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Determining metal assimilation efficiency in aquatic invertebrates using enriched stable metal isotope tracers

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Abstract

We employ a novel approach that combines pulse-chase feeding and multi-labelled stable isotopes to determine gut passage time (GPT), gut retention time (GRT), food ingestion rate (IR) and assimilation efficiency (AE) of three trace elements for a freshwater gastropod. Lettuce isotopically enriched in ^{53}Cr, ^{65}Cu and ^{106}Cd was fed for 2 h to *Lymnaea stagnalis*. The release of tracers in feces and water was monitored for 48 h, during which unlabelled lettuce was provided *ad libidum*. The first defecation of ^{53}Cr occurred after 5 h of depuration (GPT), whereas 90% of the ingested ^{53}Cr was recovered in the feces after 22.5 h of depuration (GRT). ^{53}Chromium was not significantly accumulated in the soft tissues upon exposure. In contrast, ^{65}Cu and ^{106}Cd assimilation was detectable for most experimental snails, i.e., $^{65/63}$Cu and $^{106/114}$Cd ratios in exposed snails were higher than those for controls. Food IR during the labelled feeding phase was 0.16 ± 0.07 g g^{-1} d^{-1}. IR was inferred from the amount of ^{53}Cr egested in the feces during depuration and the concentration of ^{53}Cr in the labelled lettuce. Assimilation efficiencies (±95% CI) determined using mass balance calculations were 84 ± 4% for Cu and 85 ± 3% for Cd. The ratio method yields similar AE estimates. Expanding the application of this novel stable isotope tracer technique to other metals in a wide variety of species will provide unique opportunities to evaluate the interplay between digestive processes and dietary influx of metals. Understanding the biological processes that modulate dietborne metal uptake is crucial to assess the toxicity of dietborne metals.

Keywords: Dietborne metals; Digestive processes; Gut passage time; Cadmium; Copper; Chromium; Assimilation efficiency; Food ingestion rate; *Lymnaea stagnalis*

1. Introduction

Dietary uptake of metals is increasingly recognized as an important pathway for metal accumulation (Meyer et al., 2005). Digestive processes occurring in an animal's gut influence the uptake of dietborne metals, and subsequent "dietary toxicity". Gut conditions (e.g., pH, redox potential, enzyme and surfactant activities) affect, for example, desorption of elements associated with ingested food particles, thereby enhancing their uptake (Wang et al., 1995; Mayer et al., 1997). Because molluscs (particularly bivalves and probably gastropods) process food through either a rapid intestinal or a slow glandular pathway, or both, food partitioning between extracellular and intracellular digestion can also impact metal assimilation (Decho and Luoma, 1991, 1996). But gut retention time (GRT) appears to have the most influence on element assimilation. GRT is species-specific and varies with environmental conditions. Essentially, prolonged retention of food in the digestive tract increases the uptake of metals due to more efficient assimilation (Decho and Luoma, 1991; Wang et al., 1995).

The effects of gut physiology on pollutant assimilation have been investigated mainly in marine invertebrates (e.g., polychaetes: Mayer et al., 1997; Chen and Mayer, 1998; bivalves: Decho and Luoma, 1991; Wang et al., 1995; Griscom et al., 2002). Very few studies (if any) have assessed the influence of gut retention time on metal assimilation for freshwater invertebrates. Characterizing the digestive processes that occur upon food ingestion in a wide variety of marine and freshwater species is essential to accurately predict the potential biological adverse effects of dietborne metals. Here we present a novel approach that combines pulse-chase feeding and multi-labelled stable isotope techniques for determining food GRT, food gut passage time (GPT), food ingestion rate (IR) and assimilation efficiency (AE) of three trace elements (Cr, Cu and Cd) in the freshwater

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snail *Lymnaea stagnalis*. This species has been used to investigate the effects of toxic substances on physiological processes (e.g., Gomot, 1998). We use a modified version of the ^{51}Cr:^{14}C radiotracer technique (Calow and Fletcher, 1972) and mass balance calculations to provide the first estimates of Cu and Cd AE for a herbivorous gastropod. Specifically, we ask can enriched Cr isotopes be used as inert tracers to estimate metal AE, food GPT and GRT? Can food ingestion rates be inferred from mass balance calculations using enriched metal isotope tracers?

A critical parameter for biodynamic modelling is the metal AE from ingested food (Wang et al., 1996). AE is a first-order physiological parameter that can be quantitatively compared among metals, species, food particles and environmental conditions. Dietborne metal uptake is directly proportional to AE (Wang et al., 1996; Luoma and Rainbow, 2005), which emphasizes the importance of AE for understanding and predicting metal uptake. Radioactive pulse-chase feeding techniques (i.e., involving gamma-emitting radiotracers) are most commonly used to calculate AE (Wang and Fisher, 1999). With this technique, organisms feed on uniformly radiolabelled food particles for a period of time shorter than GPT. This "feeding pulse" minimizes the possible confounding influence of isotope recycling and loss (Luoma and Fisher, 1997). Radioactivity in animals and feces is assayed after the feeding pulse and throughout depuration, during which organisms feed on non-radioactive food to purge their gut of undigested radioactive material. AE is calculated as the fraction of total ingested metal retained in the animal at the time that both digestion and assimilation are completed.

The lack of suitable radioisotopes has impeded, however, biodynamic studies of some potentially toxic metals such as Cu. Recently, enriched stable isotope methodology was used to trace Cu biodynamics and delineate Cu accumulation pathways for the freshwater bivalve *Corbicula fluminea* (Croteau et al., 2004; Croteau and Luoma, 2005). A major goal of this study is to present a novel isotope tracer approach for determining metal AE, food IR, as well as GPT and GRT. The approach offers a realistic alternative to using radioactive synthetic material refractory to digestion (e.g., latex beads: Decho and Luoma, 1991), as it involves spiking natural food with non-radioactive tracers.

2. Material and methods

2.1. Experimental organisms

Freshwater snails (*L. stagnalis* starter culture obtained from M. Grosell, University of Miami RSMAS/MBF, USA) were reared in the laboratory in moderately hard water (MOD, hardness of 80–100 mg $CaCO_3$ l^{-1}; pH of 7.8) at 15 °C. Three days prior the experiment, 20 snails of a restricted size range (mean shell size of 17 ± 0.9 mm 95% CI) were transferred to a 1 l acid-washed HDPE container filled with synthetic MOD water. Food was withheld during this period.

2.2. Labelling of food

Lettuce was exposed for 3 days to ^{53}Cr (75 μg l^{-1}), ^{65}Cu (125 μg l^{-1}) and ^{106}Cd (50 μg l^{-1}) at 15 °C, under a 16:8 h illumination. Specifically, pieces of lettuce (~1 cm^2) were placed into a 148 ml acid-washed polypropylene vial filled with deionised water spiked with commercially purchased standards (Trace Sciences International and Isoflex) isotopically enriched in ^{53}Cr (92.8%), ^{65}Cu (99.4%) and ^{106}Cd (96.5%). Because the tracer stock solutions were prepared using HNO_3, or HCl, or both, the pH of the exposure media was increased with NaOH soon after adding the lettuce. Coincidently, the pH rise enhanced the tracers' adsorption onto lettuce surfaces (Xue et al., 1988). We did not distinguish between absorbed or adsorbed metal. After exposure, lettuce pieces were thoroughly washed with Milli-Q® water (to remove weakly adsorbed metals) and offered as food for *L. stagnalis*.

2.3. Experimental procedure

Fifteen snails were individually placed in 148 ml acid-washed polypropylene containers filled with MOD water and exposed for 2 h to lettuce enriched with ^{53}Cr, ^{65}Cu and ^{106}Cd at 15 °C. Preliminary experiments indicated that Cr enriched lettuce (103 μg g^{-1}) ingested by snails took approximately 5–8 h to first appear in feces. Therefore, snails were allowed to feed on the labelled lettuce for 2 h. After feeding on the labelled food, each snail was removed, rinsed and placed in a new 148 ml acid-washed polypropylene vial filled with MOD water. Unlabelled lettuce was provided *ad libidum* during the depuration period. Media was changed after 5 and 21 h of depuration. Feces were collected after the snails fed on the labelled food and periodically over depuration, i.e., after 2, 5, 8, 12, 16, 21, 27, 36 and 48 h. Feces collected by pipette for each snail were placed individually on a piece of acid-washed Teflon sheeting and dried for 24 h at 50 °C. Snails were sacrificed (frozen) after 48 h of depuration.

Aliquots of water (5 ml) were taken immediately after labelled feeding as well as after 5, 21 and 48 h of depuration. Water samples were filtered through a 0.45 μm Millex®-HV filter (Millipore) and acidified with concentrated nitric acid (Baker Ultrex II grade, 2% final concentration). Samples of the lettuce (1–6 mg dry weight) provided during the labelled and unlabelled feeding periods were individually placed on a piece of acid-washed Teflon sheeting and dried for 24 h at 50 °C.

2.4. Control organisms

Five snails were placed individually in 148 ml acid-washed polypropylene containers filled with MOD water. Snails were fed unlabelled lettuce throughout the experiment. Collection of feces as well as water renewal and sampling were performed as described above. At the end of the experiment, snails were sacrificed (frozen).

2.5. Sample preparation and analysis

To minimize inadvertent metal contamination, labware, vials and Teflon sheeting were soaked for 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 allowed to dry at 50 °C for 3 days. Dried snails, feces and lettuce samples were weighed and digested at room temperature in Teflon vials with concentrated nitric acid (100 μl mg dry weight sample^{-1}) for 7 days (Croteau et al., 2002). Hydrogen peroxide (Baker Ultrex II grade, 40 μl mg dry weight sample^{-1}) was added prior to final dilution with ultrapure water (860 μl mg dry weight sample^{-1}). Similar weight samples of the certified reference material NIST-2976 (mussel tissue from National Institute of Standards and Technology) were submitted to the same "cold" digestion procedures during each analytical run.

Water and digested samples were analyzed for the naturally occurring stable isotopes of Cu (^{63}Cu and ^{65}Cu), Cd (^{106}Cd, ^{108}Cd, ^{110}Cd, ^{111}Cd, ^{112}Cd, ^{113}Cd and ^{114}Cd) and Cr (^{50}Cr, ^{52}Cr, ^{53}Cr and ^{54}Cr) 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 Canada; SLRS-4) were analyzed for Cu, Cd and Cr during each analytical run. To account for instrument drift and change in sensitivity, internal standardization was performed by addition of germanium (^{74}Ge) to all samples and standards, but the calibration blanks. We also reanalyzed one of our standards after every 10 samples. Deviations from standard value were, in general, less than 5% for all metals. Isotopic composition of Cu and Cd in experimental snails was expressed as the ratio of the net signal intensity (ion counts) of ^{65}Cu to ^{63}Cu and of ^{106}Cd to ^{114}Cd.

2.6. Calculation of accumulated tracer concentrations

We used an isotope tracing technique that allows tracking newly accumulated tracers, independently from background levels. The accumulated tracer concentrations (expressed as means ± 95% confidence intervals) were determined using equations derived from Croteau et al. (2004). Briefly, the relative abundance of each tracer (i.e., ^{53}Cr, ^{65}Cu and ^{106}Cd) was determined using the signal intensities of each isotope in the standards used to calibrate the ICP-MS. That is,

$$p^i = \left(\frac{\text{Intensity } {}^i\text{E}}{\sum_j^{jj} \text{Intensity } {}^j\text{E}} \right)_{\text{Standard}} \quad (1)$$

where p^i is the relative abundance of the natural isotope ^{i}E (the tracer), E is the element (metal), j and jj are the lightest and heaviest isotopes of the element E, respectively. For example, the relative abundance of ^{106}Cd isotope is

$$p^{106} = \text{Intensity} \left(\frac{{}^{106}\text{Cd}}{{}^{106}\text{Cd} + {}^{108}\text{Cd} + {}^{110}\text{Cd} + {}^{111}\text{Cd} + {}^{112}\text{Cd} + {}^{113}\text{Cd} + {}^{114}\text{Cd}} \right)_{\text{Standard}} \quad (2)$$

Eq. (1) works because p^i is constant across the range of standard concentrations. That is, the intensities measured for the various isotopes do not increase disproportionately as the total concentration of the element increases. This is critical to Eq. (3) (below). Concentrations of tracer in the experimental organisms ($[^i\text{E}]_{\hat{e}}$) were then calculated as the product of p^i and the total metal concentrations inferred by the ICP-MS software from tracer intensity ($[\text{T}^i\text{E}]$):

$$[^i\text{E}]_{\hat{e}} = p^i \times [\text{T}^i\text{E}] \quad (3)$$

For instance, concentrations of ^{106}Cd in the experimental organisms ($[^{106}\text{Cd}]_{\hat{e}}$) are

$$[^{106}\text{Cd}]_{\hat{e}} = p^{106} \times [\text{T}^{106}\text{Cd}] \quad (4)$$

Total metal concentrations inferred from the intensity of the most abundant isotope (i.e., ^{52}Cr for Cr, ^{63}Cu for Cu and ^{114}Cd for Cd) were then used to derive the original load of tracer ($[^i\text{E}]^0_{\hat{e}}$) that occurred in each sample in the absence of a spike. This is especially important for essential elements such as Cu, i.e.,

$$[^i\text{E}]^0_{\hat{e}} = p^i \times [\text{T}^k\text{E}] \quad (5)$$

where k is the most abundant isotope of the element E. In our experiment with ^{106}Cd, Eq. (5) becomes

$$[^{106}\text{Cd}]^0_{\hat{e}} = p^{106} \times [\text{T}^{114}\text{Cd}] \quad (6)$$

Finally, the net tracer uptake ($\Delta[^i\text{E}]_{\hat{e}}$) was derived from the total experimental metal concentration $[^i\text{E}]_{\hat{e}}$ (Eq. (3)) minus the pre-existing concentration of tracer ($[^i\text{E}]^0_{\hat{e}}$ from Eq. (5)),

$$\Delta[^i\text{E}]_{\hat{e}} = [^i\text{E}]_{\hat{e}} - [^i\text{E}]^0_{\hat{e}} \quad (7)$$

Consequently, net ^{106}Cd uptake ($\Delta[^{106}\text{Cd}]_{\hat{e}}$) is $[^{106}\text{C}]_{\hat{e}}$ as derived from the total experimental Cd $[^{106}\text{C}]_{\hat{e}}$ inferred from ^{106}Cd signal (Eq. (4)) minus the pre-existing load of ^{106}Cd ($[^{106}\text{Cd}]^0_{\hat{e}}$ from Eq. (6)),

$$\Delta[^{106}\text{Cd}]_{\hat{e}} = [^{106}\text{Cd}]_{\hat{e}} - [^{106}\text{Cd}]^0_{\hat{e}} \quad (8)$$

2.7. Polyatomic interferences

The analytical determination of Cr concentrations by ICP mass spectrometry could be affected by polyatomic interferences that result from the combination of sample matrice ions. The most common are argides (i.e., ArX where X = H, N, O, C, etc.). For example, organic matrices react with argon in the plasma to form polyatomic molecules that cause isobaric overlap and erroneously high results. Specifically, ^{12}C reacts with ^{40}Ar to form ^{40}Ar^{12}C, which has a mass similar to ^{52}Cr. Total oxidation of the organic constituents in sample matrices is required to avoid such confounding influences.

We tested whether our digestion procedure efficiently oxidizes the organic compounds of complex matrices (i.e., digested

Table 1
Metal concentrations ($\mu g\,g^{-1}$ or $\mu g\,l^{-1}$, ±95% CI) inferred from the natural isotopes of Cd (^{114}Cd, ^{112}Cd and ^{106}Cd), Cu (^{65}Cu and ^{63}Cu) and Cr (^{53}Cr and ^{52}Cr) in certified materials (SLRS-4, NIST-2976), control snails, unlabelled lettuce and in feces collected during the acclimation

	^{114}Cd	^{112}Cd	^{106}Cd	^{65}Cu	^{63}Cu	^{53}Cr	^{52}Cr	n
Relative abundance	28.7%	24.1%	1.25%	30.8%	69.2%	9.5%	83.8%	–
SLRS-4	0.014 ± 0.003	0.015 ± 0.003	0.26 ± 0.13	1.87 ± 0.03	1.82 ± 0.03	0.33 ± 0.14	0.38 ± 0.04	14
Certified value		0.012 ± 0.002		1.81 ± 0.08		0.33 ± 0.02		
AAS analysis						0.36 ± 0.03		7
NIST-2976								
Cold digestion	0.69 ± 0.04	0.68 ± 0.04	0.85 ± 0.23	4.17 ± 0.19	4.15 ± 0.16	1.7 ± 0.40	2.5 ± 0.52	4
Cold digestion/heat	0.66 ± 0.01	0.67 ± 0.01	0.49 ± 0.19	4.13 ± 0.12	4.11 ± 0.11	0.78 ± 0.13	1.4 ± 0.21	4
Certified value		0.82 ± 0.16		4.02 ± 0.33		0.5 ± 0.16		
AAS analysis						0.52 ± 0.11[a]		3
Control snails								
Cold digestion	3.5 ± 0.13	3.5 ± 0.13	3.9 ± 0.41	35 ± 12	35 ± 12	0.70 ± 0.37	3.7 ± 0.36	5
Cold digestion/heat	3.2 ± 0.13	3.2 ± 0.14	3.8 ± 0.28	32 ± 11	32 ± 11	0.39 ± 0.19	1.2 ± 0.34	5
AAS analysis						0.15 ± 0.12		5
Unlabelled lettuce								
Cold digestion	0.89 ± 0.12	0.90 ± 0.11	1.1 ± 0.11	9.0 ± 1.9	9.0 ± 2.0	1.5 ± 0.41	2.8 ± 0.40	5
Feces (acclimation)								
Cold digestion	4.0 ± 0.56	4.2 ± 0.52	3.5 ± 0.21	29 ± 13	29 ± 13	8.0 ± 1.8	9.5 ± 0.77	3

Samples were digested at room temperature (cold digestion). Subsamples of digested invertebrate tissues were further evaporated to dryness and reconstituted in HNO_3 prior being reanalyzed at the ICP-MS and AAS (for Cr only). Also given are sample numbers and the relative natural abundance of each stable metal isotope.
[a] Cr analyses performed *a posteriori* on samples that were "cold" digested and further evaporated by heat.

invertebrates) by heating to dryness and reconstituting with HNO_3 (5%) subsamples of "cold" digested snails and mussel tissues (NIST-2976). Concentrations of Cr in these "double" digested invertebrate samples were analyzed by both ICP-MS and flameless atomic-absorption spectroscopy (AAS, Perkin-Elmer model AA-800). Because AAS analyses use absorption of light to measure the concentration of gas-phase atoms, Cr concentrations measured by AAS are unaffected by polyatomic molecules.

Concentrations of Cr in standard reference material (mussel tissue) were over-predicted by three to five times when inferred from ^{52}Cr (Table 1). Similarly, control snail Cr concentrations were over-estimated when inferred from ^{52}Cr compared to determination by AA spectroscopy and after double digestion. To develop a correction factor for the bias, we used ^{53}Cr to infer Cr concentrations in snail and lettuce samples that had not been exposed to enriched ^{53}Cr. Comparisons with AAS analyses showed that this less abundant isotope was not affected by polyatomic interferences (Table 1). A correction factor was used to adjust background Cr concentrations (which had to be inferred from ^{52}Cr, Eq. (5)) in matrices for which concentrations inferred from both isotopes differed significantly ($p < 0.05$). The correction factor was 3.58 (±1.15, $n = 5$) for snail tissue and 1.96 (±0.32, $n = 5$) for the lettuce.

3. Results

3.1. Tracer analysis in water samples

$^{53}Chromium$ concentrations in the experimental media (water) did not vary significantly during both phases of the experiment ($p > 0.9$, Table 2). In contrast, ^{65}Cu concentrations in water after the labelled feeding period were significantly higher than those measured during the unlabelled feeding phase ($p < 0.01$). Specifically, ^{65}Cu concentrations (±95% CI) after the labelled feeding pulse were $0.35 \pm 0.05\,\mu g\,l^{-1}$ as opposed to $0.22 \pm 0.01\,\mu g\,l^{-1}$ after 5 h of depuration. Some ^{106}Cd was released as well, but quantification proved unreliable for natural waters at concentrations close to the method detection limit of approximately $5\,pg\,l^{-1}$ (i.e., ^{106}Cd is not recovered quantitatively from standard reference materials). In general, dissolved tracer concentrations during depuration did not differ between exposed and control snails ($p > 0.5$). Loss of tracers in the media during depuration was thus assumed negligible.

Despite the occurrence of dissolved metal in the exposure media, waterborne tracer uptake during the labelled feeding phase was negligible compared to that from diet. Metal influx from solution was (at the most) $11\,ng\,g^{-1}\,h^{-1}$ for ^{65}Cu, as calculated by multiplying the unidirectional metal uptake rate constant from solution (k_u) by the dissolved tracer concentration (Table 2). Assuming that exposure lasts for a maximum of 2 h (i.e., duration of labelled feeding), the experimental snails (mean weight of 39 mg) accumulated from the dissolved route a maximum of 0.84 ng of ^{65}Cu. This is two to three orders of magnitude lower than the snail metal burdens at the end of depuration (see Q^F in Table 3). Lower dissolved Cu influx was obtained using a k_u based upon free ion concentrations and calculating uptake on that basis. Likewise, dissolved Cd influx was (at the most) $0.5\,ng\,g^{-1}\,h^{-1}$, assuming the lowest reliable concentrations. Thus, the contribution of water to the overall tracer accumulation was not considered when calculating AE.

Table 2
Biodynamic parameters used to predict ^{65}Cu and ^{106}Cd accumulation in *L. stagnalis*

Parameter	Symbol	Unit	Isotope	Value ± 95% CI	*n*
Rate constant of metal uptake from dissolved phase[a]	k_u	$l\,g^{-1}\,d^{-1}$	^{65}Cu ^{106}Cd	0.74 ± 0.04 0.82 ± 0.08	–
Total metal concentration (after 2 h of labelled feeding)	$[M]_{water}$	$ng\,l^{-1}$	^{65}Cu ^{106}Cd ^{53}Cr	350 ± 51 14 ± 5 <d.l.	5
Tracer concentration in labelled lettuce[b]	$[M]_{LL}$	$\mu g\,g^{-1}$	^{65}Cu ^{106}Cd ^{53}Cr	287 ± 14 9.1 ± 0.6 30 ± 8	13
Net tracer concentration in labelled lettuce[c]	$\Delta[M]_{LL}$	$\mu g\,g^{-1}$	^{65}Cu ^{106}Cd ^{53}Cr	287 ± 14 9.1 ± 0.6 30 ± 8	13
Tracer concentration in unlabelled lettuce[b]	$[M]_{UL}$	$\mu g\,g^{-1}$	^{65}Cu ^{106}Cd ^{53}Cr	2.6 ± 0.7 0.012 ± 0.003 0.13 ± 0.02	5
Net tracer concentration in unlabelled lettuce[c]	$\Delta[M]_{UL}$	$\mu g\,g^{-1}$	^{65}Cu ^{106}Cd ^{53}Cr	0.02 ± 0.02 0.003 ± 0.001 0.009 ± 0.026	5
Mean dry weight of experimental snails	Wt	mg dry wt	–	39 ± 6.2	20

Also given are sample numbers; <d.l., below detection limit.
[a] From Croteau and Luoma (2007).
[b] See Eq. (3).
[c] See Eq. (7).

3.2. Copper and Cd ratios in the experimental snails

Fig. 1 shows $^{65/63}$Cu and $^{106/114}$Cd isotopic ratios in the experimental snails after 48 h of depuration. Tracer ratios for snails B, C and G were not different than controls (inset Fig. 1), suggesting that these snails did not feed during the labelled feeding period. Twelve experimental snails (A, D–F, H–O) were significantly enriched in both tracers compared to control. Only the enriched snails (closed circles) are considered in this study.

3.3. Egestion of tracers

The snails defecated the unassimilated tracers over similar time periods and with similar patterns (Fig. 2). Egestion peaks for ^{65}Cu and ^{106}Cd were, in general, asymmetrical, usually showing a steep front and a long, drawn-out tail. Merging the egested metal for each snail into a normalized average (central) tendency revealed single, similarly timed, egestion peaks for ^{53}Cr, ^{65}Cu and ^{106}Cd (Fig. 3).

Table 3
^{106}Cd and ^{65}Cu burdens after pulse feeding (Q^F), cumulative amount of ^{106}Cd and ^{65}Cu recovered in feces during depuration ($\sum_{Feces}$) as well as the accumulated ^{106}Cd and ^{65}Cu for each experimental snail (ΔQ)

Snail	Wt_{snail} (mg dw)	Q^F (ng snail^{-1})		$\sum_{Feces}$ (ng)		ΔQ (ng snail^{-1})	
		^{106}Cd	^{65}Cu	^{106}Cd	^{65}Cu	^{106}Cd	^{65}Cu
A	27.8	2.68	510	0.162	16.6	1.71	42.4
D	23.2	5.51	481	0.450	15.9	4.51	165
E	66.5	8.78	921	1.591	80.8	6.24	177
F	55.1	8.44	518	2.028	24.4	7.15	130
H	36.5	5.01	645	0.492	7.14	3.69	52.9
I	40.0	4.53	759	0.726	20.6	2.95	95.0
J	53.4	8.42	862	0.902	24.2	6.20	256
K	44.6	5.72	222	0.358	11.2	3.98	82.4
L	32.5	4.09	768	0.807	6.80	2.90	55.8
M	41.9	6.38	535	0.674	17.6	4.66	70.5
N	29.9	4.53	452	0.717	10.2	3.40	66.4
O	22.7	5.19	91.9	0.509	11.2	4.38	50.3
Control 1	53.0	2.18	534	0.175	11.8	0.10	<0.001
Control 2	40.5	1.57	286	0.095	8.77	0.03	<0.001
Control 3	46.1	2.21	415	0.077	16.3	0.29	−0.7
Control 4	32.9	1.39	272	0.055	5.10	0.20	−1.5
Control 5	26.6	1.30	453	0.112	13.4	0.19	−3.0

Also given is snail's dry weight (Wt_{snail}).

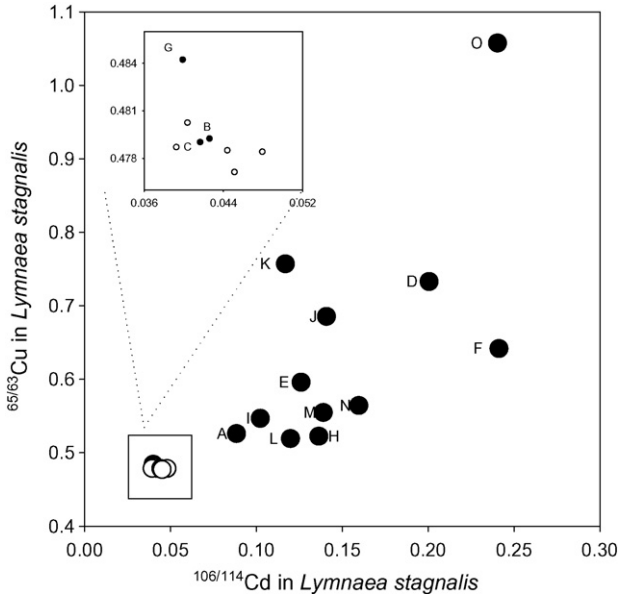

Fig. 1. Cu and Cd isotopic ratios in the experimental snails after 48 h of depuration. Open circles are for controls; solid circles are for snails exposed for 2 h to labelled lettuce. Each data point represents an experimental organism. See Table 3 for code.

The first defecation of ^{53}Cr occurred typically after 5 h of depuration. This represents the minimal gut passage time of food (GPT) for *L. stagnalis*. Using inert ^{51}Cr-labelled beads, Decho and Luoma (1991) determined GPT ranging from <1 h for *Potamocorbula amurensis* to 9.6 h for *Macoma balthica*, respectively. Gut residence time (GRT) of food in *L. stagnalis* was 4.5 times longer than of GPT (i.e., 22.5 h to defecate 90% of the ingested label, Fig. 4). The time elapsed between GPT and GRT was 8 to 28 times shorter for *L. stagnalis* compared to that for *P. amurensis* and *M. balthica* (Decho and Luoma, 1991), suggesting that gut transit time of food is much faster in snails compared to clams.

^{53}Chromium was egested at the highest rate, followed by ^{65}Cu and then by ^{106}Cd (Fig. 2). The small differences in rate and pattern among metals probably reflected concentration and assimilation differences (Figs. 3 and 4). ^{65}Cu, ^{53}Cr and ^{106}Cd concentrations in the labelled lettuce were 287, 30 and 9 μg g^{-1}, respectively (Table 2). Thus, ^{53}Cr has the highest egestion rate because this metal is poorly assimilated. The egestion rate of ^{65}Cu is lower than that for ^{53}Cr because more ^{65}Cu is assimilated. Defecation of ^{65}Cu is faster than ^{106}Cd because concentrations in the labelled food are higher for the former isotope. Tracer in the control feces reflects background isotope concentrations in the unlabelled food (which were not subtracted out in this case) (Tables 2 and 3).

Proportional rate constants of defecation ($k_{defecation}$ per h) did not vary significantly among metal (ANCOVA $p = 0.33$, Fig. 5). Thus, when compared on a similar basis, it appeared that the metals all followed the same digestive processing pathway. Similarly to rate constants of metal loss (k_e, see Croteau et al., 2004), $k_{defecations}$ were derived from the slope of the regression between ln-transformed proportional defecation over the time elapse between GPT and GRT. The $k_{defecations}$ (±S.E.) were $0.134 \pm 0.009\,d^{-1}$ for ^{53}Cr, $0.113 \pm 0.017\,d^{-1}$ for ^{65}Cu and $0.104 \pm 0.013\,d^{-1}$ for ^{106}Cd.

Table 4
Food ingestion rates (IR) calculated using Eq. (9)

Snail	$\sum^{53}Cr_{Feces}$ (ng)	$Wt_{Lettuce}$ (mg)	IR (g g^{-1} d^{-1})		
			^{53}Cr	^{106}Cd	^{65}Cu
A	10.0	0.338	0.146	0.078	0.096
D	9.09	0.307	0.159	0.302	0.360
E	10.6	0.359	0.065	0.160	0.171
F	20.2	0.683	0.149	0.301	0.164
H	12.0	0.406	0.134	0.159	0.071
I	16.6	0.559	0.168	0.125	0.125
J	70.4	2.377	0.534	0.190	0.241
K	5.80	0.196	0.053	0.130	0.095
L	12.2	0.412	0.152	0.151	0.085
M	15.7	0.531	0.152	0.178	0.094
N	3.08	0.104	0.042	0.211	0.115
O	10.0	0.338	0.178	0.302	0.121
		Mean	0.159	0.181	0.147
		95% CI	0.072	0.042	0.047

Also given are the amount of ^{53}Cr recovered in feces ($\sum^{53}Cr_{Feces}$) during depuration and the amount of lettuce ingested during the labelled feeding period ($Wt_{Lettuce}$), as in inferred from $\sum^{53}Cr_{Feces}$ and $[^{53}Cr]_{LL}$, see Table 2.

3.4. Ingestion of tracers

Food ingestion rate (IR) during the labelled feeding phase was 0.16 ± 0.07 g g^{-1} d^{-1} (Table 4). The IR during the 2 h of labelled feeding ($IR_{2\,h}$) was calculated for each experimental snail using the total amount of ^{53}Cr egested in the feces during depuration ($\Sigma^{53}Cr_{feces}$), the enriched lettuce ^{53}Cr concentration ($[^{53}Cr]_{LL}$) and the snail's dry weight (Wt_{snail}), i.e.,

$$IR_{2\,h} = \frac{\sum {}^{53}Cr_{feces}}{[^{53}Cr]_{LL} \times Wt_{snail}} \quad (9)$$

Because food was provided *ad libidum* during the labelled feeding pulse, the IR values for *L. stagnalis* likely represent a maximum. That is, snails need to forage for their food in nature. The calculations are also based on the amount of food ingested during the first two hours that followed 3 days of starvation.

Both ^{65}Cu and ^{106}Cd can be used to infer IR as well. The mass of ^{65}Cu and ^{106}Cd egested in the feces during depuration, the enriched lettuce ^{65}Cu and ^{106}Cd concentrations, the snail's dry weight along with the amount of ^{65}Cu and ^{106}Cd retained in the snail tissues after depuration were used to estimate the mass of lettuce consumed during the labelled feeding period. As shown in Table 4, employing either isotope yields, in general, similar estimates of IR.

3.5. Assimilation efficiency

Metal AE was calculated using both mass balance (Luoma et al., 1992) and ratio methods (Calow and Fletcher, 1972). Mass balance calculations (i.e., ^{65}Cu or ^{106}Cd retained divided by ^{65}Cu or ^{106}Cd ingested, using both feces and snail tissue data) yield an AE (±95% CI) of 84 ± 4% for Cu and 85 ± 3% for Cd

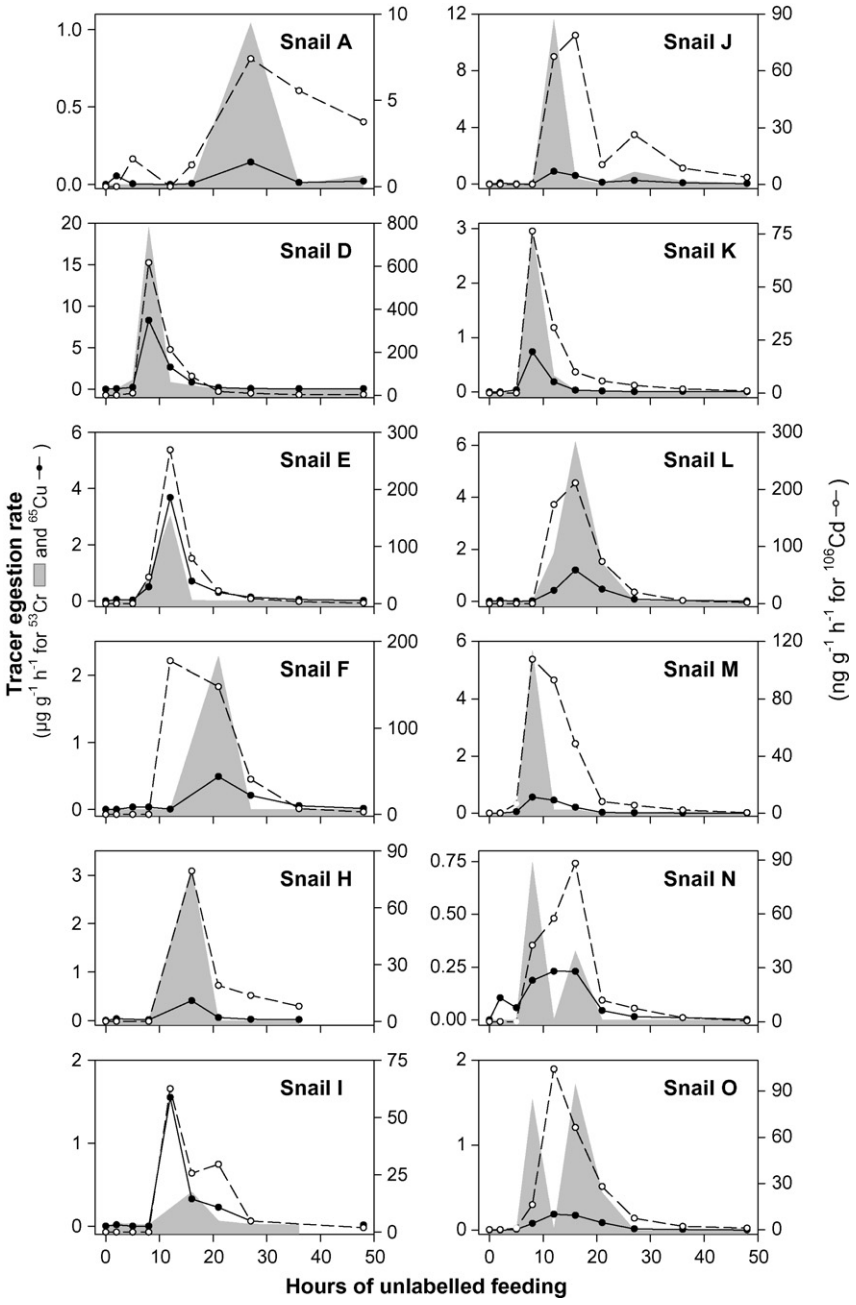

Fig. 2. Instantaneous tracer egestion rates during depuration for each experimental snail.

(Table 5). By the ratio method, ^{65}Cu AE was calculated for each snail as

$$\mathrm{AE}(\%) = 1 - \left[\frac{(^{65}\mathrm{Cu}/^{53}\mathrm{Cr})_{\mathrm{feces}}}{(^{65}\mathrm{Cu}/^{53}\mathrm{Cr})_{\mathrm{food}}}\right] \times 100 \qquad (10)$$

where $(^{65}\mathrm{Cu}/^{53}\mathrm{Cr})_{\mathrm{feces}}$ is the ratio of ^{65}Cu and ^{53}Cr net signal intensities in cumulative feces (after depuration) and $(^{65}\mathrm{Cu}/^{53}\mathrm{Cr})_{\mathrm{food}}$ is the ratio of ^{65}Cu and ^{53}Cr net signal intensities in the enriched lettuce. Estimates of AE from the ratio method were similar to estimates based upon mass balances. The ratio determinations were slightly more variable, i.e., $81 \pm 12\%$ for Cu and $78 \pm 10\%$ for Cd. The ratio method assumes that ^{53}Cr is not bioavailable to snails. This was verified *a posteriori*, i.e., snail ^{53}Cr concentrations were not different than 0 after 48 h of depuration ($0.012 \pm 0.017\ \mu\mathrm{g\ g}^{-1}$). Snails exposed for 18 h to ^{53}Cr-enriched lettuce did not accumulate ^{53}Cr (Croteau and Luoma, unpublished data). The lack of ^{53}Cr assimilation confirms that enriched stable Cr is a valuable tracer for the passage of food materials through digestive processes.

4. Discussion

Characterizing the digestive processes that occur upon food ingestion is essential to properly assessing the assimilation efficiency of dietborne metals. Among the physiological factors

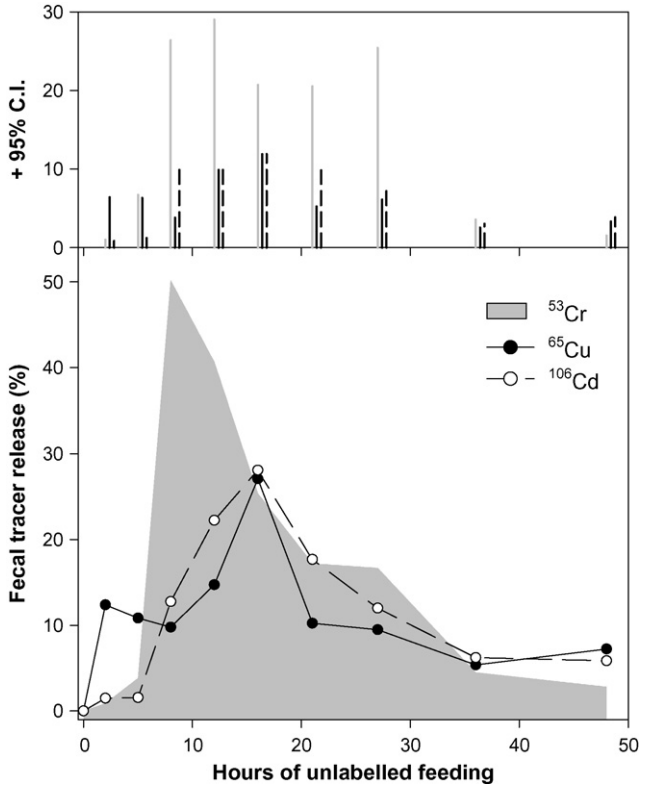

Fig. 3. Normalized average fecal release for each tracer (%) during depuration; +95% CI is shown on the upper panel for clarity.

known to influence AE is the transit time of ingested food through the gut. Specifically, extended retention of food within the digestive tract allows for more efficient digestion and absorption (Willows, 1992). For example, Ag, Zn, Co and Cd AE in the marine mussel *Mytilus edulis* increase proportionally with GRT (Wang et al., 1995). GRT can vary in response to food

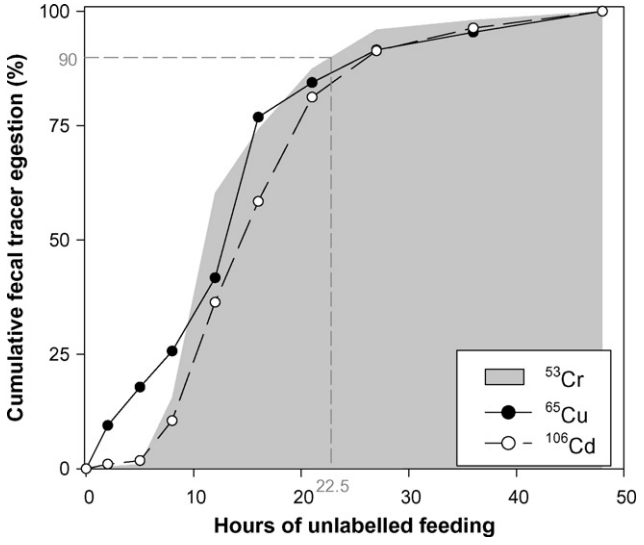

Fig. 4. Cumulative loss of ^{53}Cr in feces following 2 h of exposure to dietary tracers. Values represent cumulative percentage of ^{53}Cr in feces. Also shown are the cumulative egestion of ^{65}Cu (solid circles and line) and ^{106}Cd (open circles and dashed line).

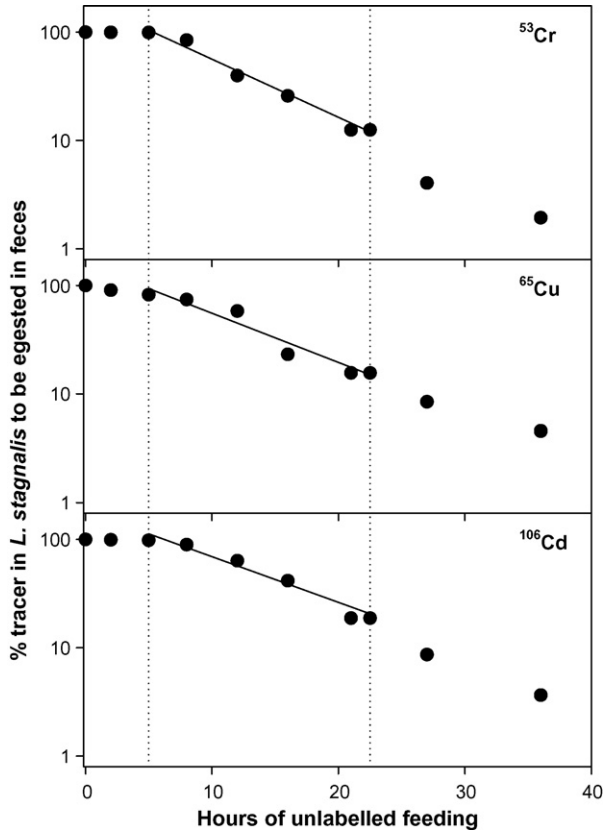

Fig. 5. Fecal release of tracers by *L. stagnalis* following 2 h of exposure to dietborne metals. Values represent percentages of tracer remaining in *L. stagnalis* to be egested in feces. Vertical dotted lines represent from left to right, GPT and GRT. Solid lines are linear regression relationships ($p < 0.01$).

concentration and quality (Wang et al., 1995, 1996), and it can be significantly different among metals (Bricelj et al., 1984). In determining AE, it is optimal to use a pulse-feeding period shorter than GPT, but very few studies verify this basic assumption (Decho and Luoma, 1991; Wang et al., 1995). Most studies also assume that AE is determined after the gut is empty, but that is not always verified. Here we show, for the first time, that enriched stable isotopes of Cr provide a simple, widely available approach for determining GPT and GRT, if determination of Cr is conducted properly and it is shown to be not bioavailable to the species of interest.

4.1. Assimilation efficiency

There are only a few measurements of Cu AE in aquatic organisms to compare with our study. The paucity of data for Cu is due to the lack of suitable tracers (Croteau et al., 2004). The AE with which *L. stagnalis* retained Cu and Cd from food (84% for Cu and 85% for Cd, Table 5) is relatively high compared to that determined for the filter-feeder bivalve *Corbicula fluminea* (38% for Cu: Croteau and Luoma, 2005; 58–72% for Cd: Lee and Luoma, unpublished) and for marine herbivorous copepods (40% for Cu: Chang and Reinfelder, 2000; 30% for Cd: Reinfelder and Fisher, 1991). A few recent studies reported correspondingly high Cd AE (84–99%) for marine predatory gastropods (Wang and Ke, 2002; Blackmore and Wang, 2004);

Table 5
AE (%) determined by the ratio method knowing that $^{65}Cu/^{53}Cr$ and $^{106}Cd/^{53}Cr$ in the labelled lettuce were 5.838 and 0.675, respectively

Snail	Cumulative intensity in feces			Ratio method		Mass balance	
	^{53}Cr	^{65}Cu	^{106}Cd	^{65}Cu	^{106}Cd	^{65}Cu	^{106}Cd
A	34,905	34,772	1,113	82.9	95.3	71.8	91.3
D	27,262	28,990	2,957	81.8	83.9	91.2	90.9
E	30,440	150,636	10,136	15.2	50.6	68.6	79.7
F	60,040	30,049	10,795	91.4	73.3	84.2	77.9
H	36,153	4,927	2,053	97.7	91.6	88.1	88.2
I	47,504	38,792	5,156	86.0	83.9	82.2	80.2
J	198,641	42,531	6,198	96.3	95.4	91.4	87.3
K	16,474	21,834	2,407	77.3	78.3	88.0	91.8
L	37,369	11,810	5,580	94.6	77.9	89.1	78.2
M	45,621	39,233	4,808	85.3	84.4	80.0	87.4
N	9,630	13,849	4,293	75.4	33.9	86.7	82.6
O	34,546	24,375	3,330	87.9	85.7	81.8	89.6
			Mean	81.0	77.9	83.6	85.4
			95% CI	12.4	10.3	4.1	3.0

Also given is the AE determined using mass balance calculations.

but apparently these are the first data for herbivorous snails. Herbivorous snails are surprisingly efficient in assimilating at least adsorbed metal from their food sources. This may be an important contributory reason why they are strong metal bioaccumulators in natural waters (Croteau et al., 2005). In addition, *L. stagnalis* breathes air (pulmonate snail). Thus, uptake of elements likely occurs through the dietary pathway, which might explain, in part, their high metal AEs. High concentrations of metallothionein and phosphate granules in the digestive glands of herbivorous gastropods (Nott and Langston, 1989; Desouky, 2006) may also accentuate bioaccumulation or be a response to traits that facilitate efficient assimilation.

The single peak of ^{53}Cr egestion (and other isotopes) observed in most snails in this experiment suggests that all the ingested food was transported, digested and assimilated along a single pathway. This contrasts to biphasic egestion that characterizes bivalves for which both intestinal and (digestive) glandular digestions are important (Decho and Luoma, 1996). Histological evidence suggests that ciliated cells whose function is to facilitate food and feces transport are especially abundant along the alimentary canal of *L. stagnalis* (Boers and Kits, 1990). If they participate in a form of intracellular digestion, it occurs without the extension of gut residence times that is typical of intracellular digestion in bivalves. Thus, snails digest food efficiently (as judged by their efficient assimilation of metals) while at the same time processing food faster than bivalves. Again, these are characteristics that might facilitate strong metal bioaccumulation. However, we cannot rule out the possibility that the remarkably high metal AEs with which *L. stagnalis* retained dietborne Cu and Cd reflect the labelling technique. Determination of metal AEs from a variety of food items is required for comprehensive characterization of species-specific AEs.

The similarity in AE between Cu and Cd for *L. stagnalis* is at first sight surprising, although it might reflect similar GRTs between elements. Wang and Fisher (1999) claimed that essential elements such as Cu are usually assimilated with higher efficiencies than nonessential elements. Both Cu and Cd were assimilated efficiently in this study. The extremely high Ca requirements for shell formation of *L. stagnalis* (Grosell and Brix, 2004) could explain in part the high Cd AE, as Cd uses Ca transporters to cross biological membranes (Simkiss and Taylor, 1995). Croteau and Luoma (2007) recently showed that Cd influx rate from solution in *L. stagnalis* is much higher than that of Cu likely because Cd has more binding sites. The high Ca demand and slow elimination of Cd has toxicological implications for this species. The animal must possess an effective mechanism to store Cd in a detoxified form, e.g., metallothioneins (Roesijadi, 1992). This detoxification mechanism might have evolved in response to the species high physiological requirement for Ca.

Lastly, the similarity in metal AE between methods (Table 5) suggests that either mass balance or ratio approached can be used to calculate Cd and Cu AE in *L. stagnalis*, once it is verified that Cr is not bioavailable. The lack of Cr accumulation and the similarity in fecal egestion rate constants among tracers verified the basic assumptions of both approaches, i.e., the "inert" tracer should not be absorbed to any significant extent and the elements must move along the gut at a similar rate.

Our study demonstrates that enriched metal stable isotope tracers are applicable to determining basic characteristics of food processing and for use in experimental determinations of metal uptake that heretofore have relied on radioisotopes. Enriched stable isotope protocols, therefore, offer opportunities for expanding studies of metal biodynamics. In addition, the use of enriched stable isotope tracers extends the procedure to elements whose radioisotopes are unsuitable for these types of experiments. The "stable isotope" pulse-chase feeding technique described herein avoids many of the problems associated with radioactivity (Croteau et al., 2004), previous contamination history (Rainbow et al., 2003), physiological turnover, as well as metal recycling due to long-term experiments (Luoma et al., 1992). The approach further provides unique opportunities to evaluate the interplay between digestive processes and trace element AE. Understanding the biological processes that mod-

ulate dietborne metal uptake is crucial to assess the toxicity of dietborne metals.

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