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Dietary Bioavailability of Cu Adsorbed to Colloidal Hydrous Ferric Oxide

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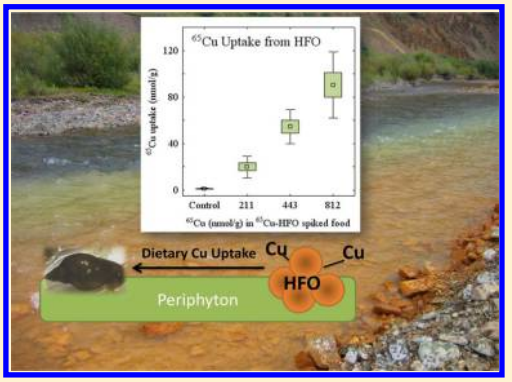
Supporting Information

ABSTRACT: The dietary bioavailability of copper (Cu) adsorbed to synthetic colloidal hydrous ferric oxide (HFO) was evaluated from the assimilation of ^{65}Cu by two benthic grazers, a gastropod and a larval mayfly. HFO was synthesized, labeled with ^{65}Cu to achieve a Cu/Fe ratio comparable to that determined in naturally formed HFO, and then aged. The labeled colloids were mixed with a food source (the diatom *Nitzschia palea*) to yield dietary ^{65}Cu concentrations ranging from 211 to 2204 nmol/g (dry weight). Animals were pulse fed the contaminated diet and assimilation of ^{65}Cu from HFO was determined following 1−3 days of depuration. Mass transfer of ^{65}Cu from HFO to the diatom was less than 1%, indicating that HFO was the source of ^{65}Cu to the grazers. Estimates of assimilation efficiency indicated that the majority of Cu ingested as HFO was assimilated (values >70%), implying that colloidal HFO potentially represents a source of dietary Cu to benthic grazers, especially where there is active formation and infiltration of these particles into benthic substrates.



INTRODUCTION

The biological availability of metals at the base of aquatic food webs is a concern because of its potential for toxicological effects on primary consumers[1−3] and transfer to higher-level consumers.[4−6] Previous studies of freshwater benthic grazers indicate that consumption of periphyton can be a dominant route of metal uptake[7,8] given the capacity of periphyton to bioconcentrate metals in forms that are highly bioavailable.[8,9] However, estimates of dietary uptake in the field are complicated by the presence of metal-enriched inorganic particles entrained in natural periphyton mats.[8] Sorption of metals to these particles will likely influence the concentration, speciation, and bioavailability of metals in the ingested material.[10−13]

Dispersion of metal-enriched particles from mining sites is a major cause of impairment to stream ecosystems worldwide.[14] The compositions of the particles are complex, and reflect site-specific geochemical and hydrological characteristics. Neutralization of acidic surface water from mines produces large quantities of metal-enriched colloidal particles (defined as particles between 1 nm and 1 μm)[15] such as hydrous iron oxides (HFO).[16] Once formed, these particles aggregate and settle onto the streambed over great distances.[17] Although previous studies have recognized that inadvertent ingestion of colloids could be an important metal exposure route to benthic organisms that can subsequently propagate to higher trophic levels,[18,19] studies evaluating their contribution to dietary metal uptake and potential risk are lacking, largely because of the difficulty in separating their contribution from that of periphyton.

Metal bioavailability from food materials can be assessed by quantifying assimilation efficiency (AE). An in vivo technique, AE represents the proportion of ingested metal that is transported across the gut membrane of an organism and retained in its soft tissue.[20] Typically AE is determined with the aid of a tracer, either a radioactive or stable isotope of the metal of interest that is used to label the food material.[20,21] Previous studies have labeled both natural and model (e.g., single algal strains, synthesized particles) materials. The decision to work with either a natural or model material tries to strike a balance between environmental realism and uncertainty in characterizing the metal bioavailability for a specific geochemical phase. With a model material, defined geochemical phases can be systematically tested and compared, and, thereby, provide insights to results obtained from complex, natural particles.

The purpose of the present study was to assess the dietary bioavailability of Cu bound to synthetic colloidal HFO. Copper is an essential micronutrient, but it is toxic at elevated body concentrations. Because interspecific differences in digestive physiology can affect the solubilization and, hence, uptake of metals bound to ingested matter,[22] AE was determined in two freshwater benthic grazers, a gastropod and a larval mayfly. The colloids were labeled with isotopically enriched ^{65}Cu to achieve environmentally relevant mass loadings,[16,17] and then mixed with a food source to simulate the entrainment of HFO in periphyton expected under natural conditions. The uptake and

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retention of ^{65}Cu from HFO was determined over a range of dietary Cu concentrations, and the bioavailability of Cu was assessed from the calculated assimilation of ingested ^{65}Cu. Desorption of ^{65}Cu from HFO and mass transfer of ^{65}Cu to diatoms was examined to support the assessment of Cu bioavailability from HFO.

EXPERIMENTAL SECTION

Test Species. Two species were selected for experimentation based on their respective natural habitats, gut morphology and physiology, and dietary assimilation of Cu. The gastropod *Lymnaea stagnalis*, the common pond snail, has a complex digestive system that utilizes extra- and intracellular digestive pathways.[23] The pH of the gut lumen is reported to be circum neutral.[24] The snail assimilates a high proportion of Cu associated with different foods and can tolerate exposures to dietary Cu concentrations up to 10 μmol/g (dry weight) before presenting signs of stress (e.g., feeding inhibition).[9] To avoid feeding inhibition, the exposures used in this study did not exceed 2.5 μmol/g. Copper concentrations in periphyton from mining sites vary greatly with the proximity to the source, and can often exceed the concentrations used in this study.[18,19] *Serratella tibialis* is a Nearctic, stream-dwelling mayfly. In general, insect larvae possess a relatively simple, tubular gut.[25] Digestion is extracellular with absorption of inorganic ions, including metals, occurring in the midgut.[26,27]

All experiments were conducted in synthetic freshwater (SFW) formulated as either soft (experiments with *S. tibialis*) or moderately hard water (experiments with *L. stagnalis*).[28] Experiments were conducted at 13 ± 1 °C and in darkness, except when the animals were handled. The experimental food source for both test animals was the diatom, *Nitzschia palea* (UTCC 160, University of Toronto). Batch cultures of *N. palea* were grown following procedures described previously.[8,9]

Collection and Acclimation of Test Animals. *L. stagnalis* was reared in the laboratory at room temperature (20 °C) in aquaria filled with moderately hard SFW. Snails were constantly fed commercial lettuce and harvested for experiments when they were approximately 8 weeks old. *S. tibialis* was collected by hand from Rock Creek, MT, USA in August 2010 and 2011. Samples were transported overnight to the U.S. Geological Survey laboratory in Menlo Park, CA where they were immediately moved to a temperature-controlled room (13 ± 1 °C) and gradually acclimated to test water (see Supporting Information (SI)). Food was withheld from both species for 24 h prior to exposure to contaminated food.

Preparation of ^{65}Cu-HFO. Synthetic colloidal hydrous ferric hydride (HFO) was prepared, based on the methods in Fuller et al.,[29] labeled with ^{65}Cu, and then aged. Labeling with ^{65}Cu was guided by a 2-site surface complexation model[30] with the intention that most Cu would be bound to the higher affinity binding site in order to minimize desorption of Cu into SFW during the exposures. The model to simulate adsorption of Cu to HFO surface hydroxyl groups requires surface complexation to two binding sites with the same reaction stoichiometry but with different affinities to account for nonlinearity of adsorption density versus dissolved Cu concentration. The model construct for Cu consists of higher affinity (strong) sites (0.005 mol sites/mol Fe, log K 2.89) and lower affinity (weak) sites comprising the remaining sites (0.205 mol sites/mol Fe, log K 0.6). An acidified ferric sulfate solution (5 mM) was diluted 10-fold with SFW resulting in a pH < 3. The pH was increased to 5 by stepwise addition of 0.1 N NaOH. An isotopically enriched ^{65}Cu solution (99.5%, Trace Sciences) was added to attain the desired Cu concentration along with 0.1 M $NaHCO_3$ to replace carbonate lost from the acidified SFW. The pH was further increased to 8.0, and then monitored over the next 48 h, with adjustment as needed. The mixture was then aged for at least 2 weeks to provide a consistent material for use in each experiment. Coagulation of the 2−6 nm HFO particles occurs over the first 4 days following precipitation to form micrometer-sized highly porous aggregates.[31] No change in the extent of Cu adsorption was observed during aging. Based on the nominal starting concentrations of ^{65}Cu and ferric oxide (0.8 and 500 μM, respectively), the model predicted that 99.7% of the added Cu would be sorbed and 2 nM would remain in solution. Furthermore, the model predicted that 60% of the sorbed Cu would be bound to the stronger binding site. Measurements of ^{65}Cu in filtered (0.1 μm) and solid phases showed that 99.1% of the ^{65}Cu was sorbed to HFO (resulting in a molar Cu/Fe ratio of roughly 1×10^{-03}, Table S1; S3 in the SI), while 6 nM was in solution. The slightly higher filtrate Cu than predicted may reflect incomplete removal of a small fraction of colloids during filtration.

Preparation of Contaminated Food. Food was presented as a diatom mat filtered onto a polycarbonate filter. Batch cultures of *N. palea* were suspended in overlying media by gentle swirling, harvested onto a polycarbonate filter (1.2 μm pore size and 47 mm diameter), and then resuspended (SFW). Diluted suspensions of ^{65}Cu−HFO were prepared and aliquots of these were dispensed into the algal suspensions to achieve the desired ^{65}Cu concentration. Untreated (control) and ^{65}Cu−HFO spiked algal suspensions were collected by filtration (as above) for the feeding experiments. Because grazing rates are lower for *S. tibialis* than *L. stagnalis*,[8,9] higher ^{65}Cu concentrations were established for *S. tibialis* to ensure that uptake of the tracer was quantifiable. Experimental methods are described in more detail in the Supporting Information.

Assimilation of Variable Dietary ^{65}Cu Concentrations (Experiment 1). To determine dietary assimilation of Cu at variable ^{65}Cu concentrations, *L. stagnalis* (n = 8 per treatment) and *S. tibialis* (n = 10 per treatment) were exposed to diatoms mixed with increasing concentrations of ^{65}Cu−HFO (see SI). Exposure times were 4 and 24 h for *L. stagnalis* and *S. tibialis*, respectively. Although an exposure time shorter than the gut passage time (i.e., initial passage of unassimilated matter) of the food bolus is necessary to determine Cu AE, the longer exposure time for *S. tibialis* was established to accommodate the slower feeding rate of this species. A previous study indicated that the gut residence time of *L. stagnalis* (i.e., complete passage of unassimilated metal) was 22 h.[32] Therefore, after the animals were exposed, they were transferred to individual containers with clean SFW and fed an uncontaminated food (lettuce for *L. stagnalis* and *N. palea* for *S. tibialis*) for 24 h to allow depuration of unassimilated ^{65}Cu. After depuration, each animal and its feces were separately collected for metals analyses.

Assimilation of ^{65}Cu Based on Three-Day Depuration (Experiment 2). The depuration period was extended to 3 days to ensure evacuation of unassimilated ^{65}Cu from the gut (estimates of AE would be positively biased if the depuration period was shorter than the gut retention time). Animals were exposed for 4 h (*L. stagnalis*, n = 36) and 24 h (*S. tibialis*, n = 36) to diatoms contaminated with ^{65}Cu−HFO. Concentrations of ^{65}Cu in the HFO-contaminated diet averaged 1285 nmol/g for *L. stagnalis* and 2271 nmol/g for *S. tibialis* (Table S3).

Following exposure, samples were collected for ^{65}Cu body burden. The remaining animals were transferred to an aquarium with SFW, fed an uncontaminated food (as above), and sampled over the next 3 consecutive days.

Transfer of ^{65}Cu from HFO to the Diatom (Experiment 3). Interpretation of the bioavailability of Cu from HFO would be biased should ^{65}Cu desorb from HFO and transfer to the diatom during the preparation of the contaminated food and the exposure. Because of the difficulty in performing a physical separation of HFO and diatoms after they were mixed, this process was examined in a two-compartment experimental system in which suspensions of ^{65}Cu−HFO and diatoms were separated by a dialysis bag (MWCO = 1 kDa (≈ 1 nm pore size) Spectra/Por, Spectrum Laboratories, Inc.), although it was recognized that the membrane could introduce artifacts not present in a system where the colloidal material was mixed directly with the diatoms. Specifically, 5 mL of ^{65}Cu−HFO was dispensed into a dialysis bag which was then sealed and inserted into a 50-mL sealable test tube (n = 2−3). Thirty mL of either moderately hard SFW (control) or a diatom−SFW suspension was added to each tube. Given the nominal pore size of the dialysis membrane, only the hydrated metal ion or inorganic complexes of this size are able to cross into the test tube. The concentrations of the colloidal suspension and the diatom suspension were designed to approximate their relative concentrations in the assimilation efficiency experiments. Samples of the ^{65}Cu−HFO suspension (unfiltered and 0.1 μm filtrate) were collected to establish the initial concentration and phase distribution of ^{65}Cu and Fe. The tubes were then loaded onto an end-over-end rotator (12 rpm) which was placed into a temperature-controlled (13 °C) room in constant darkness for up to 24 h. Samples were collected at 4 and 24 h—the respective exposure times for *L. stagnalis* and *S. tibialis*—from the dialysis bags (total and 0.1 μm filtrate) and the diatom suspension (diatoms and 1.2 μm filtrate) for determination of ^{65}Cu, Fe, and DOC (1.2 μm filtrate of diatom suspension). Concentrations of ^{65}Cu associated with extraneous sources of Cu in the experimental system (e.g., diatoms and contamination) were corrected using ^{63}Cu, as described below.

Metals and DOC Analysis. Diatoms, the soft tissue of *L. stagnalis*, the whole body of *S. tibialis*, and feces were prepared for metals analyses by methods described previously.[8,9,32] Briefly, diatoms were collected on filters and dried at 40 °C. The soft tissue and feces of individual *L. stagnalis* and *S. tibialis* were collected in separate sealable polytetrafluoroethylene (PTFE) cups and then dried at either 40 °C (*L. stagnalis*) or by freeze-drying (*S. tibialis*). Dry weights for each sample type were determined to the nearest 0.001 mg (Sartorius model M2P microbalance). Samples were digested in sealed PTFE cups with addition of 16 N HNO_3 followed by H_2O_2 at room temperature for 7 days. Iron was determined by ICP-OES and ICP-MS, while ^{65}Cu and ^{63}Cu were determined by ICP-MS.

DOC was determined by high-temperature catalytic combustion on a Shimadzu TOC-V CPH. Potassium phthalate was used as the standard. Low-DOC water (blanks less than 40 μg carbon/L) was generated from a double-deionization unit with additional ultraviolet treatment (Milli-Q Gradient, Millipore Corporation).

Calculation of ^{65}Cu Concentrations. The total ^{65}Cu concentration in a sample ([^{65}Cu]total) was calculated as the product of the relative abundance of ^{65}Cu (p65) determined using the signal intensities for each isotope in calibration standards (eq 1) and the ^{65}Cu concentration ([^{65}Cu]) (eq 2). Because Cu is an essential element, each animal has a background concentration of ^{65}Cu ([^{65}Cu]bkgd) that needs to be accounted for to determine ^{65}Cu acquired from contaminated food. This background level of ^{65}Cu was estimated as the product of the ^{63}Cu concentration ([^{63}Cu]) and (p65) (eq 3).

$$p65 = \text{Intensity}\left(\frac{^{65}\text{Cu}}{^{65}\text{Cu} + {}^{63}\text{Cu}}\right)_{\text{standard}} \quad (1)$$

$$[^{65}\text{Cu}]\text{total} = p65 \times [^{65}\text{Cu}] \quad (2)$$

$$[^{65}\text{Cu}]\text{bkgd} = p65 \times [^{63}\text{Cu}] \quad (3)$$

The concentration of ^{65}Cu acquired from ^{65}Cu−HFO (Δ[^{65}Cu]) was estimated by the difference between the total ^{65}Cu ([^{65}Cu]total) and the background ^{65}Cu ([^{65}Cu]bkgd) following the exposures (eq 4).

$$\Delta[^{65}\text{Cu}] = ([^{65}\text{Cu}]\text{total} - [^{65}\text{Cu}]\text{bkgd}) \quad (4)$$

The same approach was used to correct for extraneous sources of ^{65}Cu (complementary to [^{65}Cu]bkgd) in the dialysis system (see Experiment 3 in the SI).

Estimations of Cu Assimilation Efficiency and Ingestion Rate. Assimilation efficiency of ^{65}Cu was determined either from the mass balance of ^{65}Cu retained in the animal and recovered in its feces following depuration (eq S1) or by the proportion of ^{65}Cu retained by the animal after depuration (eq S2).[20] Ingestion rate, IR (g/g/d), was calculated for each individual from the total ^{65}Cu it ingested, (Σ Δ^{65}Cu tissue,feces in nmol), its dry tissue weight (d.w. in g), the measured ^{65}Cu concentration of the ^{65}Cu−HFO−diatom preparation ([^{65}Cu]food, nmol/g), and the exposure time (T in day) (eq 5).

$$\text{IR} = \frac{(\sum \Delta^{65}\text{Cu tissue, feces})}{([^{65}\text{Cu}]\text{food} \times \text{d.w.} \times T)} \quad (5)$$

Quality Assurance. Metal contamination was minimized by cleaning all glass and plastic used in the experiments, sample preparation, and analysis following procedures previously described.[32] Quality assurance samples included procedural blanks, standard reference material (Tort-2, lobster hepatopancreas, National Research Council Canada), and external standards. Nitric acid and hydrogen peroxide (Baker Ultrex II) were used to digest the samples and for procedural blanks. Instruments were calibrated with standard solutions at least daily. Signal drift in the ICP-OES was followed by periodically analyzing standards, while for the ICP-MS drift was compensated for by the use of an internal germanium standard and by periodically analyzing standards. Variation in signal intensity of ^{65}Cu was quantified from the signal intensities of standards containing the natural abundances of ^{63}Cu and ^{65}Cu. The method detection limit for ^{65}Cu and ^{63}Cu was 1.5 nM, while the Fe detection limits were 900 nM by ICP-MS and 64 nM by ICP-OES. The percent recovery of total Cu in standard reference material (Tort-2) was 111 ± 3 (mean ± 95% CI, n = 5).

Data Analysis. Statistical analyses of the data were performed with Statistica (ver. 10). Data for Δ^{65}Cu uptake and Fe from experiment 1 were analyzed by linear regression. These data were transformed ($\log_e$) to constrain the variance. Controls were excluded in the analysis of Δ^{65}Cu because

uptake of Δ^{65}Cu was not possible (see eq 4). Due to variance among the AE data, these data were analyzed by Spearman Rank Order Correlation (AE within species) and the Mann−Whitney test (AE between species). Results for experiment 2 were analyzed using either ANOVA or the Kruskal−Wallis test. Some animals ($\leq$ 2/treatment, except for 4/treatment for *S. tibialis* on day 1 of Experiment 2) did not consume the contaminated food (indicated by no increase in Δ^{65}Cu body burden immediately following feeding). These were removed from statistical analyses. Differences were considered significant at a type I error rate of $\alpha = 0.05$.

RESULTS

Assimilation of Variable Dietary ^{65}Cu Concentrations (Experiment 1). Concentrations of ^{65}Cu in diatoms not treated with ^{65}Cu−HFO (controls) ranged from 2 to 29 nmol/g (Table S1). The range of values likely reflected differences in the age and biomass of the cultures. Mean concentrations of ^{65}Cu in the treated diatoms ranged from 211 to 812 nmol/g for *L. stagnalis* and from 582 to 1844 nmol/g for *S. tibialis*. Linear regression of the ^{65}Cu uptake (Δ^{65}Cu/individual) against ^{65}Cu in treated diatoms was significant for both species ($p < 0.05$; $R = 0.76$, $F(1,20) = 22.3$ for *L. stagnalis* and $R = 0.50$, $F(1,23) = 7.8$ for *S. tibialis*; Figure 1). Dry tissue weight (mg), background

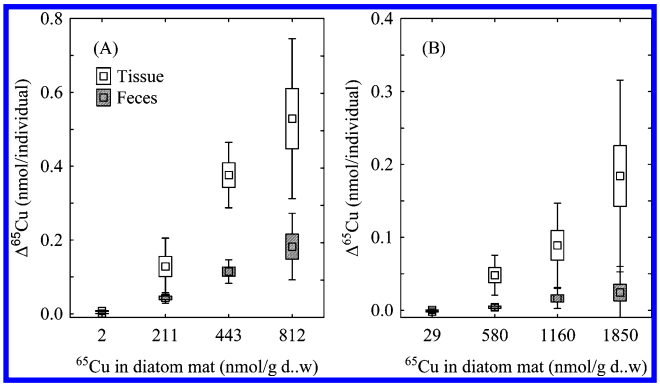

Figure 1. Box plots of Δ^{65}Cu in soft tissues and feces (shown as Δ^{65}Cu/individual) of *L. stagnalis* (A) and *S. tibialis* (B) relative to dietary ^{65}Cu. Data are the mean (square), mean ± standard error (box), and mean ± standard deviation (whiskers), n = 7−9.

concentrations of ^{65}Cu, and ingestion rates remained relatively constant over the range of dietary ^{65}Cu concentrations ($p > 0.05$, linear regression, Table S2). The amounts of Δ^{65}Cu in feces were much less than in the animals' bodies. Mass balance estimates of AE were uniform among treatments for both species and were somewhat lower ($p < 0.05$, Mann−Whitney test) for *L. stagnalis* (0.76 ± 0.09; mean ± SD, n = 22) than for *S. tibialis* (0.87 ± 0.22, n = 25). Additionally, the similarities in ingestion rates and AE among ^{65}Cu−HFO treatments suggest that acute toxic effects on feeding behavior and digestive processes did not occur.

Iron, also, increased in the animals exposed to ^{65}Cu−HFO ($p < 0.05$, $R = 0.45$, $F(1, 27) = 6.7$ for *L. stagnalis* and $R = 0.69$, $F(1, 32) = 28.7$ for *S. tibialis*), with average concentrations roughly 3- to 5-fold greater than controls (Table S2 in the SI). Based on the total ^{65}Cu consumed ($\Sigma\ \Delta^{65}$Cu tissue,feces) and the Cu/Fe molar ratio of the colloid, we estimate that approximately 12−20% of the total Fe consumed was retained. Its presence suggested that either some Fe was assimilated or the evacuation of the colloidal particles from the gut was not complete within the 24-h time frame (i.e., gut retention time > the depuration period). The latter condition could inflate estimates of ^{65}Cu assimilation.

^{65}Cu Assimilation Efficiency (Experiment 2). Test species were depurated for 3 days to ensure complete depuration of unassimilated Cu (see results from Experiment 1). After feeding, the average concentration of Δ^{65}Cu ($\Delta[^{65}Cu]$) in *L. stagnalis* was 79 ± 33 nmol/g on day 0 (Figure 2A; Table S4). Over the next 3 days of depuration,

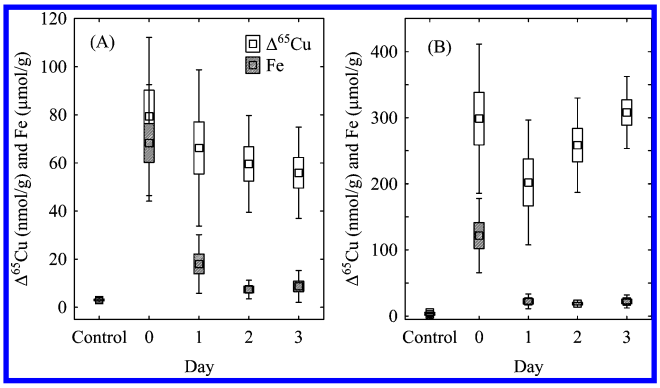

Figure 2. Box plots of Δ^{65}Cu and Fe concentrations in tissues of *L. stagnalis* (A) and *S. tibialis* (B) over the course of 3 days following ingestion of ^{65}Cu-labeled HFO. The Fe concentration in a control (not exposed) group is shown for reference (value for *L. stagnalis* from Experiment 1). Data are the mean (square), mean ± standard error (box), and mean ± standard deviation (whiskers), n = 6−9.

$\Delta[^{65}Cu]$ decreased to 56 ± 19 nmol/g; however, these differences were not significant (ANOVA, $F(1, 31) = 1.3$). For *S. tibialis*, the $\Delta[^{65}Cu]$ was 299 ± 133 nmol/g on day 0 (Figure 2B; Table S4), and, like *L. stagnalis*, $\Delta[^{65}Cu]$ did not change significantly during the depuration period (ANOVA, $H(3, 27) = 2.3$). The variation in $\Delta[^{65}Cu]$ among days was likely due to differences in ingestion rates during the pulse feeding (Table S4). In particular, ingestion rates for *S. tibialis* sampled on the first day of depuration were lower than other days with no measurable Δ^{65}Cu in 4 of 10 animals (those 4 animals were not included in the statistical analysis). The concentration of dissolved ^{65}Cu (0.45 μm filtrate) was slightly elevated in the test water following the exposure of *S. tibialis* (2.1 ± 0.5 nM, n = 3); however, its contribution to the observed body concentrations predicted from biokinetic data[8] was ≤3%. Therefore, no correction to the observed body burdens was made to accommodate dissolved uptake. In general, over the 3-day depuration period neither species lost a significant portion of the ^{65}Cu acquired from the diatom mats.

Although Fe from HFO is potentially available, the retention patterns for both species supported a gut retention time of about 2 days. In *L. stagnalis*, the concentrations of Fe on the second and third day of depuration were comparable to those of unexposed snails ($p > 0.05$; Kruskal−Wallis with multiple comparisons test), and 97% of the total Fe recovered in the feces during the depuration period was recovered by day 2, indicating that depuration of unassimilated Fe was essentially complete by the second day of depuration. Iron concentrations in *S. tibialis* also decreased sharply during the first day of depuration and then stabilized at approximately 20 μmol/g (Figure 2b; Table S4), which was roughly 4-fold greater than the control and represented approximately 8% of the Fe consumed. The recovery of Fe in feces for this experiment was

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58% of the calculated loss from the animal. Recovery of Fe was hampered by the difficulty in collecting the small quantities of the material and quantification of Fe in the samples. Unrecovered feces depurated by the mayflies potentially inflates the mass balance calculation for ^{65}Cu, however.

Assimilation efficiency (AE) of Cu was estimated from the mass balance of Δ^{65}Cu (eq S1) and as the proportional retention of Δ^{65}Cu (eq S2) on the last day of depuration (Table 1). AE varied somewhat with the method of calculation, especially for *L. stagnalis*. The close agreement between the AE estimates for *S. tibialis* suggests that practically all of the depurated Cu was recovered. Relative differences in AE between species were inconsistent for the methods of calculation; however, the results generally indicated that the majority of ^{65}Cu ingested by both species was assimilated.

Table 1. Estimates of Cu Assimilation Efficiency from HFO (Mean ± SD)

species	*n*	mass balance[a]	tissue retention[b]
L. stagnalis	9	0.99 ± 0.01	0.71 ± 0.24
S. tibialis	8	0.93 ± 0.03	0.94 ± 0.03

[a]From eq S1. [b]From eq S2.

Mass Transfer of ^{65}Cu from HFO to Diatoms (Experiment 3). The large majority of ^{65}Cu remained bound to HFO (Table 2), with less than 1% of the initial ^{65}Cu in the colloidal suspension transferred to the diatoms. Desorption of ^{65}Cu from HFO was evident by the presence of elevated Δ^{65}Cu in the 0.1 μm filtrates after 4 and 24 h relative to the initial (0 h) samples of the bulk colloidal suspensions. Increases in $\Delta[^{65}Cu]$ in the 0.1 μm filtrate were accompanied with an increase in $\Delta[^{65}Cu]$ in the 1.2 μm filtrate and in the diatoms. Total concentrations of Fe in the colloidal suspensions were unchanged in Experiment 3a, but in Experiment 3b varied in a manner that was difficult to interpret. The concentrations of DOC (mg C/L) in the diatom filtrates from Experiment 3b were stable: 3.5 (n = 1) initially, 3.5 ± 0.3 (mean ± SD, n = 2) at 4 h, and 3.6 ± 0.1 at 24 h (n = 2). The ^{65}Cu/Fe ratio was less in the plus diatom groups than in either the control or initial colloidal suspensions, indicating a disproportionate loss of total ^{65}Cu from the colloidal suspension, which was consistent with the increased $\Delta[^{65}Cu]$ in the diatom suspension. The mass differences of total Δ^{65}Cu in the colloidal suspensions between what was added (initial) and what remained after 24 h were 372 pmol Δ^{65}Cu in Experiment 3a and 1030 pmol in Experiment 3b, or 8% and 23%, respectively, of the initial ^{65}Cu. Recovery of this ^{65}Cu in the diatom suspensions (Σ filtrate and diatoms) was 76% in Experiment 3a and less than 1% in Experiment 3b. The difference in recovery was largely influenced by the difference in $\Delta[^{65}Cu]$ in the 1.2 μm filtrates between the two experiments. Replicates of these filtrates and the diatoms were, in general, highly variable. Mass transfer of Δ^{65}Cu to the diatoms—estimated as the quotient of Δ^{65}Cu bound to diatoms at 24 h (8.1 and 2.6 pmol for Experiments 3a and 3b, respectively) and the initial ^{65}Cu in the HFO suspensions (4355 and 4422 pmol)—was <1%.

DISCUSSION

The dietary bioavailability of metals sorbed to colloidal HFO has not been well studied, and, thus, remains a source of uncertainty in ecological risk assessments. The results of the present study imply that HFO is potentially an important source of Cu (and other metals) to benthic grazers given its capacity to bind Cu in a form that is highly bioavailable. The relatively high assimilation of Cu from HFO is notable because it compares to and in some cases exceeds Cu assimilated from various algal and detrital food sources by freshwater and marine

Table 2. Concentrations of ^{65}Cu Derived from HFO ($\Delta[^{65}Cu]$) and Fe in the HFO Colloidal (Total and 0.1 μm Filtrate) and the Diatom (Diatom and 1.2 μm Filtrate) Suspensions for Both Dialysis Experiments (Mean ± SD)

experiment and group	time (h)	compartment	sample	$\Delta[^{65}Cu]$ (nM)	[Fe] (μM)	Δ^{65}Cu/Fe	^{65}Cu Δ (pmol)
experiment 3a							
initial	0	colloid	total	871 ± 38	745 ± 37	1.2×10^{-03}	4355 ± 188
			0.1 μm filtrate	<MDL[a]	1.1 ± 0.2		
control (minus diatom)	24	colloid	total	853 ± 16	733 ± 3	1.2×10^{-03}	4265 ± 80
			0.1 μm filtrate	3.1 ± 0.02	0.60 ± 0.04		15.3 ± 0.1
		diatom	diatom	NA[b]	NA[b]		
			1.2 μm filtrate	<MDL[a]	0.63 ± 0.01		
plus diatom	24	colloid	total	797 ± 5	716 ± 17	1.1×10^{-03}	3983 ± 27
			0.1 μm filtrate	13 ± 4	0.69 ± 0.05		66 ± 22
			diatom	0.27 ± 0.23	0.23 ± 0.13		8.1 ± 6.8
			1.2 μm filtrate	9.1 ± 7.4	1.2 ± 0.6		274 ± 221
experiment 3b							
initial	0	colloid	total	884 ± 2	841 ± 10	1.1×10^{-03}	4422 ± 12
			0.1 μm filtrate	4.0 ± 0.5	0.72 ± 0.05		23 ± 3
plus diatom	4	colloid	total	797 ± 30	902 ± 12	9.0×10^{-04}	3987 ± 151
			0.1 μm filtrate	9.0 ± 2.0	0.96 ± 0.013		45 ± 10
		diatom	diatom	0.01 ± 0.001	0.07 ± 0.004		0.28 ± 0.04
			1.2 μm filtrate	<MDL[a]	0.42 ± 0.05		
plus diatom	24	colloid	total	678 ± 49	802 ± 16	8.0×10^{-04}	3392 ± 247
			0.1 μm filtrate	14 ± 2	0.82 ± 0.11		70 ± 9
		diatom	diatom	0.09 ± 0.05	0.11 ± 0.03		2.6 ± 1.6
			1.2 μm filtrate	0.1 ± 0.33	0.6 ± 0.03		0.5 ± 1.7

[a]Less than method detection limit. [b]Not applicable.

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invertebrates.[8,9,32−34] This finding is unusual, given that most previous studies indicated that metals (e.g., As, Cd, Zn) bound to living components of the ingested material are more bioavailable than inorganic phases.[11,35,36] The specific influence of iron oxides is equivocal. For example, when sediments have been amended or manipulated to increase Fe oxide content, metal bioavailability has been reduced[11−13,36] or not changed.[35,36] These patterns have been explained as the influence of physical structure and binding affinity of different geochemical phases on rates of metal dissolution in the digestive track and, hence, assimilation.[10,22,37] Metals that are weakly bound to surface sites are likely more accessible to competing ligands and acid secreted by the gut of an animal than metals embedded within the mineral structure and to strong binding sites (e.g., sulfide). The observed assimilation of Cu could reflect its surface binding to amorphous HFO.[38]

Interspecific differences in digestive physiology also will influence the quantity of metal solubilized from a particle and thus its availability for uptake.[22] Strong differences in Cu AE between the two species examined in the present study were not evident, however. AE varied somewhat with the particular experiment and method of calculation, and this masked any potential interspecific difference. The high AE of Cu adsorbed to HFO indicated that most of the ^{65}Cu adsorbed to HFO was solubilized during digestion, and then assimilated. Although the mechanisms of uptake were not studied, the results implied that both species possessed digestive agents with a stronger binding affinity for Cu than HFO (Log K 0.6 and 2.89 for HFO weak and strong binding sites, respectively) in sufficient concentrations to complex most of the ingested Cu. This is plausible in view of studies on Cu binding to gut ligands extracted from marine deposit feeders.[37] Furthermore, the high assimilation of Cu observed in the present study suggests dissolution and uptake of Cu was minimally constrained by reaction kinetics despite the relatively short gut retention times of the animals.[39]

Unlike Cu, relatively little of the ingested Fe was retained by either animal. The depuration pattern for Fe indicated that the gut retention time of HFO was about 2 days. This time frame is consistent with results of Croteau et al.[32] who determined the gut passage time and gut retention time in *L. stagnalis* with the biologically inert tracer Cr. We speculate that either much of the HFO was not dissolved during digestion and/or uptake of solubilized Fe was regulated.[40]

Desorption of ^{65}Cu from HFO and uptake by diatoms would confound the interpretation of Cu uptake from HFO. Should mass transfer of ^{65}Cu occur, the estimated Cu AE would in fact reflect the combined assimilation from HFO and the diatoms. The results of the dialysis experiment suggest that the effect of mass transfer of ^{65}Cu to the diatoms was minor, although the results were conditional on the physical and chemical properties of the experimental system. The presence of diatoms in the dialysis system facilitated desorption of Cu from HFO and transfer of dissolved Cu into the filtrate where a portion of Cu was available for uptake by the diatoms. Presumably, uptake of ^{65}Cu by the diatom would be proportionate to the delivery of free ion (Cu^{2+}) to binding sites on the algal membrane.[41] Transfer of ^{65}Cu to the diatom suspension could be limited by dissociation of ^{65}Cu from the HFO and by the rate of flux of the free ion (Cu^{2+}) or complexed Cu across the dialysis membrane. This process would be mediated by complexation of ^{65}Cu by DOC in the diatom filtrate. Speciation calculations using WHAM[42] (see Speciation Modeling in the SI) indicated that virtually all ^{65}Cu in the filtrate of the diatom compartment was complexed. Diffusion to and from the membrane was probably not a rate limiting step because the system was constantly mixed, thus reducing the thickness of the diffusion layer on either side of the membrane. Diffusion through the membrane could retard the delivery of ^{65}Cu to the algal suspension, however. Moreover, sorption of ^{65}Cu to the membrane could alter the behavior of ^{65}Cu relative to the system where the colloidal material was mixed directly with the diatoms. Similarly, sorption of dissolved ^{65}Cu to the dialysis membrane might have played a role in the poor recovery in the diatom suspension of the mass difference in bulk ^{65}Cu between the start and finish of the experiments. Additionally, a relatively smaller amount of ^{65}Cu could have been lost with the sorption of the colloidal particles to the membrane, as indicated by the small loss of Fe from the system.

Although the processes discussed above might have limited equilibration of ^{65}Cu in the dialysis experiment, less than 1% of the mass of ^{65}Cu originally introduced to the dialysis system as ^{65}Cu−HFO was recovered in the diatoms, an amount so small that its effect on the estimated AE was negligible. Even if one were to assume that all of the unaccounted ^{65}Cu was potentially available for uptake by the diatoms, the maximum mass transfer of ^{65}Cu ranged between 2 and 23%. At those levels, the influence of the diatom on the AE estimated from the present study can be inferred from Cu AEs reported in previous studies. Using *N. palea* as the food source, Croteau et al.[9] reported a mean Cu AE of 0.72 for *L. stagnalis* while Cain et al.[8] reported a mean Cu AE of 0.83 for *S. tibialis*. Given that these values were comparable to AEs estimated for the present study, Cu bound to the diatoms would have had little effect on the estimated assimilation of Cu from HFO. Thus, the results of the dialysis experiment suggested that under the experimental conditions, the source of ^{65}Cu was predominantly HFO and AE estimates were unaffected by mass transfer of ^{65}Cu to the diatoms.

The synthetic HFO tested in this study was designed to simulate one type of colloidal particle that forms during the neutralization of acid drainage. Obviously, naturally formed colloids are more complex than the HFO used in this study.[17] A number of factors, such as mass loading, sorption of other metals, the influence of natural organic matter on the physical structure of the colloid,[43] formation of mixed mineral phases (e.g., aluminum/iron oxides),[16,44] and aging,[35] could alter the distribution, speciation, and bioavailability of sorbed metals. Also, the contribution of colloidal metals to measured metal concentrations in periphyton will vary with the proximity to the source and with hydrology.[17,19] These processes can be studied with synthetic materials and applied to understanding the bioavailability of natural particles. Additionally, studies of metal assimilation of natural colloids combined with geochemical characterization including spectroscopic and microscopic techniques[45,46] will be instrumental in firmly establishing the importance of these particles to metal bioaccumulation and risk.

■ ASSOCIATED CONTENT

Supporting Information

Methods for acclimating test animals, preparing contaminated diet, equations for AE, ^{65}Cu mass transfer from HFO to diatoms, speciation modeling, equilibrium dialysis ^{65}Cu and Fe concentrations of HFO-diatom preparations, and additional results for experiments 1 and 2. This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. D.J.C., M.-N.C., and C.C.F. designed research; C.C.F. prepared synthetic colloids; D.J.C. and M.-N.C. performed assimilation efficiency and ^{65}Cu mass transfer experiments; D.J.C., M.-N.C., and C.C.F. analyzed data; and D.J.C. wrote the paper.

Notes

The authors declare no competing financial interest.

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