rate constant (k_e) was used to assess the relative steady-state concentration of Cd in insect tissues relative to the dissolved Cd concentration. Steady-state concentrations and the time required to reach steady state were calculated from these rate constants assuming a dissolved Cd concentration of 0.5 μg Cd/L. Cd concentrations predicted to accumulate in larvae over 1 year were modified to only consider Cd associated with potentially metal-sensitive fractions (Table 3). [b] A K_e value of 0.001 was assigned to *R. morrisoni* for modeling purposes.

TABLE 3. Subcellular Compartmentalization of ^{109}Cd in Three Aquatic Insect Taxa

	fractions				
	detoxified	metal sensitive			
taxon	MT-like proteins (MTLP)	non- MTLP proteins	organelles	microsomes	cell debris
Rhithrogena morrisoni	69.4	6.8	4.1	5.0	14.7
Ephemerella excrucians	35.8	33.0	5.2	10.5	15.4
Rhyacophila sp.	2.9	20.4	2.3	10.0	64.4

Cd per gram per day. The corresponding uptake rate constants are listed in Table 2. Species varied in their abilities to efflux Cd over a 10 day period. Most notably, the mayfly *Rhithrogena morrisoni* exhibited no efflux over 10 days, whereas the caddisfly *Rhyacophila* had a k_e value of 0.137, meaning that during the slow phase of efflux, this species was able to eliminate 13.7% of its body burden per day. The mayfly *Ephemerella excrucians* also exhibited slow efflux (Table 2). There was no relationship between body weight and Cd accumulation ($p = 0.14$) or efflux ($p = 0.57$) among the three taxa. Cd efflux also varied widely among species, and was not related to body weight (Figure 3, Table 1).

Subcellular Cd compartmentalization also varied widely among taxa (Figure 3, Table 3) and species varied in their ability to protect vulnerable cellular sites via the complexation of Cd with metallothionein-like proteins (MTLP). *Rhithrogena*

had the highest proportion of its Cd associated with MTLP (69%), whereas *Rhyacophila* only partitioned 3% of its body burden in this pool. There was no statistically significant relationship between the percentage of the Cd body burden in the MTLP pool and k_u ($p = 0.94$) or k_e ($p = 0.62$) among taxa.

Accumulation ratios derived from k_u and k_e estimates ranged from 309 for *Rhyacophila* to 54 000 in *Rhithrogena* (Table 2). Because *Rhithrogena* did not efflux Cd over the 10 day depuration phase, a k_e value of 0.001 was assigned to this species. Estimates for the length of time required to reach steady-state concentrations varied from 41 days for *Rhyacophila* to over 5000 days in *Rhithrogena*. These estimates are important, because 5000 days exceeds the lifetime of *Rhithrogena*. Therefore, to make relevant comparisons among species, we modeled accumulation over a 1 year time frame. This time frame allows for steady-state concentrations to be reached in *Rhyacophila,* however, it would reduce the Cd concentration (relative to steady state) accumulated in *Ephemerella* slightly, while reducing the concentration accumulated in *Rhithrogena* by over 90% (Table 2).

A dissolved Cd sensitivity index ranged over 2 orders of magnitude among taxa (Table 2). The index suggested that *Ephemerella excrucians* would be the most susceptible species to dissolved Cd, largely because of the relatively fast rates of Cd into potentially sensitive subcellular compartments. *Rhithrogena morrisoni* would be modest in this regard, largely because uptake into potentially sensitive compartments was so slow. *Rhyacophila* would be expected to be highly tolerant to dissolved Cd, because it had relatively slow uptake and efficient efflux.

Discussion

Physiological traits are potentially strong predictors of species responses to anthropogenic stressors (*29*) including contaminants (*30*). Our integrated studies show that toxicity tests and observations in metal-polluted streams appear to rank species sensitivity in a way that can be largely explained by physiological traits such as metal uptake, efflux, and compartmentalization. However, toxicity tests appear to greatly underestimate the sensitivity of insects to metal contamination.

Physiological traits can be revealed using short-term standardized protocols (*31*) at environmentally relevant exposure concentrations. Such approaches might be highly useful for developing mechanistically based, stressor specific tolerance values for aquatic insects that can be used in bioassessment. In experiments lasting 15 days, we can identify species that are likely to be sensitive or tolerant to dissolved Cd exposures. Furthermore, biokinetic parameters help reconcile the differences between field and toxicity studies with aquatic insects.

For example, in the field, *Ephemerella infrequens* was highly responsive to trace metal pollution (Figure 1). However, in short-term (7 d) toxicity assays, such sensitivity was not as apparent (Figure 2.). These different results can, in part, be reconciled by examining metal biodynamics and compartmentalization. In our experiments, *Ephemerella excrucians* had a very fast Cd accumulation rate, while exhibiting rather slow loss (Figure 3). Taxa possessing this combination of traits would likely be sensitive to metals in nature, because high concentrations of metals would accumulate in tissues and eventually cause toxicity. We might expect a taxon with this combination of accumulation and loss dynamics to respond to metals in short term acute toxicity tests. Yet this was not the case in the 7 day microcosm experiments, in *E. infrequens*. where EC_{50} concentrations were more than 50 fold higher than those based on field data. One possible explanation for this discrepancy could be that these larvae might effectively sequester metals in the short term, via metallothionein-like proteins or other storage mechanisms. However, our compartmentalization studies with *E. excrucians* showed that approximately half of the body burden that this species accumulated was in potentially sensitive or biologically active compartments. A more likely explanation for this discrepancy is that based on our biodynamic parameters, we estimate that it would take approximately 400 days for metal concentrations in the tissues of this species to reach steady state. Ideally, steady state should be approached in evaluating chronic toxicity. Because steady-state concentrations of Cd in potentially sensitive compartments are predicted to be quite high in this taxon, we would predict it to be highly sensitive to metals in nature.

The caddisfly *Rhyacophila brunea* was unresponsive to metals in both field and toxicity experiments. Our experiments with *Rhyacophila* sp. revealed that this taxon has very slow Cd uptake, coupled with effective efflux capabilities. Steady state predictions based on dissolved exposures suggest that this taxon would not be expected to accumulate high Cd concentrations in its tissues, even at steady state, which would be approached in approximately 41 days. Approximately 32% of this taxon's Cd body burden was associated with potentially sensitive compartments. The apparent tolerance of *R. brunea* in both field and toxicity assays might largely explained by these physiological characteristics. Interestingly, the ability to efflux Cd rapidly has been observed in other metal-tolerant trichopterans including *Hydropsyche californica* (*32*) and *Hydropsyche bettini* (*33*).

The mayfly *Rhithrogena hagenii* was similar to *Ephemerella infrequens*, in that it exhibited strong responses to metal exposures in nature, but its sensitivity in toxicity assays was not as apparent. EC_{50} concentrations were approximately 42-fold higher in the laboratory than in the field. Our experiments with *Rhithrogena morrisoni* revealed physiological traits quite unlike those of the previous two taxa described above. While Cd uptake from solution was very slow in this taxon, it showed an inability to efflux accumulated metal. This inability to efflux Cd would be particularly deleterious in a contaminated environment, because larvae would accumulate metals over their entire developmental period. *Rhithrogena* appears to counteract this shortcoming by sequestering the vast majority of its Cd burden in the metallothionein-like protein pool. This taxon was remarkable in this regard, as it sequestered almost 70% of its Cd in MTLPs, far more than any of the 21 species we have examined (Buchwalter, unpublished data). Biodynamic parameters suggest that the dissolved route of exposure is not likely to result in high concentrations of Cd in potentially sensitive compartments in this taxon, so the limited responses to metal exposures in mesocosm exposures by *R. hagenii* is somewhat surprising. However, we speculate that a taxon with this combination of traits would be sensitive to metal exposures in nature because it would be a life-long metal accumulator with a potentially expensive physiological coping mechanism in hyper MTLP expression. The dietary route of exposure is likely more important than the dissolved route in this taxon, because dissolved uptake is relatively slow, and the periphyton that this taxon feeds on in nature can accumulate concentrations of metals that are several orders of magnitude higher than dissolved concentrations (*34*). While few studies have examined the influence of route of exposure on k_e values, most of the existing invertebrate data suggest that k_e estimates are similar regardless of exposure route (*35*) (Martin et al., unpublished). We, therefore, expect this species to accumulate high concentrations of metals in potentially sensitive compartments incurring a detoxification cost or even failure.

It is somewhat surprising that rankings based on physiological experiments with dissolved Cd correlated so well with results of field biomonitoring and mesocosm studies in

which larvae were exposed to mixtures of Cd, Cu, and Zn. While accumulation rates of dissolved Cd and Zn appear remarkably similar in a given insect (*13*), we have yet to examine zinc efflux. Very little is known about Cu dynamics in stream insects, due to lack of a suitable radioisotope, but see ref *22*. Despite these data gaps, the use of physiological models to predict or rank metal sensitivity differences among taxa appears to be a promising alternative to toxicity testing with aquatic insects.

Toxicity tests with aquatic insects have generally failed to provide useful insect sensitivity rankings to date, because so many species appear unresponsive (*11*). Those species that do respond to metals in short term toxicity tests seem to respond at concentrations much higher than what has been observed in nature. Conducting toxicity assays for longer periods of time would seem to be one possible solution to this issue. Ideally, to fully evaluate the chronic toxicity of trace metals, steady-state concentrations should be approached in the test organisms. Estimates of the time required to reach steady state in our tests ranged from 41 days in *Rhyacophila*, to 399 days in *Ephemerella* to >5500 days in *Rhithrogena*. Excluding *Rhithrogena*, the average time to steady state in 20 insect taxa evaluated to date was 148 days. We suggest that it would be very difficult to maintain a chronic assay for even 40 days without significant control mortality in most aquatic insect species without outstanding facilities.

There is a reason very few chronic data exist for trace metal toxicity to aquatic insects. With the notable exception of laboratory cultures of various *Chironomus* species, most aquatic insects do not thrive for extended periods of time in the lab. Many species have complex life histories, specific (often unknown) dietary requirements, and various temperature, water flow, and substrate needs. Some species molt frequently, and in the absence of other stressors, molting alone can result in significant control mortality, making chronic toxicity assays difficult to interpret. Comparative physiological studies such as those described here might be better suited for generating species sensitivity distributions, which can be validated with field based observations.

Because insects typically make up the overwhelming majority of the macrofaunal species pool in lotic systems, it is important that their sensitivities to contaminants such as trace metals are adequately understood. Field based biomonitoring and bioassessment can be powerful tools for identifying impaired communities. Several authors have argued that experimental approaches are needed to establish causal relationships (*13*, *14*). Understanding the physiological basis for sensitivity differences among species could significantly improve the interpretive power of bioassessment data. It will, therefore, be important to further develop our understanding of physiological variation within and among different phylogenetic groups so that physiologically based studies can be more readily applied to bioassessment data.

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