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Application of enriched stable isotope technique to the study of copper bioavailability in *Daphnia magna*

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Abstract

The biokinetics of Cu in *Daphnia magna*, including dissolved uptake, assimilation and efflux, has been determined using a gamma ^{67}Cu radiotracer methodology. However, this gamma emitting radioisotope is not readily available due to its very short half-life. In the present study, we employed a stable isotope tracer (^{65}Cu) to determine the Cu biokinetics and compared our results to those determined using ^{67}Cu. The dissolved uptake rate constant of ^{65}Cu was 3.36 L/(g·day), which is higher than that of ^{67}Cu (1.32 L/(g·day)). With increasing food concentrations from 2×10^4 to 1×10^5 cells/mL, the Cu assimilation efficiency (AE) decreased from 46% to 11%, compared to a decrease from 27% to 16% when determined using ^{67}Cu. The efflux of Cu from *Daphnia magna* was quantified following both dissolved and dietary uptake. The efflux of waterborne Cu was comparable to that of dietborne Cu and the efflux rate constant (0.32–0.52 day^{-1}) was higher than that determined by ^{67}Cu (0.19–0.20 day^{-1}). By considering different water properties and handling procedure between the two experiments, we believe that these differences are reasonable. Overall, this study demonstrated that the enriched stable isotope tracer technique is a powerful tool to investigate metal bioavailability and maybe a good alternative to radioactive measurements.

Key words: stable isotope; *Daphnia magna*; copper; bioavailability

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Introduction

Daphnia magna (*D. magna*) is a freshwater zooplankton which is widespread throughout many lakes. It has been extensively used to investigate metal bioaccumulation patterns because of its importance in the food chain (connected with phytoplankton and carnivores). The important processes of metal bioaccumulation in *D. magna* (i.e., aqueous uptake, dietary assimilation and elimination) could be revealed by a biokinetic model. The biokinetics of metals, including Cd (Yu and Wang, 2002a; Guan and Wang, 2006), Zn (Guan and Wang, 2004), Se (Yu and Wang, 2002a), Ag (Lam and Wang, 2006), Ca (Tan and Wang, 2008), Hg and MeHg (Tsui and Wang, 2004a, 2004b) have already been systematically investigated in *D. magna*. Previously, we used a radioisotope (^{67}Cu) to quantify the dissolved Cu uptake, assimilation efficiency and efflux in *D. magna* and found that Cu accumulation was dominated by dietary uptake (Zhao et al., 2009). However, the above studies were all performed using gamma radioisotopes. Although radioisotope analysis enjoys high sensitivity and is a nondestructive analytical method, it suffers from some shortcomings which limit its wide application, such as the lack of suitable radioisotopes for some metals, difficulties in obtaining, storing and maintaining the radioisotopes, and handling/disposal hazards of radioactive waste.

Recently, with the rapid development of inductively coupled plasma-mass spectrometry (ICP-MS), enriched stable isotopes are increasingly used in metal research. Croteau and Luoma (2004, 2005) have employed the stable isotope tracer technique to reveal Cu bioaccumulation in the freshwater bivalve, *Corbicula*. With this method, they have also characterized the dissolved Cu and Cd uptake (Croteau and Luoma, 2007) and predicted the dietborne Cu, Cd and Ni toxicity in the freshwater snail (*Lymnaea stagnalis*) (Croteau and Luoma, 2009). Komjarova and Blust (2009) have studied the effect of Na, Ca and pH on the simultaneous uptake of Cd, Cu, Ni, Pb and Zn in the water flea, *D. magna.*

In the present study, we used the enriched stable isotope ^{65}Cu to study Cu biokinetics in *D. magna*. We determined and compared the uptake constants from the dissolved phase, assimilation efficiency and efflux rate constants.

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1 Materials and methods

1.1 Organisms, exposure medium, stable isotope and labware

D. magna obtained from the Hong Kong University of Science and Technology have been cultured in our laboratory for approximately two years. They were maintained in water taken from Jingmi River in Beijing. The water was filtered through 1.2 μm Whatman glass fiber (GF/C Whatman, Maidstone, UK) before use and renewed every two days. The daphnids were fed daily with the green algae, *Chlamydomonas reinhardtii*, at a concentration of 10^5 cells/mL (counted under the microscope using a hemocytometer). The green algae, also obtained from the Hong Kong University of Science and Technology, were cultured in an artificial WC medium (Guillard and Lorenzen, 1972). Before feeding, algae were centrifuged to remove WC medium, and then resuspended in filtered water. They were then stored in a refrigerator at 4°C before use. Both the daphnids and algae were kept in the growth chamber at 23.5°C with a light:dark cycle of 14:10 hr.

The medium used in all experiments was artificial, simplified Elendt M7 medium (Samel et al., 1999) (without EDTA, heavy metals and vitamin; SM7). The pH was adjusted to 8.0–8.4 by adding 2% HNO_3 or NaOH. The medium was equilibrated overnight prior to the experiments.

The stable copper isotope ^{65}Cu (99.2%) used in the present study was purchased from Isoflex Company (San Francisco, USA). The ^{65}Cu concentrations in the exposure medium and the digested samples of organisms were determined by ICP-MS (TJA PQ ExCell, ThermoFisher Scientific, America). All of the chemicals used in our experiments were analytical grade.

Before each exposure, 14-day-old adult daphnids with similar body size were collected from the stock cultures and acclimated in SM7 for 2–3 hr without food to evacuate their gut contents. During this exposure, the density of the daphnids was 0.1 individual per mL. In each experiment, there were three replicates per treatment. To minimize inadvertent metal contamination, labware was soaked for at least 24 hr in 14% HNO_3, rinsed 4–6 times with ultrapure water and dried at room temperature before use.

1.2 Determination of dissolved uptake, assimilation and efflux of Cu in *D. magna*

Methods to determine the dissolved uptake, assimilation and efflux of Cu in *D. magna* were based on earlier work using ^{67}Cu (Zhao et al., 2009), but with some modifications.

1.2.1 Dissolved uptake of Cu in *D. magna*

Acclimated daphnids were transferred to 600-mL polypropylene beakers (NALGENE) containing SM7 media spiked with different ^{65}Cu concentrations (2.5, 7.5, 30, 45, 75 and 110 μg/L). At 0, 2, 4, 8 and 12 hr, 10–15 daphnids were removed from the medium, rinsed twice with ultrapure water and dried at 80°C for 12 hr. The dry weight was determined by a Sartorius BS210S analytical balance (Goettingen, Germany). Then the samples were digested at 110°C in 68% HNO_3 for 12 hr. At the same time, two samples of certified oyster were also digested. Finally, the volume of samples was adjusted to 5 mL with ultrapure water and the ^{65}Cu concentration in the samples was measured by ICP-MS. Before and after the exposure, water samples were collected from each beaker to determine the "practical exposure concentrations" as the average of the two concentrations. The parameters in the dissolved uptake of Cu were calculated as the following Eqs. (1) and (2) (Lam and Wang, 2006):

$$\mathrm{DCF} = \frac{C_{^{65}\mathrm{Cu\ in\ }D.\ magna}}{C_{^{65}\mathrm{Cu\text{-}d}}} \quad (1)$$

where, DCF is the weight concentration factor, $C_{^{65}\mathrm{Cu}}$ (μg/g dw) is the ^{65}Cu concentration in *D. magna*, $C_{^{65}\mathrm{Cu\text{-}d}}$ (μg/L) is the dissolved ^{65}Cu concentration.

$$I = k_\mathrm{u} \times C_\mathrm{w} \quad (2)$$

where, I (μg/(g dw·hr)) is the Cu influx rate into *D. magna*, k_u (L/(g dw·hr)) is the uptake rate constant from the dissolved phase and C_w (μg/L) is the Cu concentration in the dissolved phase.

The relationship between DCF and k_u can be described as Eq. (3).

$$\mathrm{DCF} = k_\mathrm{u} \times t \quad (3)$$

where, t (hr) is exposure time.

1.2.2 Cu assimilation in *D. magna*

Cu assimilation was quantified at different food concentrations (2×10^4, 5×10^4 and 1×10^5 cells/mL). To label the algae *C. reinhardtii*, the cells in the log phase were transferred to modified WC medium (without Cu, Zn, or EDTA) spiked with ^{65}Cu with an initial concentration of 2×10^5 cells/mL. The spiked ^{65}Cu concentration was 200 μg/L. After 3 days of growth, the cells were collected by centrifugation and resuspended twice in SM7 media to remove the weakly bound stable isotope. The algal density was counted under a microscope using a hemocytometer. Two milliliters of the labeled algae was filtered onto a 1 μm polycarbonate membrane to measure the cellular Cu concentration, which was finally about 0.12 mg/g dry weight. After labeling algae with ^{65}Cu, 14-day-old adults with similar body size were transferred to SM7 and fed with labeled food in the dark for 15 min. Then the daphnids were transferred into SM7 medium containing unlabeled algae at a concentration of 10^5 cells/mL and depuration was started. The depuration lasted for 42 hr, and the water and food were refreshed every 6 hr. The ^{65}Cu concentrations retained in daphnids were determined by the same method as described in the uptake experiments. The Cu assimilation efficiency (AE) was calculated as the y-intercept of the linear regression between the percentage of ^{65}Cu retained in the daphnids (in natural log) and the time of depuration during the slow efflux phase.

1.2.3 Efflux of waterborne and dietborne Cu from daphnids

In this experiment, the elimination of Cu from the daphnids was quantified following both dietary and dissolved uptake. For the dietary phase exposure, *C. reinhardtii* were labeled using the same approach as in the assimilation experiment. The labeled algae were fed to the daphnids at the density of 10^5cells/mL. The food was added twice daily during the uptake period and the water was renewed to minimize the potential uptake of Cu from the aqueous phase. For the dissolved phase exposure, the SM7 medium was spiked with ^{65}Cu at a concentration of 10 μg/L to obtain a ^{65}Cu concentration in daphnids similar to that after dietary exposure. The daphnids were exposed for 18 hr per day, and no food was added during this period to prevent absorption of Cu onto the food particles. Afterwards, the daphnids were transferred to unspiked SM7 medium containing unlabeled food at a cell density of 10^5 cells/mL for another 6 hr. The daphnids were then rinsed in unspiked SM7 medium for 5 min to remove the loosely adsorbed algae and finally transferred back to the spiked SM7 medium to begin another 24 hr cycle.

Both exposures lasted for 3 days. The daphnids were then collected and depurated for another 3.5 days in SM7 containing unlabeled *C. reinhardtii* at 10^5 cells/mL. During the depuration, 10–15 daphnids were collected every 12 hr to measure the ^{65}Cu retained in *D. magna*. At the same time, the water and food were renewed. The efflux rate constant (k_e) of Cu was calculated from the slope of the linear regression between the natural log of the percentage of ^{65}Cu retained and the time of depuration between 2 and 3.5 day. The biological retention half-lives ($t_{1/2}$) of Cu were calculated as $t_{1/2} = 0.693/k_e$.

1.3 Calculation of accumulated tracer concentrations

We first calculate the relative abundance of ^{65}Cu isotope (p^{65}) using the intensity of each isotope in the standards to calibrate the ICP-MS as Eq. (4).

$$p^{65} = \text{Intensity}\left(\frac{^{65}\text{Cu}}{^{65}\text{Cu} + {}^{63}\text{Cu}}\right)_{\text{Standard}} \quad (4)$$

Signal intensity (p^{65}) is calibrated against total copper standards of varying concentration. Then the total concentrations of ^{65}Cu in the experimental samples ([^{65}Cu]) is calculated as the product of p^{65} and the total metal concentrations inferred from the ^{65}Cu intensity ([T^{65}Cu]), i.e.,

$$[^{65}\text{Cu}] = p^{65} \times [\text{T}^{65}\text{Cu}] \quad (5)$$

To calculate the "background" concentrations of ^{65}Cu in samples (the intrinsic concentration, $[^{65}\text{Cu}]_0$), the total metal concentrations inferred from the ^{63}Cu intensity ([T^{63}Cu]) is used, i.e.,

$$[^{65}\text{Cu}]_0 = p^{65} \times [\text{T}^{63}\text{Cu}] \quad (6)$$

Finally, the net concentrations of ^{65}Cu in samples is the total concentrations of ^{65}Cu ([^{65}Cu]) minus the background concentrations of ^{65}Cu in samples ($[^{65}\text{Cu}]_0$) (Croteau et al., 2004)

$$\Delta[^{65}\text{Cu}] = [^{65}\text{Cu}] - [^{65}\text{Cu}]_0 \quad (7)$$

1.4 Statistical analyses

Data were expressed as the mean ± standard error. One-way analysis of variance (ANOVA) was used to compare data when necessary. Statements of significant differences were based on accepting $p < 0.05$. All statistical comparisons were performed with SPSS 13.0.

2 Results and discussion

2.1 Dissolved uptake of Cu in *D. magna*

2.1.1 Dry weight concentration factor (DCF)

Generally, at different exposure concentrations, the DCFs Cu in *D. magna* increased in an approximately linear pattern during the 12 hr exposure period (Fig. 1). There was no evidence of saturation or steady-state accumulation within the exposure. As the exposure concentrations increased, the differences of DCFs among exposure concentrations became smaller. No obvious difference in DCF was found when the Cu concentration was > 33 μg/L ($p > 0.05$, one-way ANOVA), although the DCFs of 114 μg/L were a little lower (Fig. 1). Decreasing DCF with increasing Cu concentration from 2.3 to 33 μg/L suggested that daphnids might regulate Cu uptake; however, it should be noted that the quantified DCF may also include metal sorption onto the daphnid's body (Tsui and Wang, 2004a). Decreasing DCFs with increasing exposure concentrations were also found with Se, Hg and MeHg in daphnids (Yu and Wang, 2002a; Tsui and Wang, 2004a). This is possibly because at higher concentrations, the binding sites available on or in the animals were closer to saturation,

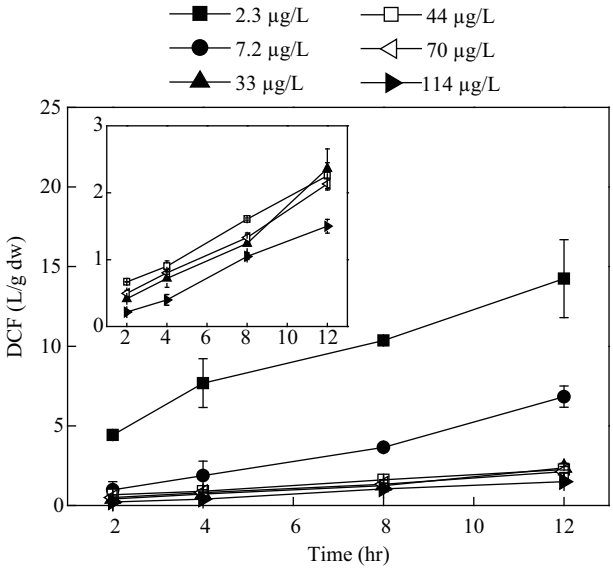

Fig. 1 Dry-weight concentration factor (DCF) in *Daphnia magna* exposed to different Cu concentrations in a 12-hr period. The inset specially shows the DCFs at higher exposure concentrations. Data are the mean ± standard deviation ($n = 3$).

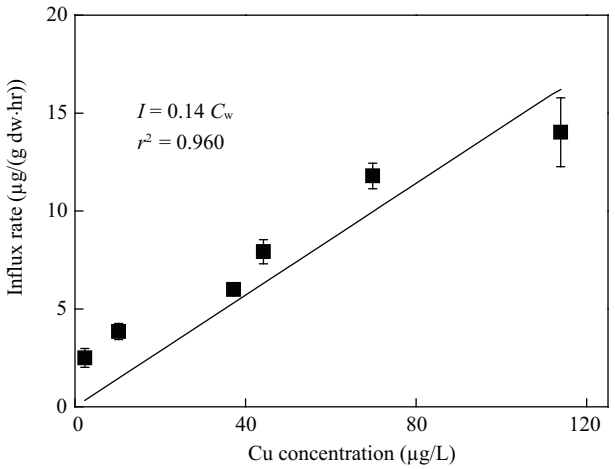

Fig. 2 Relationship between Cu exposure concentrations (C_w) and dissolved influx rate (I) of Cu in *Daphnia magna.* Data are the mean ± standard deviation (n = 3).

and a relative excess of metal dissolved was left in solution (Tsui and Wang, 2004a).

2.1.2 Influx rate of dissolved Cu and the uptake rate constant (k_u)

The relationship between the Cu influx rate from the dissolved phase and the exposure concentrations to *D. magna* is shown in Fig. 2. The power coefficient describing the relationship between the influx rate and its concentration was only 0.537 for Cu. The uptake rate constant (k_u) of Cu was therefore calculated as the slope of the linear regression between the influx rate and the Cu ambient concentration, with the assumption of a zero y-intercept (Yu and Wang, 2002a).

Clearly, there was a significant positive relationship between the dissolved Cu concentrations and the influx rate (r^2= 0.960, $p < 0.01$). Therefore, the uptake rate constant was 0.14 L/(g·hr) (3.36 L/(g·day)). The k_u (3.36 L/(g·day)) in our present study was higher than the k_u (1.32 L/(g·day)) obtained by the radioisotope tracer (Zhao et al., 2009). This might be due to different exposure solutions. The exposure medium to determine in the dissolved uptake in the previous study with ^{67}Cu was freshwater collected from an uncontaminated brook (pH 8.2, Ca^{2+} = 600 μmol/L, Mg^{2+} = 0.13 mmol/L, dissolved organic carbon (DOC) = 104 μmol/L, P = 0.28 μmol/L, SO_4^{2-} = 0.25 mmol/L), which had higher DOC than the SM7 experimental uptake media used in present experiments. Because of complexation between DOC and the bioavailable Cu species, the overall Cu uptake may have decreased (Kramer et al., 2004).

2.2 Cu assimilation

After 15 min of pulse feeding, the percentage of Cu retained in daphnids over the depuration period at different food concentrations is shown in Fig. 3. The ingested Cu was lost rapidly during the first 24 hr, followed by a gradual loss. The calculated assimilation efficiency (AE) at different algae concentrations are shown in Table 1.

Obviously, there was a significant decrease among Cu AEs at different food concentrations ($p < 0.05$, one-way ANOVA). With the increase of food concentration from

Table 1 Assimilation efficiencies (AE) of Cu in *D. magna* at different algae concentrations

Food concentration (cells/mL)	2×10^4	5×10^4	1×10^5
AE (%)	45.9 ± 2.6	27.5 ± 5.7	10.9 ± 2.1

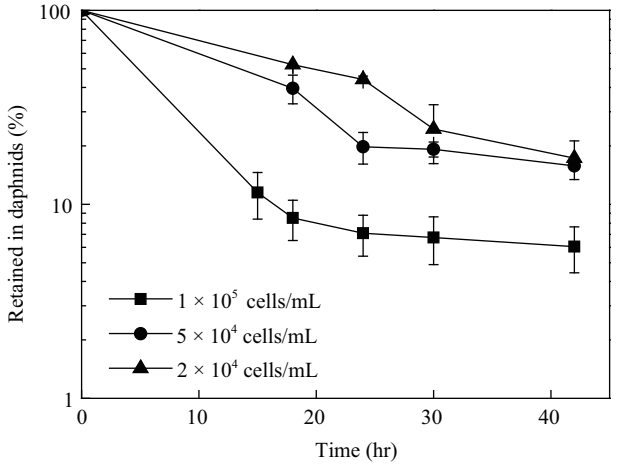

Fig. 3 Percentage of Cu retained in *D. magna* over the depuration period at different food concentrations. Data are mean ± standard deviation (n = 3).

2×10^4 to 1×10^5 cells/mL, the ingestion rate (IR) increased from 0.68 to 0.95 g/(g·day), and the AE decreased from (45.9 ± 2.6)% to (10.9 ± 2.1)%.

For the same type of food (*C. reinhardtii*), the decrease of AE with increasing food concentrations was also observed with other metals such as Cd, Cr, Se, Zn, Hg and MeHg (Yu and Wang, 2002a; Tsui and Wang, 2004a). Urabe and Watanabe (1991) suggested that at low food concentrations, IR was an important factor affecting the AEs in daphnids. In addition, Xu and Wang (2001) suggested that, at low food concentrations, the food particles may stay longer in the gut, which may then lead to a higher AE.

By comparison, when the food (*C. reinhardtii*) concentration increased from 2×10^4 to 1×10^5 cells/mL, the AEs obtained by radioisotope tracer decreased from (26.6 ± 2.9)% to (15.9 ± 2.2)% (Zhao et al., 2009), which was within the range of our present measurements (45.9% ± 2.6% to 10.9% ± 2.1%). Given the differences in the exposure medium (freshwater collected from an uncontaminated brook versus SM7 medium), this difference between the two studies was considered acceptable.

2.3 Efflux of Cu

After 3 days of aqueous or dietary accumulation of Cu, the daphnids continuously released Cu over the following 3.5 days of depuration (Fig. 4). The Cu release from the daphnids was faster during the first 2 days of depuration, followed by a gradual loss for both exposures (0.79 day^{-1} versus 0.52 day^{-1} for dissolved uptake and 0.95 day^{-1} versus 0.32 day^{-1} for dietary uptake). At the end of 3.5 days, the percentage of waterborne and dietborne Cu retained in daphnids was (10.2 ± 0.9)% and (8.0 ± 1.0)%, respectively.

The efflux rate constant (k_e) of Cu was calculated from

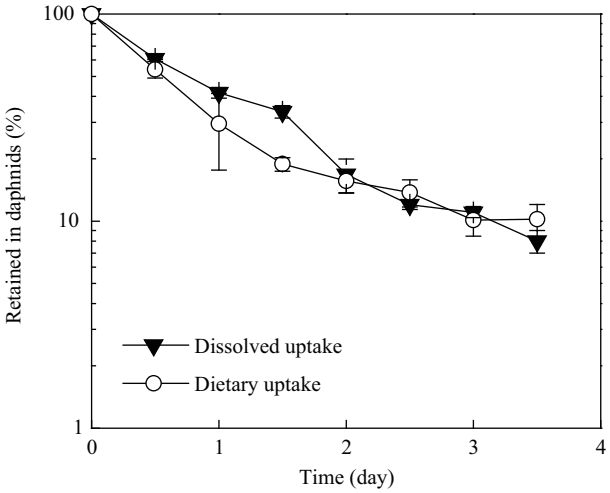

Fig. 4 Retention of Cu in *D. magna* during 3.5 days depuration at the food concentration of 10^5cells/mL following 3 days of water and dietary exposure. Data are the mean ± standard deviation (n = 3).

the period of slower loss (2–3.5 days). The waterborne Cu efflux rate constant (k_{ew}) was (0.52 ± 0.06) day^{-1} and the biological retention half life ($t_{1/2}$) was (1.34 ± 0.16) days, whereas the efflux rate constant of dietborne Cu (k_{ef}) was (0.32 ± 0.04) day^{-1} and the biological retention half life ($t_{1/2}$) was (2.2 ± 0.2) days. Indeed, there was no significant difference (one-way ANOVA, $p \approx 1$) in the last four time points for both exposure treatments (Fig. 4).

Previous studies suggested that food concentration had no significant influence on metal efflux in daphnids (except for Se) (Tsui and Wang, 2007), which is consistent with our results. Additionally, Chang and Reinfelder (2002) found that there was no significant difference between k_e of waterborne and dietborne Cu in marine copepods fed on diatoms. They inferred that the efflux of Cu was mainly controlled by the physiological conditions, whereas the food type or route of exposure did not have much influence on Cu efflux.

However, a higher k_e from the waterborne pathway was found for Cd, Cr (daphnids) and Zn (marine copepod), in contrast to that previously observed for Ag (daphnids, marine copepod), and for Cd and Co (marine copepod) (Lam and Wang, 2006; Yu and Wang, 2002b; Wang and Fisher, 1998). There were no significant differences in the efflux rates of Se and Zn in *D. magna* following aqueous and dietary uptake (Yu and Wang, 2002b). Yu and Wang (2002b) found that the relative contributions of metal loss from daphnids through molt, offspring release (neonates), regeneration into the water and fecal egestion were different between aqueous and dietary exposure. Ng and Wang (2005) also suggested that the subcellular distribution of metals was dependent on the exposure route in mussels. In general, metal partitioning was more dynamic during dissolved exposure than during dietary exposure. Consequently, the differences in k_e between different species as well as between different metals might be due to differing distributions of metals in tissues.

Although the k_e values determined by stable and radioactive tracer were of the same order of magnitude, our k_e values (0.32–0.52 day^{-1}) were higher than those measured by radioisotope tracer (0.19–0.20 day^{-1}) (Zhao et al., 2009). This might be due to the different exposure methods. To calculate the k_{ew} and k_{ef}, waterborne and dietborne exposures were carried out separately, whereas the combined waterborne and dietborne exposure was used to determine a single k_e in the radioisotope experiment (Zhao et al., 2009). The combined exposure might cause a more complicated subcellular distribution resulting in a slower efflux. This hypothesis requires additional experiments for verification. In addition, different batches of daphnids and algae may further complicate the comparison between the two studies.

Radioisotope analysis is a non-destructive method, which reduces the experimental error caused by individual differences. For the stable isotope analysis method, several daphnids were collected and digested at each timepoint, so the variation of the experimental results may be larger than that of radioisotopes. Despite the relatively larger variation, the quantification with stable isotope was still acceptable. Moreover, access to stable isotopes is much easier and more convenient than for the radioactive isotope, especially for Cu. Therefore, the stable isotope (^{65}Cu) is a good alternative to the radioactive one (^{67}Cu) in quantifying the biokinetics and bioaccumulation of Cu in daphnids.

3 Conclusions

For the first time, we successfully determined the accumulation and efflux of Cu in daphnids using a stable isotope tracer technique. In this study, all of the parameters were well quantified and were comparable to those obtained by a radioisotope method. Although some shortcomings exist, the stable isotope tracer, which is non-radioactive, provides an alternative to the radioisotope, especially for Cu biokinetics.

Acknowledgments

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