Environ. Sci. Technol. **2004**, *38,* 5002−5009

Stable Metal Isotopes Reveal Copper Accumulation and Loss Dynamics in the Freshwater Bivalve *Corbicula*

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Characterization of uptake and loss dynamics is critical to understanding risks associated with contaminant exposure in aquatic animals. Dynamics are especially important in addressing questions such as why coexisting species in nature accumulate different levels of a contaminant. Here we manipulated copper (Cu) stable isotopic ratios (as an alternative to radioisotopes) to describe for the first time Cu dynamics in a freshwater invertebrate, the bivalve *Corbicula fluminea*. In the laboratory, *Corbicula* uptake and loss rate constants were determined from an environmentally realistic waterborne exposure to ^{65}Cu (5.7 μg L^{-1}). That is, we spiked deionized water with Cu that was 99.4% ^{65}Cu. Net tracer uptake was detectable after 1 day and strongly evident after 4 days. Thus, short-term exposures necessary to determine uptake dynamics are feasible with stable isotopes of Cu. In *Corbicula*, ^{65}Cu depuration was biphasic. An unusually low rate constant of loss (0.0038 d^{-1}) characterized the slow component of efflux, explaining why *Corbicula* strongly accumulates copper in nature. We incorporated our estimates of rate constants for dissolved ^{65}Cu uptake and physiological efflux into a bioaccumulation model and showed that dietary exposure to Cu is likely an important bioaccumulation pathway for *Corbicula*.

Introduction

Trace element dynamics in aquatic organisms provide insights into species-specific mechanisms of bioaccumulation (*1*−*3*) and can explain trophic transfer of metals and metalloids into food webs (*3*−*5*). The protocols for determining trace metal dynamics quantify unidirectional uptake rate constants from solution (*6*), assimilation efficiencies (defined as the fraction of metal incorporated into animal tissue from ingested food (*7*)), and efflux rate constants (defined as the proportional rate constant for the unidirectional loss of metals from animal soft tissue via physiological elimination (*1*)). These can be combined into biodynamic models (*1, 8, 9*) to determine trace element bioaccumulation from aqueous and dietary sources (*6, 10*). Usually, these metal-specific and species-specific physiological parameters are determined using radioisotopes, because a unique marker is necessary to quantify unidirectional fluxes. However, complicated logistics, handling, and waste issues limit the use of radioisotopes to laboratories that have trained handlers and can maintain permits. In addition to health hazards associated with radioactivity, the lack of a suitable radioisotope can be a problem. For example, the study of copper (Cu) dynamics is impeded by radioisotopes that are either difficult to prepare (e.g., ^{67}Cu) or have relatively short half-lives (e.g., ^{64}Cu). Consequently, despite its prevalence in the environment (*11*) and its potential toxicity (*12*−*14*), only a few studies have properly quantified Cu accumulation dynamics in marine invertebrates (e.g., refs *15* and *16*). None have dealt with freshwater organisms.

Here we present a simple method for determining Cu bioaccumulation dynamics using stable isotopes. Stable isotope manipulations offer many of the advantages of radioisotopes without some of the problems (Table 1). We use this methodology to develop the first estimates, of which we are aware, for Cu biodynamics for a freshwater invertebrate based upon unidirectional fluxes. We also present simplified calculations for determining the quantity of spiked ^{65}Cu that was accumulated and justify simplified computations for analyzing multicompartmental loss dynamics. Finally, we incorporated the estimated Cu uptake and efflux parameters into a bioaccumulation model to determine whether diet is an important source of Cu for this animal in nature.

Recent developments in inductively coupled plasma-mass spectrometry (ICP-MS) technologies allow detection of naturally occurring, low abundance stable isotopes. Solutions and standards enriched in individual isotopes are commercially available. Thus, manipulation of stable isotope ratios in exposure media is possible (e.g., ref *18*). To provide the equivalent of a radioisotope tracer, it is preferable that an isotope of proportionately low abundance is spiked into the test media. Then it should be possible to follow the spike, independently, by using isotope ratios to account for the "background" concentrations. However, it first must be demonstrated that the challenges of both radioisotope and stable metal methodologies can be overcome. For example, does the organism take up the stable isotope in direct response to a change in the concentration in the media? If so, then it should be relatively straightforward to use manipulation of the abundance of one isotope to study uptake. Can changes in isotope ratios in biological tissues be detected after short exposures to the manipulated ratios in water or food? If so, conversions of the data to dynamic rate constants should also be feasible (*1*). Finally, can interfering fluxes be accounted for by background ratios in media and experimental animals, using easily manageable computations? A major goal of this study is to address these basic questions.

Methods

Experimental Organisms. We collected the freshwater bivalve *Corbicula fluminea* (hereafter referred as *Corbicula*) from a shallow basin in the Sacramento-San Joaquin River Delta (Franks Tract: 38° 05′N, 121°35′W) using an Ekman grab in June 2003. Clams of 5- to 15-mm shell length were held in acid-washed HDPE containers filled with Franks Tract water and transported to the laboratory in coolers. Clams were acclimatized to deionized water for 2 days at 15 °C in a glass aquarium. Cultures of the freshwater cryptophyte *Cryptomonas ozolini*, the chlorophyte *Chlorella vulgaris*, and the diatom *Cyclotella meneghiniana* were fed to clams during the acclimation period and during the experiments (see details below). Algal cultures were maintained at 15 °C in a DY−V media that included macronutrients (N, P, and Si), vitamins, trace metals (Fe and Zn), and a chelating agent (EDTA).

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10.1021/es049432q

TABLE 1. Some Advantages and Drawbacks Inherent to Using Stable Isotopes and Gamma Emitting Radioisotopes as Tracers in Metal Dynamics Studies

	advantages	disadvantages
stable isotope	most elements have 2 or more stable isotopes low detection limits by ICP-MS lack of handling/disposal hazards discriminate background from added amount of tracer with some mathematical manipulation can quantify unidirectional efflux by mathematically eliminating biases from background ratios pure stable isotopes are relatively inexpensive (compared to radioisotopes) allow the simultaneous measurement of uptake and elimination	destructive analysis (i.e., spiked tissue needs to be digested prior to be analyzed) trace element clean techniques and low detection limits require diligence in analysis requires mathematically accounting for natural versus spiked portion of isotope
radioisotope[a]	very low detection limits (gamma detector highly sensitive) allow the assessment of metal dynamics in the same individual over time nondestructive analysis inexpensive, rapid analysis easy to discriminate background from added amount of tracer unique tracer eliminates bias from efflux, allowing quantification of unidirectional efflux	suitable radioisotopes not commercially available for some metals (e.g., ^{67}Cu difficult to prepare and has short half-lives) handling/disposal hazards associated with radioactivity challenging to obtain and maintain radioisotope handling licenses experiments restricted to a limited number of elements by overlap in energy peaks or use of high activities (i.e., only a few laboratories have access to germanium−lithium detectors that allow simultaneous measurement of many isotopes). radioisotopes are relatively expensive (compare to pure stable isotope samples) high specific activity isotopes difficult to obtain (e.g., ^{110m}Ag, ^{203}Hg) low specific activity isotopes imply unrealistic exposure concentrations
stable isotope and radioisotope	conduct experiments on individuals allow the labeling of multicompartments (for some elements) sensitive detection limits allow pulse-chase experiments and short-term dissolved exposures, that minimize recycling and biases	uniform labeling will often need to be assumed

[a] See also Cornelis's (*17*) discussion of these issues.

^{65}Cu Uptake Experiment. One hundred five clams were placed individually into 15-mL acid-washed LDPE vials, which had been conditioned (i.e., rinsed with the experimental solution) to reduce loss of ^{65}Cu onto the walls and filled with deionized water spiked (5.6 μg L^{-1}) with a commercially purchased Cu standard isotopically enriched in ^{65}Cu (99.4%). Clams were fed a 1 h pulse of cultured, unspiked algae every day in order to be more representative of natural conditions; at a biomass (monitored using in vivo chlorophyll-*a* fluorescence (*12*)) representative of Franks Tract (i.e., ∼0.4−6 μg L^{-1} chlorophyll-*a* (*19*)). One hour of feeding allowed *Corbicula* to filter the algal solution at least 2 times (assuming a filtration rate of 4 mL mg^{-1} h^{-1} and a mean weight of 8.3 ± 1.0 mg for our experimental *Corbicula* (*20*)). Following feeding, each individual was transferred to a new acid-washed vial that had been conditioned and filled with deionized water spiked with ^{65}Cu. Deionized water was used to maximize exposure to free copper ion, assuming that *Corbicula*'s ^{65}Cu uptake would follow the precepts of the free ion activity model (*21, 22*). Restricting the feeding period to 1 h per day and replacing the ^{65}Cu-spiked water every day, minimized fecal scavenging and backflux of isotope to the media. After 1, 2, and 4 days of ^{65}Cu waterborne exposure, 10 to 15 clams were sacrificed, and soft tissues were removed from shells and frozen. We selected these time intervals to determine if detection of uptake was possible from the relatively short-time exposure necessary to ensure that ^{65}Cu concentrations in *Corbicula* would be minimally influenced by efflux (*1*).

^{65}Cu Loss Experiment. Following the 4-day exposure to ^{65}Cu, the remaining *Corbicula* were placed individually into 15-mL acid-washed LDPE vials filled with unspiked deionized water. Clams were once again fed a mixture of algae for 1-h each day, and water was replaced daily after feeding. At 1, 2, 4, 6, 9, and 14 days, 10 clams were sacrificed and frozen.

Control Organisms. To provide background concentrations of ^{65}Cu in clams, 10 clams were sacrificed before the beginning of the experiments. As a control, 20 clams were placed individually into 15-mL acid-washed LDPE vials filled with unspiked deionized water. Clams were fed a mixture of algae for 1-h each day and water was refreshed after feeding as described above. At the end of both the uptake (4 days) and loss experiments (18 days), 10 control clams were sacrificed and frozen.

Sample Preparation and Analysis. To minimize inadvertent Cu contamination, labware, vials, and Teflon sheeting were soaked for 24 h in 15% nitric acid, rinsed several times in ultrapure water (Milli-Q system water, >18 Mohm cm^{-1}), and allowed to dry under a laminar-flow hood prior to use.

Partially thawed *Corbicula* were dissected to remove soft tissue, placed individually on a piece of acid-washed Teflon sheeting, and allowed to dry at 40 °C for 3 days. Dried clams were then weighed (Sartorius M2P electronic microbalance) and digested at room temperature in Teflon vials with concentrated nitric acid (Baker Ultrex II grade, 100 μL mg dry weight sample^{-1}) for 7 days (*23*). Hydrogen peroxide (Baker Ultrex II grade, 40 μL mg dry weight sample^{-1}) was added prior to final dilution with ultrapure water (760 μL mg dry weight sample^{-1}). Samples of similar weight from the certified reference material TORT-2 (lobster hepatopancreas from National Research Council of Canada, NRCC) were submitted to the same digestion procedures during each analytical run. Copper concentrations measured in TORT-2 were within the certified range.

Water samples and digested clams were analyzed for both naturally occurring stable isotopes of Cu, ^{63}Cu, and ^{65}Cu, by inductively coupled plasma-mass spectrometry (ICP-MS). Specifically, all samples, blanks, and standards were introduced by direct injection (peristaltic pump; spray chamber) into an Elan 6000 ICP-MS (single-detector; quadrapole) by Perkin-Elmer. Two replicates were measured for each sample. A replicate consisted of 32 individual measurements that were averaged. External standards, serially diluted from ultrapure, single-element stock, were used to create calibration curves for each isotope. Certified reference riverine water samples (NRCC; SLRS-4) were analyzed for Cu during each analytical run. Copper concentrations derived from signal intensities for both Cu stable isotopes (i.e., ^{63}Cu and ^{65}Cu) were within the certified range. To check for the instrument drift and change in sensitivity, we reanalyzed one of our standards after every 5 samples. Isotopic composition of Cu in samples was expressed as the ratio of the net signal intensity (ion counts) of ^{65}Cu to ^{63}Cu.

Calculation of Accumulated Tracer Concentrations. We first determined the relative abundance of ^{65}Cu isotope (i.e., p^{65}) using the signal intensities of each isotope in known standards (i.e., those used to calibrate the ICP-MS) (eq 1). All standards and reference materials fell within <1% of the natural ratio that has been routinely measured on this instrument.

$$p^{65} = \text{Intensity}\left(\frac{^{65}\text{Cu}}{^{65}\text{Cu} + {}^{63}\text{Cu}}\right)_{\text{Standard}} \tag{1}$$

Signal intensity (p^{65}) was calibrated against total copper standards of varying concentration. Concentrations of ^{65}Cu in (the unknown) experimental *Corbicula* ($[^{65}\text{Cu}]_{\hat{e}}$) were calculated as the product of p^{65} and the total Cu concentrations inferred by the ICP-MS software from ^{65}Cu isotope intensity ($[\text{T}^{65}\text{Cu}]$), i.e.,

$$[^{65}\text{Cu}]_{\hat{e}} = p^{65} \times [\text{T}^{65}\text{Cu}] \tag{2}$$

Typically, total metal concentrations determined by ICP-MS can be derived from signal intensities for any (or all) metal isotopes set as analytes in the software method, assuming that there is no isotopic fractionation in the sample. For example, Cd and Cu concentrations could be inferred respectively 8 and 2 times, since Cd has 8 stable isotopes and Cu has 2. (In practice, concentrations for many multi-isotopic elements are often derived from only one isotope, due to isobaric interference that may render certain isotopes as ineffective analytes. In fact, one original purpose for analyzing multiple isotopes on ICP-MS was to determine if isobaric interferences exist.) For the purposes of this study we determined total Cu from both ^{65}Cu and ^{63}Cu.

To independently follow the spike of ^{65}Cu it is necessary to separate out the concentration of ^{65}Cu that occurred in each sample in the absence of a spike (e.g., the "background" that existed prior to exposure to the spiked solution). For this, we used the total Cu concentrations inferred from the intensity of ^{63}Cu ($[\text{T}^{63}\text{Cu}]$) to derive the original load of ^{65}Cu($[^{65}\text{Cu}]^{0}_{\hat{e}}$, eq 3).

$$[^{65}\text{Cu}]^{0}_{\hat{e}} = p^{65} \times [\text{T}^{63}\text{Cu}] \tag{3}$$

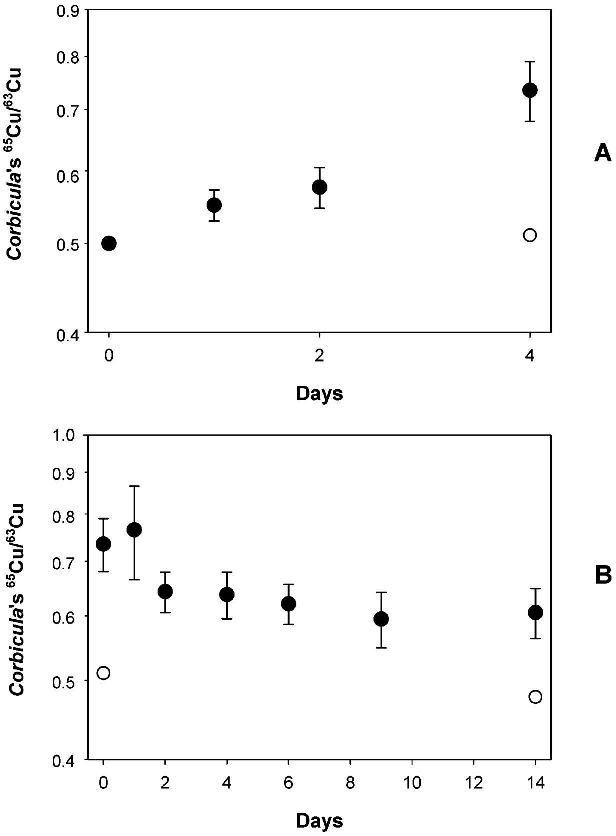

FIGURE 1. Temporal changes in *Corbicula*'s Cu stable isotope ratios (±95% C.I.) during (a) the waterborne ^{65}Cu uptake and (b) loss experiments. Open symbols represent control values.

Net ^{65}Cu uptake ($\Delta[^{65}\text{Cu}]_{\hat{e}}$) is then $[^{65}\text{Cu}]_{\hat{e}}$ as derived from the total experimental Cu inferred from ^{65}Cu signal (eq 2) minus the preexisting load of ^{65}Cu (i.e., $[^{65}\text{Cu}]^{0}_{\hat{e}}$ from eq 3)

$$\Delta[^{65}\text{Cu}]_{\hat{e}} = [^{65}\text{Cu}]_{\hat{e}} - [^{65}\text{Cu}]^{0}_{\hat{e}} \tag{4}$$

Statistical Analysis. We used *t* tests to compare ^{65}Cu concentrations in *Corbicula* at different experimental times. The significance of the relationship between *Corbicula*'s ^{65}Cu enrichment and the duration of exposure was tested by linear regression. Estimates for each model term in the ^{65}Cu uptake and loss experiments were made by nonlinear regression.

Results

As shown in Figure 1A, *Corbicula* enrichment in ^{65}Cu was progressive and significantly related to the duration of exposure ($r^2 = 0.98$, $p < 0.01$). After 1 day of exposure, *Corbicula*'s $^{65/63}$Cu ratio was significantly different from those in the unexposed animals ($p < 0.001$). Short exposures to environmentally realistic concentrations not only caused a net change in the isotope ratio in the animal but also were detectable within a period reasonable for determining unidirectional uptake dynamics.

Depuration of ^{65}Cu following 4 days of uptake from the dissolved phase occurred exponentially over time (Figure 1B). ^{65}Copper was rapidly lost from *Corbicula*'s tissue during the first 2 days of depuration ($p < 0.05$), and then no significant difference was observed in *Corbicula*'s $^{65/63}$Cu ratios from day 2 to 14 ($p > 0.1$).

Modeling ^{65}Cu Accumulation. To characterize *Corbicula*'s ^{65}Cu bioaccumulation dynamics, we first used eqs 1−4 to transform *Corbicula*'s Cu isotope ratios into ^{65}Cu concentrations. We then treated *Corbicula* as a single compartment

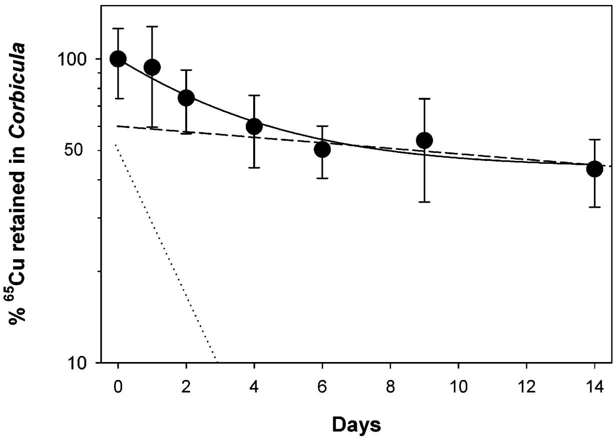

FIGURE 2. Depuration of ^{65}Cu in *Corbicula* following 4 days of exposure to waterborne metal. Values represent percentage of ^{65}Cu in clams (mean ± 95% C.I.). Dotted and dashed lines represent the two exponential terms of eq 11, i.e., the dotted line represents the first exponential term with a shorter half-time and a higher rate constant, whereas the dashed line represents the second exponential term with a longer half-time and a lower rate constant. The sum of these two exponential terms gives the model curve (solid line) obtained using eq 11 and the parameters k_1 and k_2 given in Table 2.

for the accumulation of ^{65}Cu and assumed that the accumulation of tracer was proportional to the free ^{65}Cu-ion concentration ([$^{65}Cu^{2+}$]) (*22*) and that overall uptake and loss followed first-order kinetics (e.g., ref *9*). The rate of change in *Corbicula* ^{65}Cu concentrations was expressed as the difference between ^{65}Cu entering and leaving the clam

$$\frac{\mathrm{d}\Delta[^{65}\mathrm{Cu}]_{\hat{e}}}{\mathrm{d}t} = k_u[^{65}\mathrm{Cu}^{2+}] - k_e\Delta[^{65}\mathrm{Cu}]_{\hat{e}} \qquad (5)$$

where k_u (L g^{-1} d^{-1}) is the uptake rate constant (*6*) and k_e is the proportional rate constant of loss (d^{-1}). Growth was assumed to be negligible compared to k_e since ^{65}Cu uptake and elimination lasted only 18 days and food availability was kept low.

Landrum et al. (*9*) emphasized that k_e can only be expressed in units of d^{-1} (allowing generalization to other circumstances) if it is determined from log-transformed, proportional loss (C_t/C_0) over time, where C_t is the concentration at a given time and C_0 is the concentration when unidirectional loss began. From Figure 2 it is clear that log-transformed, proportional elimination of ^{65}Cu from *Corbicula*'s tissues can be reduced to a biphasic exponential trend. Almost half (37%) of the accumulated tracer was lost within the first 4 days of depuration. Then loss was very slow through the following 10 days of depuration (Figure 2).

To simplify treating ^{65}Cu biodynamics as two compartments, we assumed that all exchanges follow first-order kinetics and that the compartments behaved independently. Uptake and loss for each compartment, separately, then would be given by

$$\frac{\mathrm{d}\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}}{\mathrm{d}t} = k_u[^{65}\mathrm{Cu}^{2+}] - k_1\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1} \qquad (6)$$

$$\frac{\mathrm{d}\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2}}{\mathrm{d}t} = k_{12}\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1} - k_2\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2} \qquad (7)$$

where $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}$ and $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2}$ (μg g^{-1} d.w.) are the ^{65}Cu concentrations in compartment 1 (C_1) and 2 (C_2), respectively, k_1 and k_2 (per day) are rate constants for ^{65}Cu efflux for each compartment, and k_{12} (d^{-1}) is the rate constant of ^{65}Cu uptake in C_2. To determine $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}$ at any given time, we summed the integrated form of eqs 6 and 7 (assuming that [$^{65}Cu^{2+}$] and $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}$ are constant over time):

$$\Delta[^{65}\mathrm{Cu}]_{\hat{e}} = \underbrace{\frac{k_u\,[^{65}\mathrm{Cu}^{2+}]}{k_1}\left(1 - \mathrm{e}^{-k_1 t}\right)}_{\text{Fast exchanging pool}} + \underbrace{\frac{k_{12}\;\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}}{k_2}\left(1 - \mathrm{e}^{-k_2 t}\right)}_{\text{Slow exchanging pool}} \qquad (8)$$

Because we could follow unidirectional ^{65}Cu loss (accounting for background ^{65}Cu influx with the isotope ratios) and assuming no influx of ^{65}Cu beyond that accounted for by [$T^{63}Cu$] (eq 3), the rate of loss of ^{65}Cu concentrations within each compartment can be expressed by

$$\frac{\mathrm{d}\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}}{\mathrm{d}t} = -k_1\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1} \qquad (9)$$

$$\frac{\mathrm{d}\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2}}{\mathrm{d}t} = -k_2\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2} \qquad (10)$$

Summation of the integrated forms of eqs 9 and 10 gives the overall ^{65}Cu concentration in *Corbicula* at a given point in time during the loss experiment, i.e.,

$$\Delta[^{65}\mathrm{Cu}]_{\hat{e}} = \Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}\,\mathrm{e}^{-k_1 t} + \Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2}\,\mathrm{e}^{-k_2 t} \qquad (11)$$

The rate constants were derived from the “fast” and “slow” phases of ^{65}Cu elimination as determined by mathematical stripping (Figure 2 (*24*)). Briefly, the rate constant associated with the slow exchanging pool was first determined from the slope of the straight line drawn through the last few points (days 6−14) of the ln-transformed proportional loss data plotted against time (Figure 2, dashed line). Then, the rate constant associated with the fast exchanging pool was determined from the slope of the straight line drawn through the points representing the difference between each earlier measured value and that predicted by the curve for the slow exchanging compartment (Figure 2, dotted line).

The depuration rate constant associate with C_1 was $k_1 = 0.319$ d^{-1} compared to $k_2 = 0.004$ d^{-1} (Table 2). The biological half-lives of ^{65}Cu in C_1 and C_2 were thus 2.5 and 182 days, respectively. Compartmental analysis of each pool indicates that at the beginning of depuration, 56 and 44% of ^{65}Cu were in the fast and slow exchanging compartment, respectively. The proportion of total ^{65}Cu in C_1 decreased to 31% after 4 days of depuration and declined to less than 3% after 14 days. After 100 and 360 days of depuration, we estimated that 30 and 11% of the initial load of ^{65}Cu would remain in *Corbicula*'s tissues (sequestered in C_2), respectively. As shown in Figure 2, the model curve obtained using the above estimated values shows a good fit of *Corbicula*'s loss of ^{65}Cu.

The assumption that ^{65}Cu is compartmentalized into independent pools within *Corbicula*, upon accumulation, allowed us to simplify the differential equations required to describe bioaccumulation dynamics. Such a treatment is consistent with the traditional approach to this type of transport (e.g. refs *24* and *25*). Where others assumed that compartments are not independent (linkages between compartment must be described (*26*, *27*)), resolution of the resulting differential equations requires “integrator software” as well as numerical methods specifically designed to both solve and fit to experimental data (e.g., ref *26*).

TABLE 2. Estimated Values (±SE) of Dynamics Parameters Used for or Derived from Modeling ^{65}Cu Accumulation and Elimination in *Corbicula*[a]

parameter	symbol	unit	value
Fast Exchanging Compartment			
rate constant of ^{65}Cu loss	k_1	d^{-1}	0.319 ± 0.251
rate constant of ^{65}Cu uptake from dissolved phase into C_1	k_u	$L\ g^{-1}\ d^{-1}$	0.224 ± 0.038
free ^{65}Cu ion concentration	$[^{65}Cu^{2+}]$	$\mu g\ L^{-1}$	5.2
^{65}Cu biological half-life	$tb_{1/2}$	d	2.5
proportion of ^{65}Cu retained after 4 days of uptake	$\%[^{65}Cu]_{ê}^{c_1}$	%	56
Slow Exchanging Compartment			
rate constant of ^{65}Cu loss	k_2/k_e	d^{-1}	0.0038 ± 0.054
rate constant of ^{65}Cu uptake from C_1 into C_2	k_{12}	d^{-1}	0.223 ± 0.038
^{65}Cu biological half-life	$tb_{1/2}$	d	182
proportion of ^{65}Cu retained after 4 days of uptake	$\%[^{65}Cu]_{ê}^{c_2}$	%	44
^{65}Cu Exchange Rates			
^{65}Cu uptake rate	^{65}Cu−UR	ng ^{65}Cu $g^{-1}\ d^{-1}$	$t_{0,14} = 38, 79$
^{65}Cu loss rate	^{65}Cu-LR	ng ^{65}Cu $g^{-1}\ d^{-1}$	$t_{0,14} = 1432, 772$
net change in ^{65}Cu	Δ^{65}Cu	ng ^{65}Cu $g^{-1}\ d^{-1}$	−1394 to 693

[a] Also given are ^{65}Cu exchange rates during our ^{65}Cu loss experiment on the basis of parameter estimates at $t = 0$ and $t = 14$.

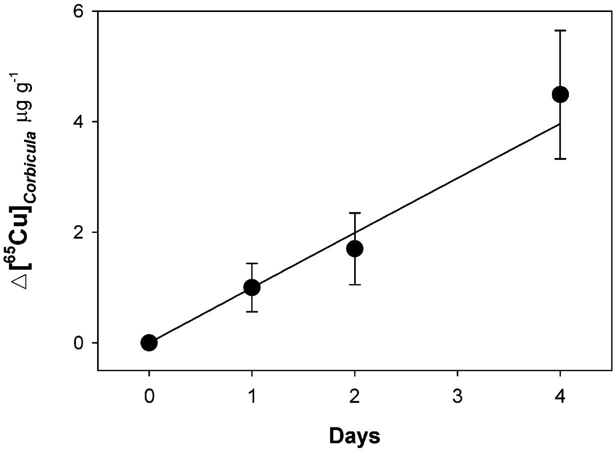

FIGURE 3. Temporal changes in accumulated *Corbicula*'s ^{65}Cu concentrations (± 95% C.I.) during short-term waterborne exposure to ^{65}Cu. Experimental data are represented by symbols (mean ± 95% C.I.). Line represents model curve obtained with eq 12 and the parameters k_u and k_e as well as the value of $[^{65}Cu^{2+}]$ given in Table 2.

Using the value of the elimination rate constant for C_1 (k_1, Table 2) and the concentration of $^{65}Cu^{2+}$ estimated by the speciation model WHAM (1.0 (*28*)), we could determine a rate constant of ^{65}Cu uptake of 0.224 ± 0.038 L g^{-1} d^{-1} (± SE) by fitting our experimental data for the first day of measurable uptake (day 1) to the integrated form of eq 5, that is,

$$\Delta[^{65}Cu]_{ê} = \frac{k_u[^{65}Cu^{2+}]}{k_e}(1 - e^{-k_e t}) \qquad (12)$$

where we assumed that at $t = 0$, $\Delta[^{65}Cu]_{ê}$ equals zero and that $[^{65}Cu^{2+}]$ was constant (i.e., nominal $[^{65}Cu^{2+}] = 5.2\text{-}\mu g\ L^{-1}$). That is, WHAM estimates that 92% of the ^{65}Cu in the exposure media was present as free ions (assuming a pH 6.5). The model curve obtained using eq 12 and the estimated values of k_u and k_1 (Table 2) shows a good fit to *Corbicula*'s ^{65}Cu uptake data (Figure 3). Then, using the values of k_u, k_1, k_2, $[^{65}Cu^{2+}]$, and $\Delta[^{65}Cu]_{ê}^{c_1}$ from Table 2, we estimated the rate constant of ^{65}Cu uptake from C_1 into C_2 (i.e., k_{12}) by fitting (using a nonlinear regression model) our data points (Figure 3) to eq 8.

An important assumption for eq 8 is that ^{65}Cu concentration in C_1 is constant over time. This appears to be reasonable for natural conditions, where applications of the model are most important. As shown in Figure 4, ^{65}Cu concentration in C_1 reaches a plateau within 10 days. So it seems reasonable that ^{65}Cu concentration in the fast exchanging pool ($\Delta[^{65}Cu]_{ê}^{c_1}$) would be near steady state, in most exposures under natural conditions (especially since the life span of *Corbicula* is 4 yrs (*29*)). Of course, the size of C_1 will vary if $[Cu^{2+}]$ varies, because the rate constants k_u and k_1 are both species and metal specific constants (*4*).

We can also use eq 8 to determine the implications of using short exposures for estimating uptake rates. For this, we inserted in eq 8 the values of our rate constants (k_u, k_1, k_{12}, k_2, Table 2) as well as that of $[^{65}Cu^{2+}]$ used during our uptake experiment, to determine uptake of ^{65}Cu into C_1 and C_2 for different exposure time. We used a step-by-step procedure to first calculate $\Delta[^{65}Cu]_{ê}^{c_1}$, which is needed to determine ^{65}Cu uptake in C_2. The computation made using more than 1500 exposure times (with time steps varying from 6 s to 2 h) showed that C_2 is negligible at the beginning of the ^{65}Cu uptake experiment (Figure 4). The relative proportion of ^{65}Cu accumulated in C_1 was slightly lower than 100% (i.e., 99.1%) after 1 h of exposure and reached 82% after 1 day. Because the slow exchanging compartment contributed minimally to the overall ^{65}Cu load during the first hours of uptake, a one-compartment model is appropriate to depict *Corbicula*'s ^{65}Cu uptake (Figures 1A and 3). Similarly, using the first measurable uptake data to estimate k_u (e.g., those for 1 day of exposure) is an appropriate way to estimate the unidirectional flux of metals from solution.

Two compartment loss dynamics complicate analysis of biodynamics, both mathematically and in terms of generalizations (the latter because two compartment analysis carries an implicit assumption about exposure time). However, two compartment analyses may not be necessary to simulate most conditions in nature if, as Cutshall (*25*) showed C_1 declines in importance as time of exposure increases. Recent biodynamic papers use a single compartment, assuming that exposures in nature are long and that the slow compartment will usually dominate loss (*6*). We tested the conditions under which biodynamics of Cu in *Corbicula* are dominated by the physiological turnover of metals in the slowest exchanging pool. As shown in Figure 4, the relative proportion of ^{65}Cu accumulated in C_1 decreased rapidly. At k_2 equals 0.004 d^{-1}, the relative proportion of ^{65}Cu accumulated in C_1 is less than 5% after 100 days of exposure; C_1 is proportionately irrelevant after such long exposures. Organisms are chronically exposed to metals in nature, as determined by biological generation times and geochemical factors such as association with sediments that buffer

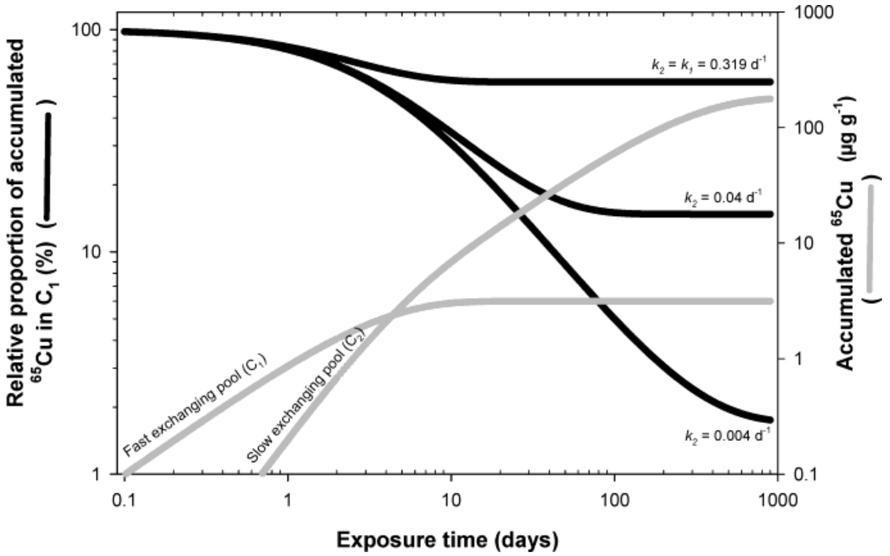

FIGURE 4. Relative proportion of ^{65}Cu accumulated in C_1 (black lines) determined over time using eq 8 and the rate constants k_u, k_1, and k_{12} and the values of $[^{65}\mathrm{Cu}^{2+}]$ and $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{C_1}$ (Table 2) for different values of k_2. Also shown, predicted ^{65}Cu concentrations (gray lines) in C_1 and C_2 over time calculated using eq 8 and the above parameters and values (Table 2).

fluctuations. On this basis, employing rate constants from the most slowly exchanging compartment is a valid assumption.

In organisms with faster rate constants of loss, the same principle holds, but the time before C_1 becomes of minor importance changes. Increasing the value of the loss rate constant for the slowest exchanging pool increased the proportion of ^{65}Cu accumulated in the fast exchanging pool. When k_2 equals k_1, a plateau is reached. This scenario likely represents the case of a unique exchanging pool (see refs *6* and *25*) (Figure 4).

We assumed that ^{65}Cu uptake by *Corbicula* during our ^{65}Cu loss experiment was negligible. We tested this assumption by using eq 5 and values of k_u and k_1 to estimate ^{65}Cu uptake and loss rates during our ^{65}Cu loss experiment (at both $t = 0$ and $t = 14$). Assuming that recycled ^{65}Cu could increase $[^{65}\mathrm{Cu}^{2+}]$ to a maximum concentration of 0.35 μg L^{-1} (i.e., concentration calculated from the amount of ^{65}Cu lost by *Corbicula* during each day of loss, knowing *Corbicula*'s weight, considering an exposure volume of 15-mL and assuming a lack of ligands that could complex ^{65}Cu ions), we calculated that rate constants of loss would have been affected by less than 2−10% of the reported values (Table 2).

Predicting Cu Accumulation in *Corbicula* in Nature. If the only source of uptake were the dissolved metal for clams in a natural system, we can use our model parameters to estimate Cu concentrations that might be expected in *Corbicula* in Franks Tract (California). Under steady-state conditions, eq 5 becomes

$$\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{\mathrm{ss}} = \frac{k_u}{k_e}[^{65}\mathrm{Cu}^{2+}] \qquad (13)$$

where $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{\mathrm{ss}}$ is the ^{65}Cu concentration in clam soft tissues (μg g^{-1}) at steady-state. The free Cu ion concentration was estimated using the speciation model WHAM (*28*) assuming that the lake water has a pH near 8 (*30*), a dissolved organic carbon concentration of 2.7 mg L^{-1} (*31*), and a total dissolved Cu concentration of 2 μg L^{-1} (32). Measured Cu concentrations in clams were from 45 to 155 μg g^{-1} depending on size (*32*). Those concentrations were 7 times higher than predicted by eq 13. This suggests that Cu accumulation by *Corbicula* in nature is driven mainly by a dietary-pathway (i.e. cannot be explained by dissolved uptake), which has been demonstrated for other metals and bivalve species (e.g., Se and Cr (III) in *Mytilus edulis* (*6*, *33*)). Similarly, the importance of food for the uptake of Cu by marine copepods has recently being highlighted by Chang and Reinfelder (*16*).

Discussion

In *Corbicula*, the rate constant k_2 ultimately acts as a bottleneck for ^{65}Cu efflux and controls physiological loss (*1*). If we set k_2 equal to k_e, we can compare *Corbicula* ^{65}Cu loss rate constant to those found for other metals in crustaceans and bivalves. We found that the Cu efflux rate constant measured for *Corbicula* was 15−20 times lower than that of marine copepods (0.06 to 0.08 d^{-1} (*16*)). This means that loss of Cu is consistent with the body of literature that suggests that metal elimination rates in copepods are, in general, an order of magnitude higher than in marine bivalves (Table 3). The k_e for Cu in *Corbicula* is similar to k_e's for Cd and Zn in the coastal oyster *Saccostrea glomerata* but somewhat lower to k_e's for Ag, Cd, Co, Se, and Zn found in other bivalves (Table 3), although rate constants of loss show in general no pattern of variation among elements and bivalve species (*4*). Extremely low metal efflux rates, like those seen for Cu in *Corbicula*, typically cause the accumulation of metals to high levels (e.g., Cd, Se, and Zn in *S. glomerata* (*34*); Zn in barnacles *Balanus amphitrite* (*35, 36*)).

Our estimate for k_u appears to be an order of magnitude lower than the Cu-specific uptake rate constant calculated by Chang and Reinfelder (*16*) for marine copepods (5.1 L g^{-1} d^{-1}, Table 3). The higher surface area ratio of copepods compared to that of bivalves would likely enhance diffusion and transport, which would help to explain in part higher k_u found by Chang and Reinfelder (*16*). Ranking the k_u values reported for marine copepods indicates that uptake rate constants for the dissolved phase increased in the order of $\mathrm{Se} < \mathrm{Co} <\sim \mathrm{Cd} < \mathrm{Zn} < \mathrm{Cu}$ and Ag (Table 3). While integrating our k_u value to those reported for marine bivalves (*6*, *34*), however, we found faster accumulation of dissolved Cd and Zn relative to that of Cu in bivalves.

The paucity of data on Cu accumulation dynamics in aquatic animals reflects mainly the lack of a suitable radioisotope for this metal. The unidirectional fluxes that drive bioaccumulation are difficult to determine unambiguously without using tracer methodologies (i.e. using stable metals or metalloids) because the background of stable metal in tissues, and the high variability typical of tissue concentrations, make detection of small changes difficult. For

TABLE 3. Some Trace Element Uptake Rate Constants from the Dissolved Phase (k_u) as Well as Efflux Rate Constants (k_e) (After Dissolved Uptake)

organism	element	k_u (L g^{-1} d^{-1})	k_e (d^{-1})	reference
marine copepods (*Acartia* sp. and *Temora* sp.)	Cu	5.1	0.06−0.08	(*16*)
marine copepod (*Temora longicornis*)	Ag	10.4	0.173	(*42*)
	Cd	0.694	0.108	(*42*)
	Co	0.606	0.122	(*42*)
	Se	0.024	0.155	(*42*)
	Zn	3.29	0.108	(*42*)
estuarine oyster (*Crassostrea rivularis*)	Cd	0.719	0.014	(*34*)
	Se	0.060	0.034	(*34*)
	Zn	2.050	0.014	(*34*)
coastal oyster (*Saccostrea glomerata*)	Cd	0.534	0.004	(*34*)
	Se	0.064	0.013	(*34*)
	Zn	1.206	0.003	(*34*)
marine mussel (*Mytilus edulis*)	Ag	1.79	0.019	(*6*)
	Cd	0.365	0.011	(*6*)
	Co	0.124	0.018	(*6*)
	Se	0.035	0.026	(*6*)
	Zn	1.04	0.020	(*6*)
marine bivalves (*Crassostrea virginica,*	Ag	ND[a]	0.01−0.04	(*43*)
Macoma balthica,	Cd	ND[a]	<0.01−0.05	(*43*)
Mercenaria mercenaria,)	Co	ND[a]	0.05−0.1	(*43*)
Mytilus edulis	Se	ND[a]	0.01−0.07	(*43*)
	Zn	ND[a]	<0.01−0.07	(*44*)
freshwater mussel (*Dreissena polymorpha*)	Ag	3.6−7.2	0.084	(*44*)
	Cd	2.3−3.2	0.011	(*44*)
	Co	0.05−0.10	0.035	(*44*)
freshwater clam (*Corbicula fluminea*)	Cu	0.224 ± 0.038	0.004 ± 0.054	this study

[a] ND, not determined.

example, rate constants of loss cannot be accurately determined after stable metal spikes, because a background of stable metal occurs within all media and influx from that background cannot be accounted for. Thus rates determined by tissue analysis (or changes in media) will appear slower than unidirectional rates. Landrum et al. (*9*) called these "elimination rate constants", representing fractional elimination, while the animal is still exposed to the element. Such "constants" are valid only for the experimental conditions they describe. They represent a combination of mechanisms (influx and efflux) and thus cannot be generalized over a variety of conditions. Unidirectional "depuration" constants (*9*) or rate constants of loss (the traditional terminology from radioecology (*25*)) are both generic and mechanistic descriptors, if the loss curves are analyzed correctly.

Some studies have overcome this difficulty using novel methodologies, usually adapted, however, to a narrow range of conditions. For example, Chang and Reinfelder (*15*) used siliceous tests of diatom cells as an unassimilated tracer (i.e., ratio method (*37*)) to determine Cu assimilation efficiency (AE) in marine copepods. Although their conclusions were limited to diatoms, incorporation of AE into a kinetic bioaccumulation model highlighted for the first time that dietary uptake, and therefore bioaccumulation dynamics cannot be ignored for Cu (*16*).

Manipulation of stable isotope ratios appears to provide an alternative to radiotracers, in determining trace metal biodynamics, without some of the drawbacks inherent to using radionuclides (Table 1). Recently, Evans et al. (*18*) showed the feasibility of following bioaccumulation with stable Cd isotopes. They used two stable Cd isotopes to measure, simultaneously, uptake and loss of Cd from caddisfly larvae (*Hydropsyche*). They showed that ^{114}Cd and ^{113}Cd behaved independently across a range of conditions (including total metal concentration) and successfully differentiated the processes. Their approach differed from ours in that they were not able to obtain k_u's comparable to other studies. Their uptake experiments were conducted over a 9-day period, so it is likely that the tissue concentrations they report were influenced by efflux (*1*). They also did not subtract background from each measurement so they could not obtain unbiased k_e's. Eimers et al. (*38, 39*) used the same two stable isotopes to differentially labeled sediment and water and successfully demonstrated separate uptake from each. But their conclusions were also based upon net outcomes and were influenced by their choice of the relative concentrations at which they labeled food and water (e.g. refs *3* and *40*).

If stable isotope methodologies are to be widely used, it is also preferable that the equations necessary to differentiate net uptake of the manipulated isotope from the preexisting isotope ratios are as straightforward as possible. Evans et al. (*18*) applied the principle of isotopic dilution (*41*) to transform stable Cd concentrations measured on the ICP-MS into tracer concentrations within organisms. Although the details of their conversion are not fully described, isotope dilution typically mandates complicated arithmetic and duplication of the analysis (i.e., sample and a spiked-sample), which could be problematic while dealing with small samples (e.g., individual invertebrates). The calculations proposed herein (eqs 1−4) directly and concisely convert metal concentrations measured at the ICP-MS into accumulated tracer concentrations.

Overall, refining an approach that applies metal stable isotopes to trace Cu bioaccumulation dynamics allowed us to determine for the first time unidirectional Cu influx and efflux rate constants in a freshwater organism and to compare these fluxes to other metals. The refinement stems in part from the use of baseline Cu isotopic ratios determined from standards to convert metal concentrations measured at the ICP-MS into tracer concentrations. Our experimental results suggested that a slow rate constant of loss for Cu likely explains the elevated Cu levels found in these molluscs in nature. Metal uptake from diet might act as a major pathway for Cu accumulation in *Corbicula* in nature. The refined approach described herein expands the access to tracer methodologies and offers potential to help investigating biodynamics of Cu and other metals in a wide variety of species. Predictive models, forecasts of bioaccumulation under different circumstances, understanding of bioaccumulation strategies of different species, and delineation of exposure pathways for a variety of species are all possible outcomes of greater use of these methods.

Acknowledgments

The technical assistance of F. Parchaso and G. E. Moon with sample collections is gratefully acknowledged. Funding was providing by grant to S. N. Luoma and others from the CALFED Bay Delta Program and the U.S. Geological Survey Toxic Substances Research Program, with some support to S.N. L. from a W. J. Fulbright Distinguished Scholar award. M.-N. Croteau was supported by a postdoctoral fellowship from Fonds de Recherche sur la Nature et les Technologies (Québec). Critical comments from A. R. Stewart and A. Tessier are greatly appreciated.

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Received for review April 15, 2004. Revised manuscript received July 6, 2004. Accepted July 15, 2004.

ES049432Q