Novel and Nontraditional Use of Stable Isotope Tracers To Study Metal Bioavailability from Natural Particles

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · MARCH 2013

Impact Factor: 5.33 · DOI: 10.1021/es400162f · Source: PubMed

3 AUTHORS, INCLUDING:

Daniel J Cain
United States Geological Survey
37 PUBLICATIONS **1,298** CITATIONS
SEE PROFILE

Christopher C Fuller
United States Geological Survey
87 PUBLICATIONS **4,858** CITATIONS
SEE PROFILE

Environmental Science & Technology

Article

pubs.acs.org/est

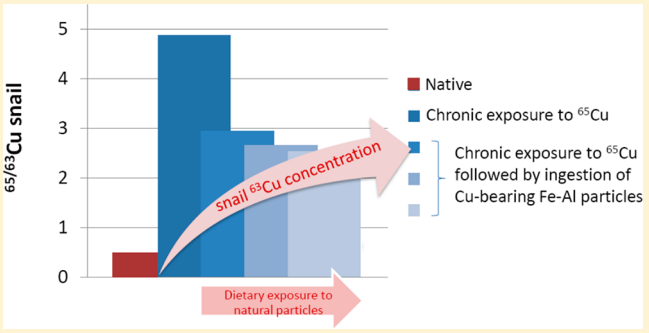
Novel and Nontraditional Use of Stable Isotope Tracers To Study Metal Bioavailability from Natural Particles

Marie-Noëlle Croteau,* Daniel J. Cain, and Christopher C. Fuller

U.S. Geological Survey, MS 496, 345 Middlefield Road, Menlo Park, California 94025, United States

Supporting Information

ABSTRACT: We devised a novel tracing approach that involves enriching test organisms with a stable metal isotope of low natural abundance prior to characterizing metal bioavailability from natural inorganic particles. In addition to circumventing uncertainties associated with labeling natural particles and distinguishing background metals, the proposed "reverse labeling" technique overcomes many drawbacks inherent to using radioisotope tracers. Specifically, we chronically exposed freshwater snails (*Lymnaea stagnalis*) to synthetic water spiked with Cu that was 99.4% ^{65}Cu to increase the relative abundance of ^{65}Cu in the snail's tissues from ∼32% to >80%. The isotopically enriched snails were then exposed to benthic algae mixed with Cu-bearing Fe−Al particles collected from the Animas River (Colorado), an acid mine drainage impacted river. We used ^{63}Cu to trace Cu uptake from the natural particles and inferred their bioavailability from calculation of Cu assimilation into tissues. Cu assimilation from these particles was 44%, indicating that 44% of the particulate Cu was absorbed by the invertebrate. This demonstrates that inorganic particulate Cu can be bioavailable. The reverse labeling approach shows great potential in various scientific areas such as environmental contamination and nutrition for addressing questions involving uptake of an element that naturally has multiple isotopes.



INTRODUCTION

Tracers are invaluable tools to investigate the fate of minerals and trace elements in various biological systems such as cell cultures, animals, and humans.[1−3] In environmental toxicology, nutrition, and biomedicine, tracers are commonly used to characterize the absorption, excretion, transport, and transformation of trace metals,[2−10] major cations, and nutrients.[1,11−13] The most common types of tracers are radioisotopes[14] and enriched stable isotopes.[15] Both share similar advantages such as low detection limits and the possibility to discriminate tracer from background and to work with multiple isotopes for some elements. However, only γ-emitting radiotracers allow for the repeated measurement of the tracer on the same unit (e.g., cell, individual) as analyses are nondestructive. However, complicated logistics, handling, and waste issues limit their use to laboratories that have trained handlers and can maintain permits. In addition to health and safety hazards associated with radioactivity, the lack of suitable radioisotopes can be a problem for the study of elements such as Cu for which radioisotopes either are too difficult to prepare (e.g., ^{67}Cu) or have half-lives too short (e.g., ^{64}Cu $t_{1/2}$ = 12.7 h) for proper study design (see Croteau et al.[2] for more detailed comparison of advantages and disadvantages of both tracer approaches). Enriched stable isotopes have the potential to overcome many of the shortcomings of radioactive tracers. Their application is growing rapidly as a result of new developments in mass spectrometric instrumentation, especially inductively coupled plasma mass spectrometry (ICP-MS).[2,4,7,8,10,15]

In most applications of tracers in biological systems, however, the proper labeling of the substance under study is complex. For example, a major challenge to studying the bioavailability of metals from natural particles is posed by the difficulty to properly label natural particles. Typically, labeling time is short, which favors sorption of the label onto particle surfaces.[16] But the chemical speciation and physical location of metals within a particle can vary greatly. In addition to sorption, metals can be coprecipitated with manganese and/or iron oxides, occluded in aggregates of colloidal particles, incorporated into mineral structures, or trapped under layers of organic matter. Systematically labeling each of these geochemical phases is impossible, yet they can all influence metal bioavailability. Moreover, the age of the metal−particle association plays a crucial role in controlling metal bioavailability as metals can redistribute over time to more resistant phases.[17] Aggregation of colloids over time can also limit the rate of desorption of metals.[18] Equilibration time further determines the partitioning of metals between sediment particles and the dissolved phase. For example, short

Received: January 11, 2013
Revised: February 21, 2013
Accepted: March 4, 2013
Published: March 4, 2013

equilibration time and high spiked metal concentrations can alter metal exposure pathways, resulting in flawed estimations of metal bioavailability.[19] Whether an added tracer and the background metal it represents are similarly distributed among binding sites also remains uncertain in most cases. For example, high concentrations of an enriched stable metal isotope tracer might shift the distribution of metals from its native state and as such misrepresent metal bioavailability. Thus, unless synthetic particles are prepared using a tracer to assess the influence of a specific geochemical phase (e.g., ^{65}Cu bound to colloidal hydrous ferric oxides[8]) or natural particles are allowed to age in the presence of a tracer for a period of time representative of the metal−particle contact time in nature, the most basic assumption of a tracer study is rarely met. That is, a tracer must behave in exactly the same way as the natural metal it is intended to represent.[14]

To circumvent these problems, we devised a novel tracing approach that involves enriching test organisms with an isotope of low natural abundance of the element under study prior to the onset of an experiment. Conceptually, the procedure reverses the labeling from the "source" to the "recipient", such that the test organism is artificially enriched with a rare isotope of the element under study. The source is not altered by labeling and thus is studied in its natural state. Subsequent exposure to the element from the source shifts the isotopic ratio of the element in the test organism toward its natural isotopic ratio. Here we demonstrate the technique for copper (Cu) and show its application to characterize Cu bioavailability from natural inorganic particles. Specifically, we chronically exposed freshwater snails to enriched ^{65}Cu to artificially increase the abundance of the less common Cu isotope (^{65}Cu) in the snail's soft tissues to a point where most of the snail's background Cu is ^{65}Cu instead of ^{63}Cu. The relative abundances of Cu natural isotopes are 0.692 for ^{63}Cu and 0.309 for ^{65}Cu in the standard reference material NIST 976 (commercial metal).[20] We first show that the reverse labeling has no measurable effect on growth. We then show that following a pulse-chase exposure to environmentally relevant concentrations, Cu uptake from natural inorganic particles induces detectable changes in the Cu isotopic ratios of soft tissues of isotopically enriched test animals. We provide equations to convert isotope ratios into accumulated metal concentrations, which were then used to determine biokinetic rate constants and infer Cu bioavailability. This novel "reverse labeling" technique provides a means to simply and directly assess the bioavailability of unaltered natural inorganic particles, which addresses a long-standing uncertainty in the literature.

MATERIALS AND METHODS

Isotopically Enriched Snails. Freshwater snails (*Lymnaea stagnalis*) were reared in the laboratory in a 40 L glass tank filled with 20 L of moderately hard (MOD) water.[21] Eight weeks prior to experiments, snails of a restricted size range (i.e., soft tissue dry weight of <1 mg) were transferred to an 8 L glass aquarium filled with MOD water spiked with isotopically enriched ^{65}Cu (99.4% Trace Sciences) to achieve a total Cu concentration of 10 μg L^{-1}. Snails were fed lettuce ad libitum during the exposure period. Medium was replaced every 11−12 days.

Animas River Particles (ARP). Particles were harvested in September 2006 from the Animas River (Colorado) ∼0.8 km downstream of its confluence with Cement Creek by concentrating the particulate fraction from 35 L of river water to 1 L by tangential flow ultrafiltration using a 10 kDa filter (2 nm effective pore size).[22] In samples collected from Cement Creek upstream of the confluence at this time, ∼50% of the total Fe was in the particulate phase, with little measurable Al (∼4%) and no Cu in the particulate phase (>10 kDa). After mixing and neutralization of Cement Creek water (pH 4.1) with circumneutral Animas River (pH 7.5) during transport to the sampling point, metal concentrations in the ultrafiltrate indicate that precipitation of the remaining dissolved Fe, nearly complete precipitation of Al, and ∼50% sorption of Cu to colloidal phases had occurred (Table S1, Supporting Information). Cement Creek comprises about 22% of the discharge to the Animas River and about 87, 95, and 71% of the total load of Fe, Al, and Cu, respectively. A detailed description of this site and colloid formation in the confluence can be found in Schemel et al.[22]

Particles were transported on ice to the laboratory where they were concentrated by settling and centrifugation and then stored at 4 °C. Total metal concentrations of the ARP were determined by ICP-OES analysis of a split of the recovered particles after drying and digestion in nitric acid (Table S1, Supporting Information). Prior to the uptake experiments, the particles were resuspended in 30 mL of the ultrafiltrate. The suspension was subsampled and diluted with MOD water to achieve a Cu concentration of 720 μg L^{-1}.

Uptake Experiments. To characterize the uptake of Cu from particles entrained with food, we used the benthic diatom *Nitzschia palea* as a food source. Algae were grown axenically in an S-diatom medium.[23] They were harvested onto 1.2 μm Isopore membrane filters (Millipore) and washed with soft (SO) water[21] to make algal mats. We employed the protocol described by Croteau et al.[24] to present the algae in the form the snails would ingest. Briefly, algae were resuspended into a 50 mL acid-washed Falcon tube filled with MOD water amended with different volumes of ARP. After a short gentle mixing (<1 min), the diatoms mixed with the ARP were harvested onto 1.2 μm Isopore membrane filters. Analysis of filtrates showed that the majority of ARP was retained on the filters (73−100% recovery of the Cu and Fe initially added). Small sections of the filters holding the diatoms mixed with the ARP were sampled, dried for 24 h at 40 °C, and weighed prior to metal analysis (Table S2, Supporting Information). The weight of diatoms was corrected for the mass of ARP on the filters to get Cu (μg) per gram of diatoms. The remaining filters were offered as food to *L. stagnalis*.

For each treatment, eight isotopically enriched ^{65}Cu snails were exposed to diatoms amended with ARP for 6 h in 150 mL acid-washed polypropylene vials partially submerged in a 40 L glass tank. Exposure was shorter than gut residence time (∼22 h).[25] Specifically, the animals were allowed to ingest a mass of food mixed with ARP, then removed, rinsed with MOD water, placed individually in acid-washed enclosures, and fed uncontaminated food (lettuce) ad libitum for 48 h. After depuration, snails were removed from each enclosure and frozen. The feces produced by each snail were collected in acid-washed Teflon vials and dried for 48 h at 40 °C. Aliquots of water (n = 3) were taken immediately after the dietary exposure, as well as at the end of depuration. Water samples were acidified with concentrated nitric acid.

Sample Preparation and Analysis. To minimize inadvertent metal contamination, labware, vials, and Teflon sheeting were soaked for at least 24 h in acid (15% nitric and 5% hydrochloric), rinsed several times in ultrapure water, and

dried under a laminar-flow hood prior to use. Partially thawed *L. stagnalis* were dissected to remove soft tissue, placed individually on a piece of acid-washed Teflon sheeting, and dried at 40 °C for 3 days. Dried snails, feces, and diatoms were weighed and digested in concentrated nitric acid, following the protocol described in Croteau et al.[24] Similar weight samples of the certified reference material TORT-2 (lobster hepatopancreas from the National Research Council Canada) were submitted to the same digestion procedure during each analytical run.

Water and digested samples were analyzed for the naturally occurring stable isotopes of Cu by ICP-MS (Supporting Information). All samples, blanks, and standards were introduced by direct injection (peristaltic pump; spray chamber) into the ICP-MS (single-detector; quadrupole). Analyte analysis for each sample consisted of 20 individual measurements that were averaged. External standards, serially diluted from ultrapure, single-element stock, were used to create calibration curves for each isotope. To account for instrument drift and change in sensitivity, internal standardization was performed by addition of germanium to all samples and standards except the calibration blanks. The method detection limit for ^{65}Cu and ^{63}Cu was 0.03 μg L^{-1}. Measured Cu concentrations in the TORT-2 were consistently within the certified values. We also reanalyzed one of our standards after every 10 samples. Deviations from the standard values were <5% for the analyzed Cu isotopes at all times.

Data Analysis. $^{65/63}$Cu ratios, Cu concentrations, and Cu burdens in *L. stagnalis* are the mean of six to seven individual measurements ± standard deviation (SD). Cu concentrations in the diatoms are the mean of three individual measurements ± SD. Treatment differences were tested by ANOVA with Tukey's post hoc multiple-comparisons test. The effect of reverse labeling on growth was tested by ANCOVA. Differences were considered to be significant at a type I error rate of $\alpha = 0.05$.

RESULTS

Isotopically Enriching Test Organisms with ^{65}Cu. The $^{65/63}$Cu in snails exposed for 8 weeks to 10 μg L^{-1} of ^{65}Cu increased from 0.48 ± 0.01 to 4.88 ± 0.74, which shifted the relative abundance of ^{65}Cu in the snails soft tissues (p^{65}_{snail}) (eq 1) from 32.2 ± 0.2 to 82.7 ± 2.4%. p^{65}_{snail} was determined using the net signal intensity for each isotope in the snail's tissue after the ^{65}Cu exposure:

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

The total Cu concentration in the isotopically enriched snails ([^{63}Cu] + [^{65}Cu]) averaged 94 ± 9 μg g^{-1} at the end of the 8 week exposure to 10 μg L^{-1} of ^{65}Cu, which is about 2.8 times higher than the background Cu concentration of unexposed snails.[26]

To determine if pre-exposure to ^{65}Cu induced physiological and/or biochemical changes that would subsequently affect Cu uptake by snails, we conducted a companion experiment to evaluate the growth of snails chronically exposed to 10 μg L^{-1} of ^{65}Cu. The estimated growth rate constants (k_g ± 95% CI, in day^{-1}) for the control and treated (^{65}Cu-exposed) snails were 0.034 ± 0.002 and 0.039 ± 0.003, respectively. The log-transformed dry weights of control and treated snails were not different (ANCOVA, $F(1,91) = 2.08$, $p = 0.15$), suggesting that ^{65}Cu exposure did not affect growth.

Modification of Cu Isotopic Ratios upon Exposure to Natural Inorganic Particles. The extent of ^{63}Cu enrichment in snails was greatest between the controls and the first exposure concentration. Figure 1 shows a nonlinear shift in the $^{65/63}$Cu of snails exposed to increasing dietary concentrations of Cu-bearing ARPs collected downstream of an acid mine drainage inflow to the Animas River, Colorado (ARP). Specifically, snail's $^{65/63}$Cu decreased from 4.88 ± 0.74 to 2.95 ± 0.17 when the dietary Cu concentration increased from 14 to 542 μg g^{-1} (Table S2, Supporting Information). Further increases in the dietary Cu exposure only marginally reduced the snail's $^{65/63}$Cu. The shift in the isotopic Cu ratio of the exposed snails indicates that the Cu from the natural particles mixed with diatoms was accumulated and thus bioavailable. Uptake of Cu by native snails (e.g., field-collected or laboratory-reared snails) would not have been detected under similar exposure conditions (eq S2, Supporting Information).

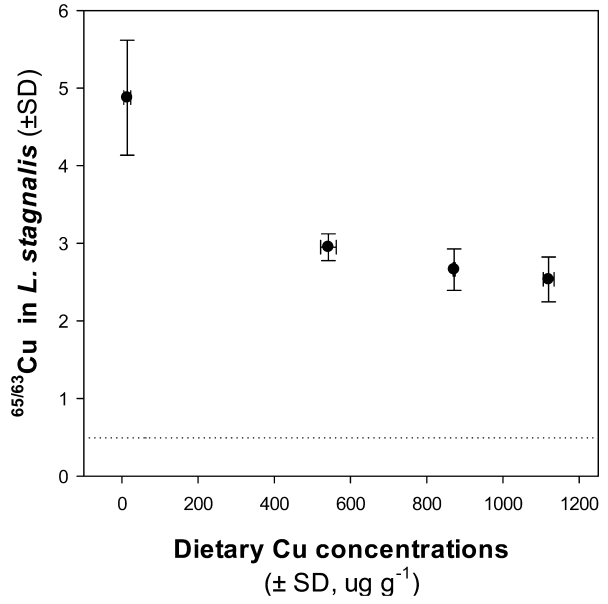


Figure 1. $^{65/63}$Cu (±SD) in *L. stagnalis* soft tissue after exposure to increasing concentrations of ARP mixed with diatoms. Each symbol represents $^{65/63}$Cu for six to seven individuals and three diatom samples (μg g^{-1} ± SD). The dotted line represents the $^{65/63}$Cu of native snails.

When the snails were exposed to diatoms amended with increasing concentration of Cu-bearing Fe–Al particles, both ^{63}Cu and ^{65}Cu were accumulated, but for every 2 atoms of ^{63}Cu taken up, only 1 atom of ^{65}Cu was accumulated (i.e., the $^{65/63}$Cu ratio of natural materials is roughly 0.5).[26] Because the Cu isotopic ratio of the experimental snails had been manipulated to favor ^{65}Cu ($^{65/63}$Cu ratio of 4.88), the uptake of Cu from the natural particles reduced the $^{65/63}$Cu ratio in the snail tissues (Figure 1). More than 98% of the ^{63}Cu in the exposure originated from the ARP, that is, the ^{63}Cu concentration in the diatoms mixed with the ARP was 9 μg g^{-1}.

Calculation of Background ^{63}Cu Concentrations. To independently follow the accumulation of ^{63}Cu that originated from the exposure to the natural particles (ARP), it was necessary to calculate the concentration of ^{63}Cu that occurred

dx.doi.org/10.1021/es400162f | Environ. Sci. Technol. 2013, 47, 3424–3431

in each sample prior to the exposure (i.e., the background ^{63}Cu concentration). For this, we used the snail background ^{65}Cu concentration ($[^{65}Cu]^0_{snail}$ in $\mu g\ g^{-1}$) and the relative abundance of ^{65}Cu in the isotopically enriched snails (p^{65}_{snail}).

Specifically, we first quantified the snail ^{65}Cu accumulation ($\mu g\ g^{-1}$) during the exposure to the ARP. For this, we used the first terms of the integrated form of a kinetic biodynamic model (eq 2)[27] in which k_{uf} (g g^{-1} day^{-1}) is the rate constant of Cu uptake from diatoms mixed with the ARP (i.e., k_{uf} is assumed to be similar for both Cu isotopes), $[^{65}Cu]_{food}$ ($\mu g\ g^{-1}$) is the ^{65}Cu concentration in the diatoms mixed with the ARP, k_e (day^{-1}) is the rate constant of Cu loss, and T_1 is the exposure duration (days) (Table 1). Loss of background ^{65}Cu by the snails is assumed to be negligible during the short uptake phase (i.e., 6 h), whereas loss of newly accumulated ^{65}Cu is assumed to be negligible compare to that of $[^{65}Cu]^0_{snail}$ during depuration.

Table 1. Values Used for Calculating Cu Background Concentrations, Cu Uptake from ARP, and the Relative Abundance of ^{65}Cu in *L. stagnalis*

parameter/variable	symbol	unit	value
rate constant of uptake from food	k_{uf}	g g^{-1} day^{-1}	0.14
rate constant of loss[a]	k_e	day^{-1}	0.026
exposure duration	T_1	day	0.25
depuration duration	T_2	day	2
dietary Cu concentration during exposure to ARP[b]	$[Cu]_{food}$	$\mu g\ g^{-1}$	14–953[c]
dietary Cu concentration during depuration and during the enrichment phase to ^{65}Cu[b]	$[Cu]_{food}$	$\mu g\ g^{-1}$	7–9[c]

[a]From Croteau and Luoma.[47] [b]Total Cu concentration. [c]Range.

$$[^{65}Cu]_{snail} = \frac{k_{uf}[^{65}Cu]_{food}}{k_e}(1 - \exp^{-k_eT_1}) + [^{65}Cu]^0_{snail}\ \exp^{-k_eT_2} \quad (2)$$

The background ^{65}Cu concentration in each experimental snail ($[^{65}Cu]^0_{snail}$) was then determined by solving eq 2 for $[^{65}Cu]^0_{snail}$ using the depuration period T_2 (days), the snail ^{65}Cu concentration measured at the end of the depuration period ($[^{65}Cu]_{snail}$), and the accumulated ^{65}Cu concentration, as determined above. Because snails were fed lettuce that had a ^{65}Cu concentration of ~2.6 $\mu g\ g^{-1}$, the ^{65}Cu accumulated during the depuration phase (0.7 $\mu g\ g^{-1}$) was also subtracted from the observed snail ^{65}Cu concentration.

The pre-existing concentrations of ^{63}Cu in each experimental snail ($[^{63}Cu]^0_{snail}$ in $\mu g\ g^{-1}$) was then determined using the calculated background ^{65}Cu concentration (as described above) and the relative abundance of ^{65}Cu in the control snails (p^{65}_{snail}, eq 1):

$$[^{63}Cu]^0_{snail} = \frac{[^{65}Cu]^0_{snail}}{p^{65}_{snail}} - [^{65}Cu]^0_{snail} \quad (3)$$

^{63}Cu Accumulation from Exposure to Natural Particles. Figure 2 shows that accumulation of <25 ng of ^{63}Cu was sufficient to induce a significant change in the snail's $^{65/63}Cu$. For example, snails exposed to the lowest Cu concentration in their diet (542 $\mu g\ g^{-1}$) accumulated 23.6 ng of ^{63}Cu after 6 h of exposure, which increased their ^{63}Cu burden from 37.3 to 61.0 ng. Correspondingly, snails accumulated roughly half that amount of ^{65}Cu, but ^{65}Cu accumulation was not detectable (P = 0.2) because of the large ^{65}Cu background concentration.

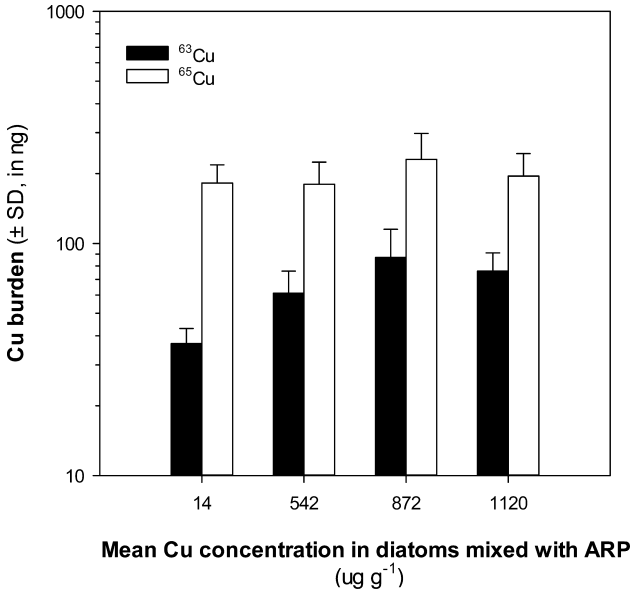


Figure 2. Snail Cu accumulation (ng ± SD) as a function of the dietary Cu concentration. The solid and open bars represent ^{63}Cu and ^{65}Cu accumulation, respectively.

Assuming no preferential assimilation of Cu isotopes by the snails and no transfer of Cu from ARP to diatoms,[8] the decrease in the snail's $^{65/63}Cu$ was thus due exclusively to ^{63}Cu accumulation from the ARP. As a result, significant Cu accumulation in *L. stagnalis* for the lowest amount of ARP mixed with food was detected only when both the accumulated Cu concentration and the Cu exposure concentrations were expressed in terms of ^{63}Cu, not in terms of the total Cu concentration (as commonly used) nor in terms of ^{65}Cu concentration (Figure S2, Supporting Information). The dose response nature of the ^{63}Cu uptake demonstrated that the ^{63}Cu was bioavailable from the ARP.

Normalization of the accumulated ^{63}Cu concentrations to account for the exposure duration reveals that ^{63}Cu influx rates into *L. stagnalis* ($\mu g\ g^{-1}\ day^{-1}$) increased linearly with ^{63}Cu exposure concentration ranging from 9 to 587 $\mu g\ g^{-1}$ (Figure 3). The Cu uptake rate constant from food (k_{uf}) for the ARP was 0.14 ± 0.03 g g^{-1} day^{-1} (±95 CI). k_{uf} was determined from the slope of the regression between ^{63}Cu uptake rate into *L. stagnalis* soft tissue after a short exposure time (to minimize the influence of efflux)[28] and the dietborne exposure concentrations (data from the linear portion of the curve). The rate constant of metal uptake represents first-order accumulation processes (i.e., metal influx solely depends on the exposure concentration). k_{uf} is expressed as μg of metal (g of tissue)$^{-1}$ day^{-1} per μg metal (g of food)$^{-1}$, or g of food per g of tissue per day.

The Cu uptake rate constant from food (k_{uf}) for the ARP was nearly 5 times lower than the k_{uf} for Cu bound to synthetic hydrous ferric oxide, but similar to the k_{uf} for CuO nanoparticles mixed with diatoms (Table 2). As shown in Figure 3, ^{63}Cu influxes into *L. stagnalis* deviated from linearity to level off around 145 μg of ^{63}Cu g^{-1} day^{-1} when ^{63}Cu exposure concentrations exceeded 587 $\mu g\ g^{-1}$. The relationship between Cu influx and dietborne Cu concentrations has also been reported to deviate from linearity when snails were offered diatoms labeled with high concentrations of Cu.[29] Experiments conducted with Zn, Ag, Ni, and Cd also showed that dietary Zn

dx.doi.org/10.1021/es400162f | Environ. Sci. Technol. 2013, 47, 3424–3431

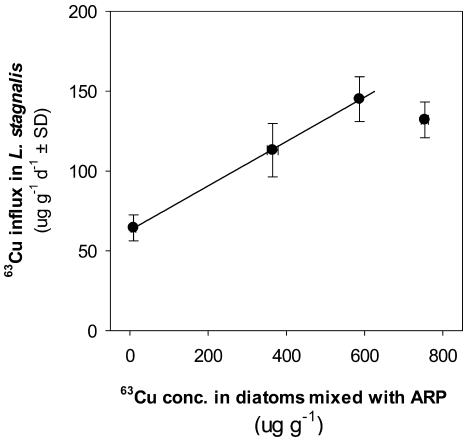


Figure 3. ^{63}Cu uptake rates (μg g^{-1} day^{-1} ± SD) in *L. stagnalis* soft tissue after dietborne exposures. Each symbol represents Cu influxes for six to seven individuals and three diatom samples (μg g^{-1} ± SD). The solid line represents the statistically significant linear regression relationship.

Table 2. Rate Constants of Dietary Cu Uptake (k_{uf} ± 95% CI) and Cu Assimilation Efficiency (AE ± SD) in *L. stagnalis* Determined for Different Food Sources and/or Cu Forms

dietary exposure to	k_{uf} (g g^{-1} day^{-1})	AE (%)	ref
lettuce pre-exposed to $CuNO_3$	0.16 ± 0.04	86 ± 2	47
diatoms pre-exposed to $CuNO_3$	0.06 ± 0.005	[72−82]	29
diatoms mixed with CuO nanoparticles	0.13 ± 0.02	41 ± 4	Croteau et al., unpublished data
diatoms mixed with synthetic Cu-HFO	0.69 ± 0.20	[71−93]	8
diatoms mixed with ARP	0.14 ± 0.03	44 ± 11	this study

and Ag can suppress influxes in *L. stagnalis*.[24,29,30] The elevated concentrations of Fe, Al, and Zn in the ARP might have acted synergistically with Cu to suppress Cu influxes (Table S2, Supporting Information).

Cu Assimilation Efficiency and Food Ingestion Rates. The reverse labeling approach allows determination of important digestive processes such as food ingestion rates (IR) and assimilation efficiency (AE). Food ingestion rates (IR) during the feeding phase (g of ingested food per g of body tissue per day) were determined by mass-balance calculations using the total amount of ^{63}Cu retained in the snails after depuration ($^{63}Cu_{snail}$ in ng), the amount of ^{63}Cu egested in the feces during depuration ($^{63}Cu_{feces}$ in ng), the ^{63}Cu concentration in the diatoms mixed with the ARP ($[^{63}Cu]_{diatoms}$ in ng g^{-1}), the snail's dry weight (wt_{snail} in g), and the exposure duration (T_1):

$$IR = \frac{(^{63}Cu_{snail} + {}^{63}Cu_{feces})}{[^{63}Cu]_{diatoms} \times wt_{snail} \times T_1} \quad (4)$$

For example, IR for snails exposed to diatoms mixed with ARP did not vary significantly among exposure concentrations; that is, IR averaged 0.26 ± 0.04 g g^{-1} day^{-1}. Food IR was, however, 5 times higher for the controls, suggesting that feeding was inhibited whenever ARP were mixed with the diatoms. Specifically, food IR averaged 1.4 ± 0.7 g g^{-1} day^{-1} for the control snails and decreased to 0.26 ± 0.04 g g^{-1} day^{-1} when the ^{63}Cu exposure concentration increased from 9 to 754 μg g^{-1} (Figure 4A). Concentration-dependent feeding inhibition has been observed in *L. stagnalis* exposed to diatoms labeled with high levels of Cu.[29]

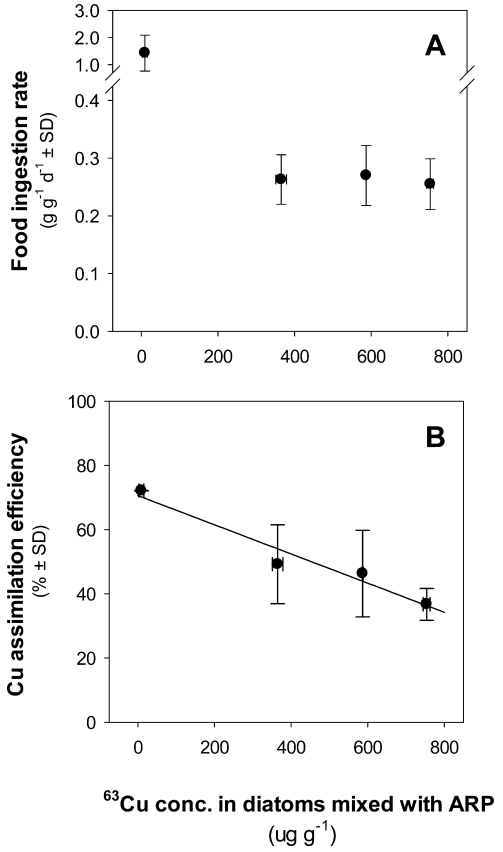


Figure 4. (A) Food ingestion rate (g g^{-1} day^{-1} ± SD) and (B) Cu assimilation efficiencies (% ± SD) in snails exposed for 6 h to diatoms mixed with increasing amounts of ARP. The solid line represents the statistically significant linear regression relationship.

Cu AE (%) for each experimental organism was calculated using mass-balance calculations and averaged by treatment (eq 5). AE represents the proportion of ingested Cu that is transported across the gut membrane and retained in soft tissue.[31]

$$\text{Cu AE} = \frac{^{63}Cu_{snail}}{^{63}Cu_{snail} + {}^{63}Cu_{feces}} \times 100 \quad (5)$$

Cu assimilation efficiency steadily declined with increasing exposure concentrations (p = 0.02, Figure 4B). Specifically, Cu AE decreased from 72 ± 0.1 to 37 ± 5% when dietary ^{63}Cu exposure concentration increased from 9 to 754 μg g^{-1}. Overall, Cu AE was 44 ± 11%, which is lower than the Cu AE reported for Cu sorbed to synthetic hydrous ferric oxide (80−90%)[8] and for Cu associated with a natural food source (e.g., 72−82% in diatoms)[29] (Table 2). The Cu AE reported for the ARP is, however, comparable to that found for Cu from CuO nanoparticles (Table 2). Mass transfer of Cu from the ARP to the diatoms prior to ingestion likely was small and, therefore, contributed little to the observed uptake and Cu AE, as shown by Cain et al.[8] for Cu sorbed to colloidal hydrous ferric oxide. The reduction of Cu influx at the highest exposure concentrations (Figure 3) could thus be explained, in part, by

the decline in AE. This suggests that the snail's physiological processes controlling the assimilation of Cu were impaired at the highest exposure concentrations. It remains unclear which constituent of the food (i.e., trace metal or major element or both) was responsible for the impairment.

DISCUSSION

Applications of tracer techniques have been invaluable to the study of processes that influence the fate of minerals and trace elements in various biological systems.[2,6−9,32] However, typical tracing requires labeling the source of contamination, which does not necessarily represent the speciation of the native metals, and may affect many kinds of assessments. For example, the physical location of metals within a particle can vary greatly, but systematically labeling each geochemical phase is impossible, yet each phase can influence metal bioavailability. The "reverse labeling" approach described herein is advantageous because it allows measurements of AE to be made on particles in their native state (i.e., no manipulation or labeling). It involves enriching test organisms in a low abundance isotope (of the metal of interest) prior to exposure to naturally contaminated particles. Our results showed that 8 weeks of exposure to 10 μg L^{-1} of ^{65}Cu increases the relative abundance of ^{65}Cu in the soft tissues of freshwater snails from ∼32% to >80%, which was sufficient to detect uptake.

The use of snails isotopically enriched in ^{65}Cu allowed detecting accumulation of 24 ng of Cu after a short dietary exposure (e.g., 6 h) to natural Cu-bearing Fe−Al particles. Greater sensitivity could be gained with a greater enrichment of the organisms with ^{65}Cu. In this study, uptake of ^{63}Cu from the lettuce probably capped the enrichment factor of ^{65}Cu. That is, we estimated that p^{65}_{snail} in this experiment could not have exceeded 90% because snails consumed food containing ^{63}Cu during the ^{65}Cu enrichment phase (Table 1). Specifically, there was approximately 5 μg g^{-1} of ^{63}Cu in the lettuce offered as food to the snails during the ^{65}Cu enrichment phase, which yielded an uptake of 13 μg g^{-1} of ^{63}Cu over the pre-exposure period (eq 2, first term). This uptake was, however, offset by a loss of 22 μg g^{-1} of ^{63}Cu (eq 2, second term). Thus, exposed snails became progressively depleted in ^{63}Cu as they were becoming enriched in ^{65}Cu. Rearing young snails in an environment that is completely free of ^{63}Cu would increase the $^{65/63}$Cu ratio. However, the experimental pre-exposure would be more demanding. For example, the isotopic composition of the food would need to be manipulated to reduce the isotopic signature of ^{63}Cu. The increased effort would likely yield only a marginal improvement in sensitivity. Finding the experimental setup that is practical and will achieve a satisfactory level of sensitivity is key to the reverse labeling approach.

Greater sensitivity could also be gained with longer pre-exposures to waterborne ^{65}Cu, higher pre-exposure concentrations, or both. However, further gain in sensitivity would be offset by the risks of eliciting toxicity because of a greater Cu accumulation by the test organisms. Greater Cu bioaccumulation could induce physiological and biochemical changes that could subsequently affect uptake, as described above. Pre-exposure conditions thus need to also take into account the test organism's "health". Importantly, our results demonstrated that pre-exposure to ^{65}Cu had no effect on the snail's growth rate, indicating a lack of sublethal effects.[33] Past exposure to metals, especially acute metal pre-exposure, can induce physiological or biochemical changes that can later affect metal uptake.[34−37] Although there is no clear consistent effect of metal pre-exposure on metal uptake, we cannot rule out the possibility that Cu uptake from the ARP in *L. stagnalis* had been somewhat influenced by the pre-exposure to ^{65}Cu. The use of low pre-exposure concentrations can minimize this confounding influence, although comparisons of bioaccumulation kinetics between native and isotopically enriched snails are needed to fully assess the effects of pre-exposure.

Geochemical and Biological Influences on Copper Bioavailability. Differences in k_{uf} among studies suggest that Cu bioavailability to *L. stagnalis* is 4 times higher for Cu bound to synthetic hydrous ferric oxide (HFO) than for Cu associated with the ARP (Table 2). In contrast, Cu bioavailability from the ARP is similar to that of CuO nanoparticles mixed with diatoms, suggesting that the geochemical nature of the particles influences the extent of Cu bioavailability. Metals sorbed to particle surfaces such as Cu on hydrous ferric oxides appear to be highly bioavailable,[8] in contrast to metals occluded in particles such as synthetic CuO nanoparticles.[26] The precise geochemical composition of the ARP is unknown, however. From the study of Schemel et al.,[22] one can hypothesize the complex nature of the ARP on the basis of the sequence leading to colloidal particle formation. That is, about half of the Fe was particulate (<10 kDa) upstream of the confluence of the acid Cement Creek (source) with the Animas River (Table S1, Supporting Information). Upon mixing, Al precipitated rapidly between pH 4 and 6.5, with the remaining Fe precipitating by the downstream end of the mixing zone where the colloid particles were harvested (see Materials and Methods). Cu sorption increased from 0 in Cement Creek to about 50% after mixing and transport to the collection site. Thus, one hypothesis is that Al is precipitated onto iron oxide that had already sorbed Cu, as some fraction of Fe is already precipitated in Cement Creek, with further sorption of Cu and precipitation of Al occurring upon neutralization. Another hypothesis is that Cu adsorbed to aluminum oxides or to a mixed aluminum−iron oxide. In either scenario, Cu may be less available because of stronger binding and/or occlusion by precipitation of Al and Fe onto existing surfaces. In laboratory studies, Al was incorporated into the iron oxide at levels of up to 30 mol % (defined as Al/Fe + Al) when present during Fe precipitation.[38] At higher Al concentrations, aluminum hydroxide phases also formed. An Al mole percent of about 70% is estimated for the Animas River assuming that half the Fe had precipitated prior to mixing of Cement Creek water into the river. Thus, the ARP likely is composed of both aluminum-substituted iron oxides and aluminum oxides, in addition to iron oxides formed in Cement Creek. Cu surface complexation equilibria and desorption kinetics for this complex mixture have not been evaluated.

In addition to geochemical factors, biological factors play a crucial role in determining metal bioavailability to a species. For example, digestive processes[39] and gut conditions such pH, Eh, and enzyme activity[40] can affect the release of ingested metals in the gut, thereby influencing their uptake. Our studied species, *L. stagnalis*, has a complex digestive system that utilizes extra- and intracellular digestive pathways,[41] although the acidity of the gut lumen is reported to be circum neutral.[42] High concentrations of pollutants in the diet can also disrupt gut function and feeding activity,[24,29,43] both of which are of significance to higher level processes such as growth and reproduction.[44] Our results show that the presence of ARP in the diet of *L. stagnalis* induced a dietary metal stress captured

by a steady decline of Cu AE with increasing exposure concentrations and by the suppression of their feeding rates, which suggests that starvation might be a mode of stress for benthic feeders.

Our results show that the reverse labeling approach offers a unique tool to unify both biological and geochemical influences when the bioavailability of metals associated with complex geochemical phases is evaluated. The approach offers the potential to systematically investigate the bioavailability of a wide variety of geochemical phases and metals, which would provide crucial insights to advance the current understanding of the influence of solid-phase geochemical speciation on metal bioavailability. For example, our study provides not only evidence that Cu associated with natural Fe–Al particles is bioavailable but also a method to explain the potential direct uptake of metals from colloids, which addresses a long-standing uncertainty in the literature (e.g., Newman and McIntosh[45] and Farag et al.[46]). Most important, the reverse labeling approach shows great potential for addressing questions involving uptake of an element that naturally has multiple isotopes in various scientific areas such as environmental contamination and nutrition.

■ ASSOCIATED CONTENT

Supporting Information

Metal concentrations in particles and water collected in the Animas River; concentrations of Fe, Cu, Al, Zn, and Pb in diatoms mixed with ARP; ICP-MS analysis; growth rate experiment, prediction of Cu accumulation in native snails after exposure to ARP, and Cu concentrations in snails after exposure to diatoms mixed with ARP. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mcroteau@usgs.gov.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Samuel Luoma and Aïda Farag for reviewing the manuscript, as well as three anonymous reviewers whose comments improved the manuscript. Support for this research was provided by the Toxics Substance Hydrology Program of the U.S. Geological Survey. We thank Briant Kimball for his assistance and guidance in collecting the ARP. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. government.

■ REFERENCES

(1) Au, A. P.; Reddy, M. B. Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *J. Nutr.* **2000**, *130*, 1329–1334.

(2) Croteau, M. N.; Luoma, S. N.; Topping, B. R.; Lopez, C. B. Stable metal isotopes reveal copper accumulation and loss dynamics in the freshwater bivalve Corbicula. *Environ. Sci. Technol.* **2004**, *38*, 5002–5009.

(3) Harvey, L. J.; Dainty, J. R.; Beattie, J. H.; Majsak-Newman, G.; Wharf, S. G.; Reid, M. D.; Fairweather-Tait, S. J. Copper absorption from foods labelled intrinsically and extrinsically with Cu-65 stable isotope. *Eur. J. Clin. Nutr.* **2005**, *59*, 363–368.

(4) Pickhardt, P. C.; Folt, C. L.; Chen, C. Y.; Klaue, B.; Blum, J. D. Impacts of zooplankton composition and algal enrichment on the accumulation of mercury in an experimental freshwater food web. *Sci. Total Environ.* **2005**, *339*, 89–101.

(5) Wang, W. X.; Fisher, N. S. Assimilation efficiencies of chemical contaminants in aquatic invertebrates: a synthesis. *Environ. Toxicol. Chem.* **1997**, *18*, 2034–2045.

(6) Reinfelder, J. R.; Fisher, N. S. The assimilation of elements ingested by marine copepods. *Science* **1991**, *251*, 794–796.

(7) Cain, D. J.; Croteau, M. N.; Luoma, S. N. Bioaccumulation dynamics and exposure routes of Cd and Cu among species of aquatic mayflies. *Environ. Toxicol. Chem.* **2011**, *30*, 2532–2541.

(8) Cain, D. J.; Croteau, M. N.; Fuller, C. C. Dietary bioavailability of Cu adsorbed to colloidal hydrous ferric oxide to freshwater benthic grazers. *Environ. Sci. Technol.* **2013**, DOI: 10.1021/es3044856.

(9) Buchwalter, D. B.; Luoma, S. N. Differences in dissolved cadmium and zinc uptake among stream insects: mechanistic explanations. *Environ. Sci. Technol.* **2005**, *39*, 498–504.

(10) Hintelmann, H.; Evans, R. D.; Villeneuve, J. Y. Measurement of mercury methylation in sediments by using enriched stable mercury isotopes combined with methylmercury determination by gas chromatography–inductively coupled plasma mass spectrometry. *J. Anal. At. Spectrom.* **1995**, *10*, 619–624.

(11) Charles, P. Calcium absorption and calcium bioavailability. *J. Intern. Med.* **1992**, *231*, 161–168.

(12) Weaver, C. M. Use of calcium tracers and biomarkers to determine calcium kinetics and bone turnover. *Bone* **1998**, *22*, 103S–104S.

(13) Van den Heuvel, E. G. H. M.; Muys, T.; Pellegrom, H.; Bruyntjes, J. P.; van Dokkun, W.; Spanhaak, S.; Schaafsma, G. A new method to measure iron absorption from the enrichment of ^{57}Fe and ^{58}Fe in young erythroid cells. *Clin. Chem.* **1998**, *44*, 649–654.

(14) Cornelis, R. Use of radiochemical methods as tools for speciation purposes in environmental and biological sciences. *Analyst* **1992**, *117*, 583–588.

(15) Stürup, S.; Hansen, H. R.; Gammelgarrd, B. Application of enriched stable isotopes as tracers in biological systems: a critical review. *Anal. Bioanal. Chem.* **2008**, *390*, 541–554.

(16) Jannasch, H. W.; Honeyman, B. D.; Balistrieri, L. S.; Murray, J. W. Kinetics of trace element uptake by marine particles. *Geochim. Cosmochim. Acta* **1988**, *52*, 567–577.

(17) Griscom, S. B.; Fisher, N. S.; Luoma, S. N. Geochemical influences on assimilation of sediment-bound metals in clams and mussels. *Environ. Sci. Technol.* **2000**, *34*, 91–99.

(18) Fuller, C. C.; Davis, J. A.; Waychunas, G. A. Surface chemistry of ferrihydrite: part 2. Kinetics of arsenate adsorption and coprecipitation. *Geochim. Cosmochim. Acta* **1993**, *57*, 2271–2282.

(19) Lee, J. S.; Lee, B. G.; Luoma, S. N.; Yoo, H. Importance of equilibration time in the partitioning and toxicity of zinc in spiked sediment bioassays. *Environ. Toxicol. Chem.* **2004**, *23*, 65–71.

(20) Böhlke, J. K.; de Laeter, J. R.; De Bièvre, P.; Hidaka, H.; Peiser, H. S.; Rosman, K. J. R.; Taylor, P. D. P. Isotopic composition of the elements, 2001. *J. Phys. Chem. Ref. Data* **2005**, *34*, 57–67.

(21) U.S. Environmental Protection Agency. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*; U.S. EPA: Washington, DC, 2002; EPA-821-R-02-012.

(22) Schemel, L. E.; Kimball, B. A.; Runkel, R. L.; Cox, M. H. Formation of mixed Al-Fe colloidal sorbent and dissolved-colloidal partitioning of Cu and Zn in the Cement Creek – Animas River confluence, Silverton, Colorado. *Appl. Geochem.* **2007**, *22*, 1467–1484.

(23) Irving, E. C.; Baird, D. J.; Culp, J. M. Ecotoxicological responses of the mayfly *Baetis tricaudatus* to dietary and waterborne cadmium: implications for toxicity testing. *Environ. Toxicol. Chem.* **2003**, *22*, 1058–1064.

(24) Croteau, M. N.; Dybowska, A. D.; Luoma, S. N.; Valsami-Jones, E. A novel approach reveals that ZnO nanoparticles are bioavailable and toxic after dietary exposure. *Nanotoxicology* **2011**, *5*, 79−90.
(25) Croteau, M. N.; Luoma, S. N.; Pellet, B. Determining metal assimilation efficiency in aquatic invertebrates using enriched stable metal isotope tracers. *Aquat. Toxicol.* **2007**, *83*, 116−125.
(26) Misra, S. K.; Dybowska, A.; Berhanu, D.; Croteau, M. N.; Luoma, S. N.; Boccaccini, A. R.; Valsami-Jones, E. Traceable nanoparticles: isotopically modified ^{65}CuO nanoparticles. *Environ. Sci. Technol.* **2012**, *46*, 1216−1222.
(27) Luoma, S. N.; Rainbow, P. S. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. *Environ. Sci. Technol.* **2005**, *39*, 1921−1931.
(28) Luoma, S. N.; Fisher, N. S. Uncertainties in assessing contaminant exposure from sediments. In *Ecological Risk Assessment of Contaminated Sediments*; Ingersoll, C. G., Dillon, T., Biddinger, G. R., Eds.; SETAC Special Publication Series: Pensacola, FL, 1997.
(29) Croteau, M. N.; Luoma, S. N. Predicting dietborne metal toxicity from metal influxes. *Environ. Sci. Technol.* **2009**, *43*, 4915−4921.
(30) Croteau, M. N.; Misra, S. K.; Luoma, S. N.; Valsami-Jones, E. Silver bioaccumulation dynamics in a freshwater invertebrate after aqueous and dietary exposure to nanosized and ionic Ag. *Environ. Sci. Technol.* **2011**, *45*, 6600−6607.
(31) Wang, W. X.; Fisher, N. S. Delineating metal accumulation pathways for marine invertebrates. *Sci. Total Environ.* **1999**, *237*−*238*, 459−472.
(32) Wang, W. X.; Fisher, N. S.; Luoma, S. N. Kinetic determinations of trace element bioaccumulation in the mussel *Mytilus edulis*. *Mar. Ecol.: Prog. Ser.* **1996**, *140*, 91−113.
(33) Newman, M. C.; Unger, M. A. Sublethal effects to individuals. In *Fundamentals of Ecotoxicology*; CRC Press: New York, 2003.
(34) Boisson, F.; Hartl, M. G. J.; Fowler, S. W.; Amiard-Triquet, C. Influence of chronic exposure to silver and mercury in the field on the bioaccumulation potential of the bivalve *Macoma balthica*. *J. Exp. Mar. Biol. Ecol.* **1998**, *311*, 315−337.
(35) Blackmore, G.; Wang, W. X. Uptake and efflux of Cd and Zn by the green mussel *Perna viridis* after metal preexposure. *Environ. Sci. Technol.* **2002**, *36*, 989−995.
(36) Shi, D. S.; Blackmore, G.; Wang, W. X. Effects of aqueous and dietary preexposure and resulting body burden on silver biokinetics in the green mussel *Perna viridis*. *Environ. Sci. Technol.* **2003**, *37*, 936−943.
(37) Rainbow, P. S.; Ng, T. Y. T.; Shi, D.; Wang, W. X. Acute dietary pre-exposure and trace metal bioavailability to the barnacle *Balanus amphitrite*. *J. Exp. Mar. Biol. Ecol.* **2004**, *311*, 315−337.
(38) Cismasu, A. C.; Michel, F. M.; Stebbins, J. F.; Levard, C.; Brown, G. E. Properties of impurity-bearing ferrihydrite I. Effects of Al content and precipitation rate on the structure of 2-line ferrihydrite. *Geochim. Cosmochim. Acta* **2012**, *92*, 275−291.
(39) Decho, A. W.; Luoma, S. N. Time-courses in the retention of food material in the bivalves *Potamocorbula amurensis* and *Macoma balthica*: significance to the absorption of carbon and chromium. *Mar. Ecol. Prog. Ser.* **1996**, *78*, 303−314.
(40) Mayer, L. M.; Schick, L. L.; Self, R. F. L.; Jumars, P. A.; Findlay, R. H.; Chen, Z.; Sampson, S. Digestive environments of benthic macroinvertebrate guts: enzymes, surfactants and dissolved organic matter. *J. Mar. Res.* **1997**, *55*, 785−812.
(41) Boer, H. H.; Kits, K. S. Histochemical and ultrastructural study of the alimentary tract of the freshwater snail *Lymnaea stagnalis*. *J. Morphol.* **1990**, *205*, 97−111.
(42) Carriker, M. R. Observations on the functioning of the alimentary system of the snail *Lymnaea stagnalis appressa* Say. *Biol. Bull.* **1946**, *91*, 88−111.
(43) Farag, A. M.; Woodward, D. F.; Brumbaugh, W.; Goldstein, J. N.; MacConnell, E.; Hogstrand, C.; Barrows, F. T. Dietary effects of metals-contaminated invertebrates from the Coeur d'Alene River, Idaho, on cutthroat trout. *Trans. Am. Fish. Soc.* **1999**, *128*, 578−592.
(44) Maltby, L.; Kedwards, T. J.; Forbes, V. E.; Grasman, K.; Kammenga, J. E.; Munns, W. R.; Ringwood, A. H.; Weis, J. S.; Wood, S. N. Linking individual-level responses and population-level consequences. In *Ecological Variability: Separating Natural from Anthropogenic Causes of Ecosystems Impairment*; Baird, D. J., Burton, G. A., Eds.; SETAC Press: Pensacola, FL, 2001.
(45) Newman, M. C.; McIntosh, A. W. Appropriateness of Aufwuchs as a monitor of bioaccumulation. *Environ. Pollut.* **1989**, *60*, 83−100.
(46) Farag, A. M.; Nimick, D. A.; Kimball, B. A.; Church, S. E.; Harper, D. D.; Brumbaugh, W. G. Concentrations of metals in water, sediment, biofilm, benthic macroinvertebrates, and fish in the Boulder River watershed, Montana, and the role of colloids in metal uptake. *Arch. Environ. Contam. Toxicol.* **2007**, *52*, 397−409.
(47) Croteau, M. N.; Luoma, S. N. A biodynamic understanding of dietborne metal uptake by a freshwater invertebrate. *Environ. Sci. Technol.* **2008**, *42*, 1801−1806.