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DELINEATING COPPER ACCUMULATION PATHWAYS FOR THE FRESHWATER BIVALVE *CORBICULA* USING STABLE COPPER ISOTOPES

MARIE-NOËLE CROTEAU* and SAMUEL N. LUOMA
U.S. Geological Survey, 345 Middlefield Road, MS465, Menlo Park, California 94025

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Abstract—Delineation of metal uptake routes in aquatic invertebrates is critical for characterizing bioaccumulation dynamics and assessing risks associated with metal exposure. Here we demonstrate that Cu stable isotopic ratios can be manipulated in both exposure media and algae to determine the efflux rate constant (k_e) and to estimate Cu assimilation efficiency (AE) from ingested food in a freshwater bivalve (*Corbicula fluminea*). The Cu AE in *Corbicula* fed ^{65}Cu-spiked *Cryptomonas ozolini* was 38%. Copper uptake routes had no significant influence on efflux; k_e of 0.004 per day characterized the slowest component of efflux following short-term exposures to ^{65}Cu in water or in both food and water. Incorporation of the physiological parameters for dietary and dissolved uptake as well as rate constants of loss into a bioaccumulation model allowed for assessing the relative contribution of water and food as Cu sources. At [$^{65}Cu^{2+}$] of 6.7 μg/L, *Corbicula* accumulated twice as much Cu from diet as from water. In most freshwater systems, the dietary pathway is likely to act as the major Cu uptake route for *Corbicula*. Extrapolation of our laboratory results to the San Francisco Bay–Delta (California, USA) indicated that our biodynamic model and the laboratory-derived parameters for dietary ^{65}Cu uptake provided a realistic representation of the processes involved in Cu accumulation by the bivalve *Corbicula*.

Keywords—Dietary exposure Uptake route Biodynamics Assimilation efficiency Copper

INTRODUCTION

Aquatic organisms are exposed to metal pollutants from dissolved and particulate phases. Delineating the routes of metal uptake is critical for characterizing bioaccumulation dynamics and assessing risks associated with contaminant exposure. The dominant exposure pathway depends on species (e.g., food selection, feeding rates) or geochemistry. For example, trace elements existing in water primarily in anionic forms (those for which oxidation state controls bioavailability: e.g., As and Se) are mainly accumulated from food [1,2]. Where metal concentrations in particles and other food items tend to be enriched orders of magnitude over concentrations of dissolved metals, dietary pathway can be an important exposure route of pollutants (although exceptions exist) [3]. The dissolved uptake pathway is expected to be more important for metals with fast uptake rates from solution, with weak particle reactivity, or under geochemical conditions favoring low partitioning to solid phases [4].

The relative contribution of food and water to the accumulation of metals in aquatic organisms can be determined by dynamic modeling using species-specific physiological parameters. These parameters include metal assimilation efficiencies from ingested food, metal uptake rate constants from the dissolved phase, and metal efflux rates [4,5]. These physiological coefficients are usually determined using radioisotopes [6]. However, suitable radiotracers are lacking for some elements, such as copper. For example, ^{64}Cu has a half-life that is too short to work with, and ^{67}Cu is difficult to prepare and thus is not easily and affordably available from commercial sources. Consequently, only a few studies have properly quantified Cu bioaccumulation dynamics [6–8]. As a result, little is known about Cu bioavailability from food. Here, we used an approach refined by Croteau et al. [6] that applies metal stable isotopes to trace Cu bioaccumulation dynamics. Manipulation of Cu stable isotopic ratios in both exposure media and algae (offered as food) allowed us to determine for the first time Cu assimilation efficiency from ingested food for the freshwater bivalve *Corbicula fluminea*. We incorporated Cu assimilation efficiency, Cu uptake from water, and efflux into a bioaccumulation model to determine whether diet is an important source of Cu for this invertebrate in nature.

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

MATERIALS AND METHODS

Experimental organisms

The freshwater bivalve *Corbicula fluminea* (hereafter referred as *Corbicula*) was collected from a shallow basin in the Sacramento–San Joaquin River Delta (Franks Tract: 38°05′N, 121°35′W) using an Ekman grab in August 2003. Clams of a restricted size range, 5 to 15 mm in shell length (mean of 11.4 ± 0.4 mm; 95% confidence interval [CI]), were held in acid-washed high-density polyethylene containers filled with lake water and were transported to the laboratory in coolers. Clams were acclimatized to artificial lake water (hardness of 10–13 mg $CaCO_3$/L), adjusted to a pH of 6, for three weeks at 15°C in a glass aquarium. The freshwater cryptophyte *Cryptomonas ozolini* was fed to clams during the acclimation period and during the experiments (see details below).

Contamination of algae as food for Corbicula

Axenic cultures of *C. ozolini* were maintained at 15°C in a DY-V media [9] that included macronutrients (N, P, and Si), vitamins, trace metals (Fe and Zn), and a chelating agent (ethylenediamine tetraacetic acid). Algae, to be used as food for *Corbicula* during the ^{65}Cu uptake experiments, were washed, filtered through a 0.4-μm polycarbonate membrane filter (Whatman Nuclepore, Maidstone, UK) (under low vacuum

pressure to minimize cell breakage: i.e., <130 mm Hg), and resuspended in synthetic water (hardness of 10–13 mg $CaCO_3$/L; pH of 6) spiked with a commercially purchased Cu standard isotopically enriched in ^{65}Cu (99.4%). Concentration of free ^{65}Cu ions in the experimental media was estimated using the Windermere humic aqueous model ([WHAM], 1.0: [10]). Cells were exposed to 12.4 μg/L of ^{65}Cu for 24 h in acid-washed polycarbonate Erlenmeyer flasks. Similar concentrations of Cu can be observed in the San Francisco Bay–Delta. Algal growth was quantified by measurements of in vivo fluorescence [11], which was directly related to algal density, as determined by counting under a microscope (r^2 = 0.99, n = 5). The ^{65}Cu concentrations in algae were measured daily after filtering culture aliquots (three replicates) onto preweighted 0.4-μm polycarbonate membrane filters that were rinsed with ultrapure water (Milli-Q® system water, >18 Mohm/cm) (Millipore, Bedford, MA, USA) and then dried at 40°C for 2 d.

^{65}Cu uptake by Corbicula

One hundred twenty-five clams were randomly and individually placed into 15-ml acid-washed low-density polyethylene vials, which had been rinsed with the experimental solution to reduce loss of ^{65}Cu onto the walls; then filled with the ^{65}Cu-spiked experimental media. Clams were fed a 1-h pulse of the cultured ^{65}Cu-spiked algae every day in order to minimize loss of bound ^{65}Cu to the water at a biomass representative of the San Francisco Bay–Delta (i.e., ~0.4–6 μg/L chlorophyll *a*, [12]). One hour of feeding allowed *Corbicula* to filter the algal solution at least two times at a filtration rate of 4 ml/mg/h [13] and a mean clam weight of 7.9 ± 0.9 mg. Following feeding, each individual was transferred to a new acid-washed vial that had been conditioned (rinsed with the experimental solution) and filled with artificial water spiked with ^{65}Cu. The frequent water renewal was designed to minimize depletion of ^{65}Cu by uptake, shell sorption, or fecal scavenging. In addition to providing salts and acid–base balance required by *Corbicula*, the use of a very soft synthetic water of pH 6 allowed maximizing exposure to free copper ion, assuming that *Corbicula*'s ^{65}Cu uptake would follow the precepts of the free ion activity model [14]. After 2 and 4 d of ^{65}Cu exposure to both water and food, 11 to 13 clams were sacrificed and frozen. These time intervals were selected because detection of uptake is possible from this relatively short time exposure [6] and also to ensure that ^{65}Cu concentrations in *Corbicula* would be minimally influenced by efflux [4].

^{65}Cu loss experiment

Following the 4-d exposure to ^{65}Cu, the remaining *Corbicula* were placed individually into 15-ml acid-washed low-density polyethylene vials filled with unspiked artificial water. Clams were fed unlabeled *C. ozolini* for 1 h each day. ^{65}Copper concentrations in unspiked algae were measured after filtering culture aliquots (three replicates) onto preweighted 0.4-μm polycarbonate membrane filters that were rinsed with ultrapure water and then dried at 40°C for 2 d. Experimental media was replaced daily after feeding. At 1, 3, 5, 8, 12, 17, and 24 d, 5 to 11 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, 30 clams were placed individually into 15-ml acid-washed low-density polyethylene vials filled with unspiked artificial water. Clams were fed unlabeled algae for 1 h each day and water was refreshed after feeding (as described above). At the end of the uptake (day 4) and twice during the loss experiments (i.e., at 8 and 24 d), 10 clams were sacrificed and frozen.

Phytoplankton ingestion rates for Corbicula

Ten clams of 7 to 9 mm in shell length (acclimatized to synthetic water for three weeks) were placed individually into 15-ml acid-washed low-density polyethylene vials. After 23 h of starvation, each clam was transferred to a new acid-washed vial filled with unspiked media to which algae (*C. ozolini*) were added at a known cell density (as inferred by the in vivo fluorescence to cell density relationship, as described above). Algal densities were similar to those used in the ^{65}Cu uptake experiments. After an hour of feeding, samples were shaken, and subsamples were taken for determination of algal density by in vivo fluorescence; clams were sacrificed and frozen. Phytoplankton settling rate was determined in five control vials (i.e., clams not added). The biomass of algae consumed by each clam was quantified using the relationship between algal cell density and weight, as determined after filtering known volumes of algal suspension (for which cell density had been determined) onto preweighted 0.4-μm polycarbonate membrane filters that were dried at 40°C for 48 h and then weighed (r^2 = 0.99, n = 4).

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, 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 d. Dried clams and filters holding algae were weighed and digested at room temperature in Teflon vials with concentrated nitric acid (Baker Ultrex II grade, 100 μl/mg dry wt sample) for 7 d [15]. Hydrogen peroxide (Baker Ultrex II grade, 40 μl/mg dry wt sample) was added prior to final dilution with ultrapure water (860 μl/mg dry wt sample). Filters were removed from digested samples, rinsed with ultrapure water, dried at 40°C for 1 d, and weighed. Samples of similar weight from the certified reference material TORT-2 (lobster hepatopancreas from National Research Council of Canada, Ottawa, ON) 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 the ICP-MS (single-detector; quadrapole). 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 (National Research Council of Canada; SLRS-4) were analyzed for Cu during each analytical run. Copper concentrations derived from signal intensities for both Cu stable isotopes were within the certified range. To check for the instrument drift and change in sen-

sitivity, we reanalyzed one of our standards after every five 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

The accumulated tracer concentrations were determined using the equations described by Croteau et al. [6]. Briefly, the relative abundance of ^{65}Cu isotope (i.e., p^{65}) was determined using the signal intensities of each isotope in standards used to calibrate the ICP-MS:

$$p^{65} = \text{intensity}\left(\frac{^{65}Cu}{^{65}Cu + {}^{63}Cu}\right)_{\text{standard}} \quad (1)$$

Concentrations of ^{65}Cu in the experimental organisms ($[^{65}Cu]_{\hat{e}}$) were then calculated as the product of p^{65} and the total Cu concentrations inferred by the ICP-MS software from ^{65}Cu isotope intensity ($[T^{65}Cu]$):

$$[^{65}Cu]_{\hat{e}} = p^{65} \cdot [T^{65}Cu] \quad (2)$$

Total Cu concentrations inferred from the intensity of ^{63}Cu ($[T^{63}Cu]$) were used to derive the original load of ^{65}Cu that occurred in each sample in the absence of a spike ($[^{65}Cu]^0_{\hat{e}}$):

$$[^{65}Cu]^0_{\hat{e}} = p^{65} \cdot [T^{63}Cu] \quad (3)$$

Net ^{65}Cu uptake ($\Delta[^{65}Cu]_{\hat{e}}$) was then $[^{65}Cu]_{\hat{e}}$, as derived from the total experimental Cu inferred from ^{65}Cu signal (Eqn. 2) minus the pre-existing load of ^{65}Cu (i.e., $[^{65}Cu]^0_{\hat{e}}$ from Eqn. 3),

$$\Delta[^{65}Cu]_{\hat{e}} = [^{65}Cu]_{\hat{e}} - [^{65}Cu]^0_{\hat{e}} \quad (4)$$

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

^{65}Copper uptake and elimination by Corbicula

Clams exposed to both dissolved and dietary ^{65}Cu showed significant enrichment in ^{65}Cu after 2 d of exposure ($p = 0.001$, Fig. 1A). Upon exposure to both waterborne and dietary ^{65}Cu, *Corbicula*'s ^{65}Cu:^{63}Cu ratios (±95% CI) increased from 0.500 (±0.002) to 0.777 (±0.131), the latter being significantly different from those in the unexposed animals ($p < 0.001$). No further significant enrichment occurred in clams exposed to ^{65}Cu for two more days ($p = 0.19$). Neither the animal size nor the time of exposure to ^{65}Cu influenced ^{65}Cu:^{63}Cu variability (Fig. 1A). Transformation of *Corbicula*'s ^{65}Cu:^{63}Cu ratios into ^{65}Cu concentrations using Equations 1 to 4 revealed that clams accumulated up to 8 μg/g of ^{65}Cu after 4 d exposure to both dissolved and particulate tracer (Fig. 2). This was almost twice the amount measured in *Corbicula* after 4 d of exposure to a similar level of waterborne ^{65}Cu ($^{65}Cu^{2+}$ concentration of 5.2 μg/L: [6]).

Copper isotopic ratios in *Corbicula* did not vary significantly over the 24 d of depuration ($p > 0.7$). $^{65/63}$Copper ratios (±95% CI) in clams pre-exposed to ^{65}Cu-enriched food and water during 4 d averaged 1.095 (±0.211) at the beginning of the depuration experiment and declined to 0.843 (±0.144) after 24 d (Fig. 1B). Neither the animal size nor the time of dep-

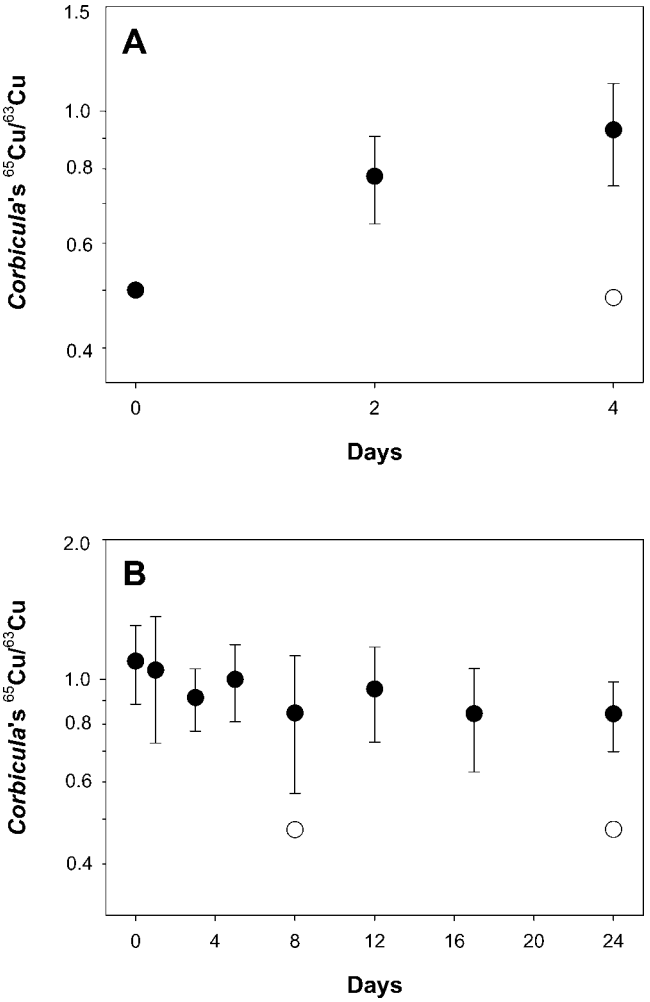

Fig. 1. Temporal changes in Cu stable isotope ratios of *Corbicula* (±95% confidence interval) during (**A**) the ^{65}Cu uptake and (**B**) loss experiments. Open symbols represent control values.

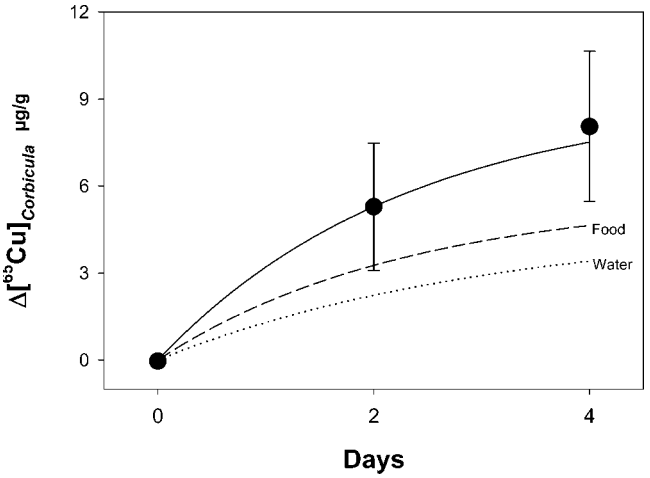

Fig. 2. Temporal changes in accumulated *Corbicula*'s ^{65}Cu concentrations (±95% confidence interval [CI]) during short-term waterborne and dietary exposure to ^{65}Cu. Experimental data are represented by symbols (mean ± 95% CI). Solid line represents model curve obtained with Equation 7 and the parameters k_u, AE, IR, and k_e, as well as the value of $[^{65}Cu^{2+}]$ and $[^{65}Cu]_{\text{food}}$ given in Table 1. Dashed and dotted lines represent contribution of each ^{65}Cu exposure route, namely food and water.

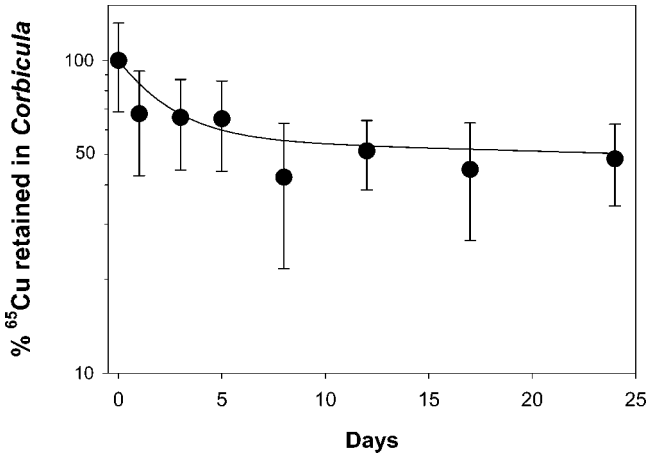

Fig. 3. Depuration of ^{65}Cu in *Corbicula* following 4-d exposure to waterborne and dietary ^{65}Cu. Values represent percentage of ^{65}Cu in clams (mean ± 95% confidence interval). Solid line represents the sum of the two exponential terms of Equation 5 and the parameters k_1 and k_2 given in Table 1.

uration influenced Cu isotopic ratio variability (Fig. 1B). As shown in Figure 3, elimination of ^{65}Cu from *Corbicula*'s tissues was characterized by a biphasic exponential trend. A similar pattern of loss was reported for *Corbicula* exposed to only waterborne ^{65}Cu [6], indicating that the route of Cu uptake had no influence on efflux. Almost one third (35%) of the accumulated tracer was lost within the first 5 d of depuration. Then loss was very slow through the following 19 d of depuration (Fig. 3).

Phytoplankton ingestion rates for Corbicula

The biomass of algae consumed by clams in an hour led to an estimated ingestion rate (IR) of 0.028 g algae/g tissue/d (Table 1). Phytoplankton settling rate was minimal in the controls. After an hour of feeding, 13 ± 2% of the initial load of algae remained in the experimental solution. Therefore, declines in algal densities did not affect the measurement of IR.

Modeling Corbicula*'s ^{65}Cu loss*

We described *Corbicula*'s ^{65}Cu loss by a two-compartment model that takes into account exchanges between compartments but allows elimination from the central compartment only. We assumed that all exchanges follow first-order kinetics. To simplify the differential equations required to describe the linkages betweens pools, we assumed also that compartments behave independently (see detailed discussion in Croteau et al. [6]). The overall ^{65}Cu concentration in *Corbicula* at any given time during the loss experiment is given by

$$\Delta[^{65}\mathrm{Cu}]_{\hat{e}} = \Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}\, e^{-k_1 t} + \Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2}\, e^{-k_2 t} \quad (5)$$

where $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_1}$ and $\Delta[^{65}\mathrm{Cu}]_{\hat{e}}^{c_2}$ (μg/g dry wt) are the ^{65}Cu concentrations in compartments 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. ^{65}Copper loss rate constants and initial ^{65}Cu concentrations in each compartment were estimated by iterative, nonlinear regression analysis. We used starting values determined by mathematical ''backstripping'' (Fig. 3; [16]), because nonlinear regression methods need reasonable initial estimates to converge [17]. Rate constants of loss (±standard error) associated with the fast exchanging pool (C_1) were $k_1 = 0.432 \pm 0.163$ per day compared to $k_2 = 0.004 \pm 0.005$ per day for the slow exchanging pool (C_2) (Table 1). The uncertainty that surrounds rate constants determined by using stable isotopes as tracers is probably greater than that observed for radioisotopes, especially for low values, because the ICP-MS quadropole is not as sensitive as a gamma detector (see table 1 in Croteau et al. [6]). The biological half-lives of ^{65}Cu in C_1 and C_2 were 1.6 and 158 d, 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 compartments, respectively. The proportion of total ^{65}Cu in C_1 decreased to 13% after 5 d of depuration and declined to less than 1% after 12 d. We calculated that 28 and 9% of the initial load of ^{65}Cu would remain in *Corbicula*'s tissues (sequestered in C_2) if depuration had continued for 100 and 360 d, respectively. The ratio of k_1 to k_2 equals 108, which reflects the greater storage capability of C_2 compared to C_1 [17]. This means C_2 will dominate Equation 5 during long periods of exposure [6].

Corbicula*'s ^{65}Cu uptake dynamics*

To determine *Corbicula*'s ^{65}Cu uptake dynamics, we treated *Corbicula* as a single compartment for the accumulation of

Table 1. Values of dynamics parameters used for, or derived from, modeling ^{65}Cu accumulation and elimination in *Corbicula*

Parameter	Symbol	Unit	Value
Rate constant of ^{65}Cu loss of C_1	k_1	d^{-1}	0.432 ± 0.163[a]
Rate constant of ^{65}Cu loss for C_2	k_2	d^{-1}	0.004 ± 0.005[a]
^{65}Cu concentrations in C_1 at the beginning of depuration	$\Delta\,[^{65}\mathrm{Cu}]_{\hat{e}}^{c1}$	μg/g	5.4 ± 1.1[b]
^{65}Cu concentrations in C_2 at the beginning of depuration	$\Delta\,[^{65}\mathrm{Cu}]_{\hat{e}}^{c2}$	μg/g	6.9 ± 0.5[b]
^{65}Cu biological half-life in C_1	$tb_{1/2}$	d	1.6
^{65}Cu biological half-life in C_2	$tb_{1/2}$	d	158
Proportion of ^{65}Cu retained in C_1 after 4 d of uptake	$\%[^{65}\mathrm{Cu}]_{\hat{e}}^{c1}$	%	56
Proportion of ^{65}Cu retained in C_2 after 4 d of uptake	$\%[^{65}\mathrm{Cu}]_{\hat{e}}^{c2}$	%	44
Rate constant of ^{65}Cu uptake from dissolved phase[c]	k_u	L/g/d	0.224 ± 0.038[a]
Free ^{65}Cu ion concentration[d]	$[^{65}\mathrm{Cu}^{2+}]$	μg/L	6.7 ± 0.04
^{65}Cu assimilation efficiency	AE	%	38 ± 10[a]
Ingestion rate	IR	g algae/g/d	0.028 ± 0.005[b]
Spiked-algae ^{65}Cu concentration	$[^{65}\mathrm{Cu}^{2+}]_{\mathrm{Sp\_algae}}$	μg/g	225 ± 41[b]
Unspiked-algae ^{65}Cu concentration	$[^{65}\mathrm{Cu}^{2+}]_{\mathrm{Usp\_algae}}$	μg/g	4 ± 0.5[b]

[a] Estimated value (±standard error [SE]). AE = assimilation efficiency; IR = ingestion rate.
[b] Measured value (±95% CI).
[c] From Croteau et al. [6].
[d] Assuming that at a pH of 6, 54.3% of the dissolved ^{65}Cu (12.4 μg/L) in the exposure media was present as free ions (i.e., major ions concentrations [μM] of 143 for Na^+, 48 for Ca^{2+}, 62 for Mg^{2+}, 6.7 for K^+, 143 for CO_3^{-2}, 110 for SO_4^{-2}, and 6.7 for Cl^-).

Table 2. Lower and upper limits (determined from 95% confidence interval) for the biodynamic parameters k_u, $[^{65}Cu^{2+}]$, ingestion rate, $[^{65}Cu]_{food}$, and k_1 used to predict ranges of Cu assimilation efficiency (AE) (Eqn. 8)

	Parameter limits		% Predicted AE	
Parameter	Lower	Upper	W/lower	W/upper
k_u (L/g/d)	0.146	0.299	46.6	30.5
$[^{65}Cu^{2+}]$ (μg/L)	6.66	6.74	38.5	38.2
IR[a] (g algae/g/d)	0.024	0.333	46.0	32.9
$[^{65}Cu]_{food}$ (μg/g)	184	266	46.9	32.4
k_1 (d^{-1})	0.032	0.831	19.2	61.5

[a] IR = ingestion rate.

^{65}Cu. The equation describing accumulation of ^{65}Cu through time by *Corbicula* is

$$\frac{d\Delta[^{65}Cu]_{\hat{e}}}{dt} = \underbrace{k_u[^{65}Cu^{2+}]}_{^{65}\text{Cu uptake from water}} + \underbrace{AE \cdot IR \cdot [^{65}Cu]_{food}}_{^{65}\text{Cu uptake from food}} - \underbrace{k_e\Delta[^{65}Cu]_{\hat{e}}}_{^{65}\text{Cu loss by efflux}} \quad (6)$$

where $[^{65}Cu^{2+}]$ is the free ^{65}Cu-ion concentration (μg/L), AE is the ^{65}Cu assimilation efficiency (g ^{65}Cu retained per gram ^{65}Cu ingested), IR is the phytoplankton ingestion rate (g algae/g/d), $[^{65}Cu]_{food}$ is the ^{65}Cu concentration in food (μg/g), and k_e is the proportional rate constant of loss (per day), respectively. Growth was negligible, and thus that term was not included in the equation.

The Cu assimilation efficiency (38%) was determined by fitting our experimental data for the first day of measurable uptake (day 2) to the integrated form of Equation 6, that is:

$$\Delta[^{65}Cu]_{\hat{e}} = \frac{(k_u[^{65}Cu^{2+}]) + (AE \cdot IR \cdot [^{65}Cu]_{food})}{k_e}(1 - e^{-k_e t}) \quad (7)$$

We employed data from very short exposure to ensure that the tissue concentration would be minimally influenced by efflux [4]. Solving Equation 7 for AE gives

$$AE = \frac{k_e\Delta[^{65}Cu]_{\hat{e}} - \{k_u[^{65}Cu^{2+}](1 - e^{-k_e t})\}}{IR \cdot [^{65}Cu]_{food}(1 - e^{-k_e t})} \quad (8)$$

The experimentally derived values used in the Equation 7 were the elimination rate constant for C_1 (k_1 = 0.432 per day), a k_u of 0.224 L/g/d [6], a $^{65}Cu^{2+}$ concentration of 6.7 μg/L, an averaged ^{65}Cu concentration in spiked algae of 225 μg/g, and the IR of 0.028 g algae/g tissue/d (Table 1). The model curve shows a good fit to *Corbicula*'s ^{65}Cu uptake data (Fig. 2). To test sensitivity to variability in the parameters, Cu AE was also determined for reasonable ranges of k_u, $[^{65}Cu^{2+}]$, IR, $[^{65}Cu]_{food}$, and k_1 (Table 2). The range of Cu AE estimates was similar to the variability in uptake.

An important assumption for Equations 7 and 8 is that ^{65}Cu taken from water and food enters *Corbicula* through a single (or central) compartment (C_1), the fast exchanging pool (Eqn. 9):

$$\frac{d\Delta[^{65}Cu]_{\hat{e}}^{c_1}}{dt} = k_u[^{65}Cu^{2+}] + AE \cdot IR \cdot [^{65}Cu]_{food} - k_1\Delta[^{65}Cu]_{\hat{e}}^{c_1} \quad (9)$$

This fast exchanging compartment is assumed to dominate the total uptake at 2 d, so the loss rate from C_1 can be used as k_e. Upon accumulation, ^{65}Cu is slowly transferred into C_2, the slow exchanging (or peripheral) compartment (Eqn. 10):

$$\frac{d\Delta[^{65}Cu]_{\hat{e}}^{c_2}}{dt} = k_{12}\Delta[^{65}Cu]_{\hat{e}}^{c_1} - k_2\Delta[^{65}Cu]_{\hat{e}}^{c_2} \quad (10)$$

where k_{12} (per day) is the rate constant of ^{65}Cu uptake from C_1 into C_2, determined by fitting (using a nonlinear regression model) our data points (Fig. 2) to the sum of the integrated form of Equations 9 and 10. As discussed in Croteau et al. [6], assuming that compartments behave independently allows direct determination of each constant and simplifies treatment of ^{65}Cu biodynamics.

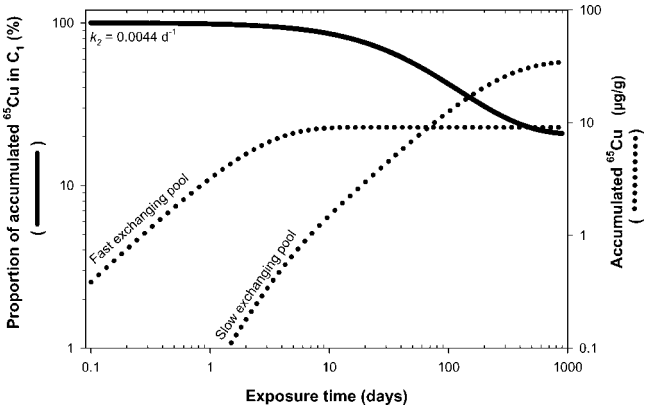

Fig. 4. Proportion of ^{65}Cu accumulated in C_1 (solid line) determined over time using the sum of the integrated form of Equations 9 and 10, the rate constants k_u, k_1, k_2, k_{12}, the values of AE, IR, $[^{65}Cd]_{food}$, $[^{65}Cu^{2+}]$ and $[^{65}Cu]_{\hat{e}}^{c1}$ (Table 1). Also shown, predicted ^{65}Cu concentrations (dotted lines) in C_1 and C_2 over time calculated using the integrated form of Equations 9 and 10 and the above coefficients.

We tested the assumption that the fast exchanging pool dominates the total uptake at 2 d by determining uptake of ^{65}Cu into C_1 and C_2 for different exposure times. For this we inserted into the integrated form of Equations 9 and 10 values of our rate constants (k_u, k_1, k_{12}, k_2), AE, and IR, as well as $[^{65}Cu]_{food}$ and $[^{65}Cu^{2+}]$. We used a step-by-step procedure to first calculate $[^{65}Cu]_{\hat{e}}^{c1}$, which is needed to determine ^{65}Cu uptake in C_2. The computation used more than 1,500 exposure times, with time steps varying from 6 s to 2 h. As shown in Figure 4, ^{65}Cu uptake in C_2 is negligible at the beginning of the uptake experiment. The relative proportion of accumulated ^{65}Cu in C_1 was 99.8% after 1 h of exposure and was 96.7% after 2 d. Because the slow exchanging compartment contributed minimally to the overall ^{65}Cu load during the early phase of uptake, we conclude that a one-compartment model is appropriate to estimate the AE of ^{65}Cu for that time period (Figs. 1A and 2).

^{65}Cu exchange rates

We also assumed that ^{65}Cu uptake by *Corbicula* during our ^{65}Cu loss experiment was negligible. We tested this assumption by inserting in Equation 6 the values for k_u, AE, IR, and k_1 as well as the ^{65}Cu concentration in the unspiked food (Table 1) to estimate rates of ^{65}Cu loss and uptake (for both dissolved and dietary pathways) during our ^{65}Cu loss experiment. We estimated that recycled ^{65}Cu could increase $[^{65}Cu^{2+}]$ to 0.9 μg/L. This is the maximum concentration calculated from the averaged 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. The ^{65}Cu-uptake rates (from both exposure pathways) were lower than loss rate by 10 to 400 times. Rate constants of loss would have been negligibly affected by the uptake of all the ^{65}Cu excreted, given dilution in the efflux media.

DISCUSSION

Assimilation efficiency of ^{65}Cu from food and efflux in Corbicula

The assimilation efficiency with which *Corbicula* retained Cu from food (38%) is similar to that determined for marine copepods fed diatoms (40%) using an inert-tracer ratio method [7]. Because there are no other measurements of Cu AE in aquatic organisms, we compared Cu AE in clams with that of other metals in the marine mussel *Mytilus edulis* [18]. The AE of Cu by *Corbicula* is within the range typical of Zn (16–48%), Se (15–72%), and Co (20–43%), somewhat higher than those typical of Cd (11–34%) and Ag (4–34%), and almost two orders of magnitude higher than that of Am (1–6%). Although metal AE is influenced by abiotic and biological factors, especially food characteristics (e.g., quantity and quality: [18,19]), Cu AE for *Corbicula* appears to be consistent with results obtained from the body of literature showing that essential elements (Cu, Zn, Se, and Co) are generally assimilated with higher efficiency than nonessential elements (see review by Wang and Fisher [19]). Most important, metal stable isotope methodology [6] allows for determination of biodynamic parameters, such as AE, that are consistent with those estimated using nonradiotracer (e.g., mass balance or ratio method) and radiotracer approaches [19].

The efflux of Cu from *Corbicula* showed a faster rate within the first days of depuration than thereafter (0.432 vs 0.004 per day for k_1 and k_2, respectively: Table 1 and Fig. 2), a result that is consistent with many previous observations in bivalves [5,6]. Exposure routes (dissolved and diet) had no significant influence on *Corbicula*'s efflux rate constants (k_e of 0.004 per day; Table 1 and [6]). So partitioning of ^{65}Cu into *Corbicula*'s exchanging pools is controlled primarily by internal physiology. Similarly, Wang et al. [5] reported that neither the duration of exposure nor the pathway of accumulation had an effect on the efflux rate constants in mussels. Our estimate for k_2 is slower than the k_e found for other metals in crustaceans and bivalves (see table 3 in Croteau et al. [6]) and might be responsible for the high Cu concentrations found in *Corbicula* in nature (i.e., up to 244 μg/g; [20]). Barnacles are another example of a species that can accumulate remarkable levels of Zn as a result of high uptake rate from solution, efficient assimilation from food, and extremely slow rate of excretion [21].

Delineating Corbicula*'s Cu uptake pathways*

Determination of ^{65}Cu influx rates from water and diet allowed for direct determination of the relative importance of Cu uptake from the dissolved phase and ingested food for *Corbicula*. Incorporating the physiological parameters for ^{65}Cu uptake and loss (Table 1) into Equation 6 showed that 64% of *Corbicula*'s ^{65}Cu was obtained from diet and 36% from water under these experimental conditions (Fig. 2). *Corbicula* accumulated twice as much ^{65}Cu from food as from water at a free-Cu ion concentration of 6.7 μg/L (pCu of 5.17). If complexation by organic ligands resulted in lower Cu^{2+} concentrations, as often happens in nature, the dietary pathway is likely to be even more important. Chang and Reinfelder [8] reported that Cu uptake from food is the main pathway of accumulation for marine copepods living in waters with moderate to low dissolved free-Cu ions concentrations (pCu ranging from 14.8 to 11.8).

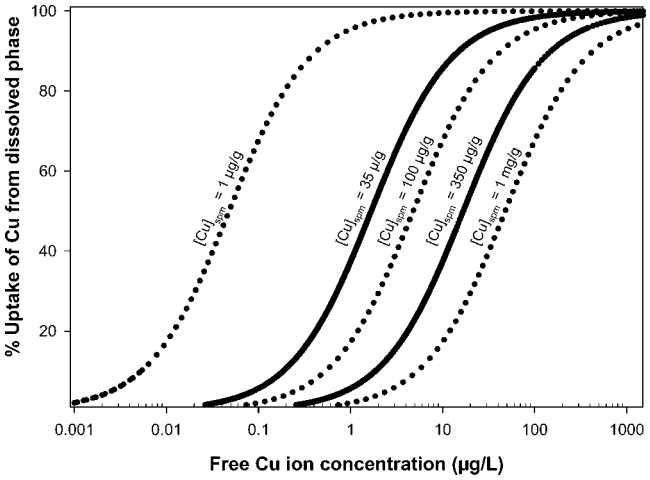

Fig. 5. Relative contribution of the dissolved uptake pathway to *Corbicula* Cu accumulation upon exposure to free ^{65}Cu ions at different concentrations of dietary Cu. Solid lines represent the range of Cu levels found in suspended particulate matter collected in Franks Tract in December 2003 (M.-N. Croteau and A.R. Stewart, unpublished data). Model curves were determined using Equation 7; the parameters k_u, AE, IR, and k_e (Table 1); and various values of $[Cu^{2+}]$ and $[Cu]_{food}$ of 1, 35, 100, 350, and 1,000 μg/g.

Partitioning between dissolved and particulate phases can vary by as much as a 50-fold measure for a metal and is crucial for determining the relative importance of uptake pathways [22]. More free Cu ion in water compared to particulate material results in greater uptake from water compared to diet (Fig. 5). For example, dissolved uptake could be a dominant uptake pathway in lakes affected by mining discharges, in which $[Cu^{2+}]$ could be as high as 44 μg/L ([23]) or in ecosystems receiving pulse discharges of Cu (e.g., algicide application), which result in temporarily high dissolved Cu levels. After a pulse, however, Cu distribution between water and particles will change over time, as Cu ions will be complexed by organic ligands (mainly). Achterberg et al. [24] showed that equilibrium between dissolved reactive and organically complexed Cu took <15 h in natural freshwaters. As a result, the relative importance of the dissolved uptake pathway would decrease as Cu concentration in suspended particulate matter increases for a given free Cu ion concentration (Fig. 5).

Predicting Cu accumulation in Corbicula *in nature*

Under steady-state conditions, Equation 6 becomes:

$$\Delta[^{65}Cu]_e^{ss} = \frac{(k_u[^{65}Cu^{2+}]) + (AE \cdot IR \cdot [^{65}Cu]_{food})}{k_e} \quad (11)$$

where $\Delta[^{65}Cu]_e^{ss}$ is the ^{65}Cu concentration in clam soft tissues (μg/g) at steady state. Copper uptake from water by *Corbicula* is probably negligible under environmental conditions in which dissolved organic matter is abundant (Fig. 5). Franks Tract (hereafter referred as FT), a tidal freshwater lake in the Delta of San Francisco Bay, is such an environment [12], with a dissolved organic carbon concentration of 2.7 mg/L [25]. We tested the validity of both our experimentally derived phys-

iological coefficients and the assumption of negligible dissolved Cu bioavailability by comparing predicted Cu bioaccumulation to Cu measured in *Corbicula*'s tissues from FT. For this, we omitted the first composite term on the right-hand side of Equation 11, assuming negligible Cu ion concentrations. We employed the rate constant of loss for the slow exchanging compartment, because chronic exposure to metals in nature is long, and the slow compartment should dominate loss [5,6]. Values of Cu AE and IR were taken from Table 1. Suspended particulate matter was collected from FT and contained 35 μg/g of Cu (M.-N. Croteau and A.R. Stewart, unpublished data). We predicted Cu concentration of 88 μg/g in *Corbicula*. The prediction falls within the range of concentrations measured in clams of similar size from FT (i.e., 45–155 μg/g: [20]). Thus, Cu accumulation by *Corbicula* in nature appears to follow the kinetics we determined in the laboratory and is driven by the dietary pathway. The results also demonstrate that quantifying the slow loss from the slow exchanging pool by regression, even though it was not significantly different from 0 (Table 1), allowed accurate prediction of metal concentrations in nature.

The results of our studies with *Corbicula* show that manipulation of stable isotope ratios can be used to quantify dietary and dissolved uptake rates in a bivalve, as well as rate constants of loss. These physiological coefficients appear to be a valid representation of the processes that control Cu bioaccumulation in *Corbicula* in a typical habitat and thus should be useful in predicting Cu bioaccumulation by this species in other environments.

Metal fractionation in biological tissues

A basic assumption for using metal stable isotopes to trace bioaccumulation dynamics is that the tracer behaves in exactly the same way as the total metal [26]. This implies that metal isotope rates are not altered during the uptake phase by biological fractionation. Otherwise, the unidirectional fluxes determined based upon this approach would either be overestimated (if the stable isotope set as tracer is preferentially assimilated) or underestimated (if any of the tracer's stable isotopes are preferentially assimilated). Bias introduced by fractionation could greatly affect model predictions, resulting in poor compatibility with field observations and, thus, could lead to erroneous evaluations of metal impacts. Little is known about fractionation of metal isotopes in organisms, but it is generally acknowledged that the degree of fractionation is inversely related to the atomic mass of an element. Heavier isotopes form bonds of greater energy that are more stable (due to their high dissociation energies) than their isotopically lighter counterparts. For example, stable isotopes of heavy elements such as those of lead (e.g., ^{204}Pb and ^{206}Pb) do not fractionate in biological tissues, while lighter elements such as carbon (e.g., ^{13}C and ^{12}C) and nitrogen (e.g., ^{15}N and ^{14}N) fractionate in plants and animals, respectively [27]. We expect, therefore, that biological fractionation of Cu stable isotopes within *Corbicula*'s tissues was negligible. The validation experiment supports this. Nevertheless, there is a need for study of metal isotopic fractionation in biological tissues with instruments suited for rigorous isotope ratio studies, such as thermal ignition mass spectrometry or multi-collector ICP-MS.

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