Environmental Science & Technology

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Measuring Metal Uptake and Loss in Individual Organisms: A Novel Double Stable Isotope Method and its Application in Explaining Body Size Effects on Cadmium Concentration in Mussels

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Cite This: https://doi.org/10.1021/acs.est.1c01582

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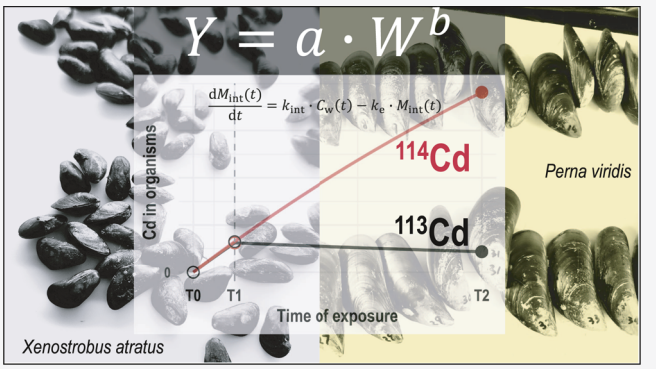
ABSTRACT: Interindividual variabilities in metal bioaccumulation confound our interpretation of the biomonitoring data. Measuring metal toxicokinetics in organism "individuals" may provide insights into the processes underlying the variabilities. Therefore, we developed a double stable isotope method that can simultaneously measure uptake and elimination of metals in individual organisms and thus the distribution of the toxicokinetic parameters. Specifically, we exposed organisms to both isotopes (^{113}Cd and ^{114}Cd; Cd = cadmium) during the first stage and to only one isotope (^{114}Cd) during the second stage. Metal uptake and elimination rate constants (i.e., k_u and k_e) were simultaneously estimated from the content of the two isotopes measured in each organism at the end of the second stage. We applied the method to investigate the interindividual variability in Cd concentrations caused by body size in two marine mussel species. Cd concentrations are higher in larger *Xenostrobus atratus* but lower in smaller *Perna viridis*. Size-dependent Cd uptake is found to be responsible for size effects on Cd concentrations in the mussels and the interspecies differences in the relationship between Cd concentration and body size. Specifically, Cd k_u increases with size in *X. atratus* (0.057−0.297 L g^{-1} d^{-1}) but decreases with size in *P. viridis* (0.155−0.351 L g^{-1} d^{-1}). In contrast, Cd k_e is not influenced by body size (*X. atratus*: 0.002−0.060 d^{-1}; *P. viridis*: 0.008−0.060 d^{-1}). Overall, we extended the applicability of the stable isotope methods to measure metal toxicokinetics in "individual" organisms, providing a readily available tool for investigating problems related to metal bioaccumulation.



KEYWORDS: *stable isotope, allometry, body size, toxicokinetics, cadmium, bivalve, biomonitoring*

1. INTRODUCTION

Monitoring the quality of coastal waters by measuring concentrations of contaminants, such as metals, in marine bivalves is widely practiced worldwide.[1] The validity of such monitoring programs relies on the assumption that there is a tight relationship between metal concentration in organisms and the level of metal contamination in the environment. However, such relationship is confounded by various factors (e.g., size, season, species, sex, and reproduction cycle), leading to substantial variabilities within and between populations and species and uncertainties in data interpretation.[1,2] Among the major confounding factors, the body size of organisms is the easiest to be measured and thus seems most promising to be corrected for.

However, the effects of body size can be complex.[3,4] For a given species, metal concentrations were usually lower in larger individuals.[3] The decrease of metal concentration with size may be explained by the mechanisms such as lower specific surface area and decreased metabolic rate with increasing size. This trend was often more obvious in essential elements (e.g., Cu, Zn) than in nonessential elements (e.g., As, Cd, and Pb).[3,5] Nonetheless, it is not uncommon to see metal concentrations increase or remain constant with increasing size, which remains unexplained by the abovementioned mechanisms. Many data have been accumulated on the effects of size; however, a unified framework to quantitatively explain (not to mention to predict) size effects is lacking. Multiple processes underlie the observed effects of size. The toxicokinetic model, which describes the multiple processes involved in bioaccumulation (e.g., uptake, elimination, and growth dilution), may be such a framework.[6−8]

In the toxicokinetic framework, to investigate interindividual variability, including that caused by body size, we need to measure the toxicokinetics in organism "individuals". This can

Received: March 10, 2021
Revised: June 16, 2021
Accepted: June 17, 2021

be achieved using a (γ-radiating) radioisotope tracer technique, which is nondestructive and can measure the same individual repeatedly, generating a time course of uptake and efflux.[8] However, in addition to the lack of suitable radioisotopes for many metals, the applications of radioisotope tracers are subject to restrictions due to safety issues and the need of disposal of radioactive wastes.[9,10] The accessibility of the technique and the scale of manageable experiments are subject to major limitation. The application of stable isotope tracers frees us from these restrictions; however, stable isotope methods have their own limitations.[10,11] The most conspicuous disadvantage of the stable isotope methods is that they are destructive, i.e., the organisms need to be sacrificed for measurements. Therefore, to use the traditional stable isotope method for generating a time course of metal uptake or loss, different individuals are sampled at different time points. It implicitly assumes all individuals are the same, leading to the loss of information on interindividual variability.

In this study, we propose a new double stable isotope method to measure metal toxicokinetics in individual organisms. Specifically, organisms will be exposed to one or two isotopes (^{113}Cd and ^{114}Cd) during different stages (Figure 1); the concentrations of these two isotopes will be measured

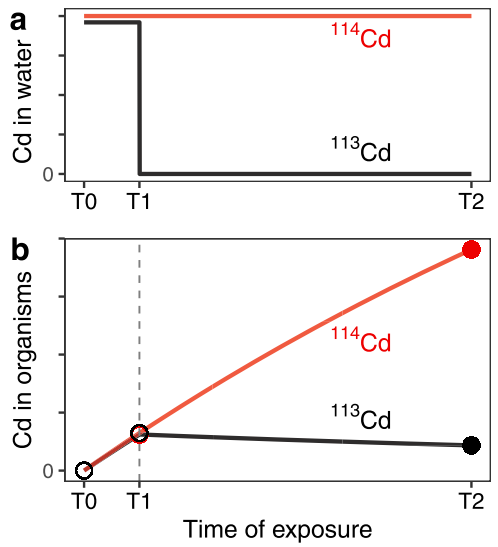


Figure 1. Experimental design for the double stable isotope method. (a) Mussels were exposed to both Cd isotopes (i.e., ^{113}Cd and ^{114}Cd) of similar concentrations until time *T*1 and then were exposed to only ^{114}Cd from time *T*1 to *T*2. (b) Content of ^{114}Cd in mussels was expected to increase continuously during the whole exposure period, whereas the content of ^{113}Cd was expected to increase during *T*0 and *T*1, and then to decrease during *T*1 and *T*2.

simultaneously in each organism. Such exposure design facilitates the full use of toxicokinetic information and enables the measurement of metal uptake and elimination simultaneously in each individual organism. Two marine mussels with different Cd concentration−body size relations are investigated: *Xenostrobus atratus* and *Perna viridis*. The black-pigmy mussel *X. atratus* is widely distributed in coastal waters of east Asia and often exists as a dominant species in the intertidal zone. Our previous study has demonstrated that Cd concentration in *X. atratus* is positively related to its body size.[12] The green mussel *P. viridis* is an important commercial species in southern China and Southeast Asia; and a negative correlation between Cd concentration and body size was observed in this species.[13] We have two major objectives in this study: (1) to develop a double stable isotope method capable of measuring Cd toxicokinetics in individuals of marine mollusks and (2) to investigate body size effects on Cd concentrations and to explain the interspecies differences, using the toxicokinetics obtained with the double isotope method.

2. MATERIALS AND METHODS

2.1. Organisms and Materials. To investigate the relationship between tissue Cd concentrations and organism body size, two mussel species were collected from eight intertidal sites around Xiamen (see Supporting Information (SI) Figure S1 for the map of sampling sites). Efforts were made to collect mussels spanning a wide range of sizes at each sampling site: *X. atratus* from six sites ranged in shell length from 4.1 to 15.6 mm; *P. viridis* from two sites had a shell length from 10.6 to 50.0 mm (Figure S2). The soft tissues in these mussels were separated from the shells, rinsed with deionized water, freeze dried, weighed, and determined for Cd concentrations (*cf.* Section 2.3).

To measure Cd toxicokinetics in individual mussels of different sizes, additional *X. atratus* (shell length 4.9−12.5 mm) and *P. viridis* (shell length 11.4−50.0 mm) were collected from pillars of Yanwu Bridge (site 3, 24.4380°N, 118.1058°E) and from waters around Dadeng Island (site 7, 24.5528°N, 118.3790°E), respectively. Before being used in experiments, the mussels were acclimated for 2 weeks to experimental conditions, i.e., 22 ± 1 °C, salinity 30, and 14 h light:10 h dark cycle. During acclimation, the mussels were fed daily with the green alga *Chlorella* sp. (Cd < 0.1 μg g^{-1} dry weight).

Seawater (salinity 30, pH 8.0, Cd around 0.03 μg L^{-1}) was collected from Tong'an Bay (24.5669°N, 118.1925°E), Xiamen. Seawater used for measuring Cd toxicokinetics was filtered through a 0.22 μm mixed cellulose ester membrane. Cd exposure solutions were prepared by adding stable isotopes ^{113}Cd and ^{114}Cd (ISOFLEX, San Francisco; see Table S1 for more information) into seawater to target concentrations, and were equilibrated overnight before use.

2.2. Cd Toxicokinetics Determination Using the Double Isotope Method. A specially designed double isotope exposure was used to simultaneously determine Cd uptake and elimination in individual mussels (Figure 1). The exposure was divided into two stages. During the first stage (*T*0−*T*1), the mussels were exposed to both ^{113}Cd and ^{114}Cd; during the second stage (*T*1−*T*2), the mussels were exposed only to ^{114}Cd of the same concentration but not to ^{113}Cd. From *T*1 to *T*2, we expected to see a decrease of ^{113}Cd and a steady increase of ^{114}Cd in the mussels. For *X. atratus*, the nominal exposure concentrations of ^{113}Cd and ^{114}Cd were both 3.0 μg L^{-1}; *T*1 and *T*2 were 2 and 14 d, respectively. For *P. viridis*, the nominal concentrations of ^{113}Cd and ^{114}Cd were both 2.5 μg L^{-1}; *T*1 and *T*2 were 0.42 d (i.e., 10 h) and 7.42 d (i.e., 178 h), respectively. The reason for using different *T*1, *T*2, and Cd isotope concentrations in the two species was the much smaller size of *X. atratus* than *P. viridis*; longer *T*1 and higher isotope concentrations were required to elevate Cd isotopes in *X. atratus* tissues for reliable analysis. The Cd concentrations used here (2.5−3.0 μg L^{-1}) were substantially lower than the no-effect concentration of Cd (34.9 μg L^{-1}) observed in the clam *Potamocorbula laevis* under similar conditions (salinity 30)[14] and were also lower than the

https://doi.org/10.1021/acs.est.1c01582
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estuarine/marine criterion continuous concentration of Cd (7.9 μg L^{-1}) recommended by the United States Environmental Protection Agency.[15]

A flow-through system was used to maintain constant concentrations of Cd isotopes in the exposure solutions (Figure S3). The exposure solution was stirred by magnetic stirrers and continuously renewed using peristaltic pumps at a flow rate that would renew the solution once every 24 h. The mussels were placed in a perforated polypropylene box or on a nylon mesh hanging in the solution to avoid being interfered by the stirrers. To avoid adsorption of Cd isotopes onto food particles and the introduction of dissolved organic matter that may affect Cd bioavailability, the mussels were taken out and fed *Chlorella* sp. in clean seawater for 40 min daily.

Before the commencement of exposure, 20 unexposed *P. viridis* and 15 unexposed *X. atratus* were dissected to determine the initial Cd concentrations. To start the exposure, 145 individuals of *X. atratus* were placed in 6 L of test solution, whereas 160 individuals of *P. viridis* were placed in 12 L of test solution. Seventy *X. atratus* and 80 *P. viridis* were sampled at time *T*1; the remaining individuals were sampled at time *T*2. The sampled mussels were rinsed immediately with 1 mmol L^{-1} EDTA (pH 8.0) to terminate Cd uptake. They were then measured for shell length and shell height and dissected; the soft tissues were washed twice in 1 mmol L^{-1} EDTA and twice in deionized water (18.2 MΩ cm), placed in clean ziplock plastic bags and freeze dried. Water samples were also collected at intervals of 3−12 h to monitor concentrations of Cd isotopes in the test solutions. The water samples were acidified by adding 100 μL of "1 + 1" HNO_3 (7.3 mol L^{-1}, prepared by mixing one volume of concentrated HNO_3 with one volume of deionized water) per 10 mL of sample.

2.3. Chemical Analysis. The dried soft tissues were individually weighed to the nearest 1 μg (Mettler Toledo XP6) for *X. atratus* and 0.1 mg for *P. viridis*. They were then digested in 65% HNO_3, first at room temperature overnight and then at 80 °C for 8 h. Concentrations of ^{111}Cd, ^{113}Cd, and ^{114}Cd in mussel samples and water samples were measured using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x). For the ICP-MS analysis, the mussel samples were diluted to have a final HNO_3 concentration around 2%, and the water samples were diluted 10 times with 2% HNO_3. Matrix effects and instrumental drift were corrected using an internal standard ^{103}Rh. Stability of the instrument performance was checked by measuring a quality control sample after every 20 measurements. The standard reference material (SRM 2976, mussel tissue) was digested and analyzed following the same procedures as tissue samples; measurements were considered acceptable when measured concentrations of ^{111}Cd, ^{113}Cd, and ^{114}Cd of the SRM fell within 90−110% of the certified values.

The concentrations of newly accumulated ^{113}Cd and ^{114}Cd in individual mussels were calculated using the following equations[14]

$$\text{new}[^{113}\text{Cd}] = ([\text{total Cd}]_{113} - [\text{total Cd}]_{111}) \times 12.22\% \quad (1)$$

$$\text{new}[^{114}\text{Cd}] = ([\text{total Cd}]_{114} - [\text{total Cd}]_{111}) \times 28.73\% \quad (2)$$

where $[\text{total Cd}]_{111}$, $[\text{total Cd}]_{113}$, and $[\text{total Cd}]_{114}$ are total Cd concentrations reported by the ICP-MS instrument, when selecting the isotopes ^{111}Cd, ^{113}Cd, and ^{114}Cd as the analyte, respectively. It should be noted that, although the values measured by ICP-MS were the "real" concentrations of each selected isotope, the reported values were the concentrations of "total Cd" because the calibration standards (Agilent, part number 5183-4688) were not prepared with pure isotopes but were a mixture of Cd isotopes of natural relative abundance (Table S1). As a result, the reported total Cd concentration is the real concentration of the isotope divided by its relative isotopic abundance in the calibration standard (12.22 and 28.73% for ^{113}Cd and ^{114}Cd, respectively). ^{111}Cd is an unspiked isotope; the reported ^{111}Cd concentration thus indicates the concentration of background Cd in mussels.

In short, "$[\text{total Cd}]_{113} \times 12.22\%$" and "$[\text{total Cd}]_{114} \times 28.73\%$" are the current concentrations of ^{113}Cd and ^{114}Cd in mussel tissues after exposure; "$[\text{total Cd}]_{111} \times 12.22\%$" and "$[\text{total Cd}]_{111} \times 28.73\%$" are the background concentrations of ^{113}Cd and ^{114}Cd existing before exposure, respectively. The concentrations of the newly accumulated ^{113}Cd and ^{114}Cd are thus calculated by subtracting the "background concentrations" from the "current concentrations". More detailed explanations on stable isotope data analysis are available in our previous paper.[14]

2.4. Toxicokinetic Modeling and Data Analysis. A one-compartment first-order toxicokinetic model was used to describe Cd bioaccumulation processes in the mussels (Figure S4). The variation of Cd mass in each mussel is a balance between Cd uptake and elimination[6,7]

$$\frac{dM_{int}(t)}{dt} = k_{int} \times C_w(t) - k_e \times M_{int}(t) \quad (3)$$

where $M_{int}(t)$ (μg) is the mass of ^{113}Cd or ^{114}Cd in mussels at time t; $C_w(t)$ (μg L^{-1}) is the concentration of ^{113}Cd or ^{114}Cd in the exposure solution, k_{int} (L d^{-1}) is the internalization rate constant, and k_e (d^{-1}) is the elimination rate constant. Accordingly, the concentrations of Cd isotopes [$C_{int}(t)$, μg g^{-1}] in mussel tissues and the uptake rate constant (k_u, L g^{-1} d^{-1}) of Cd are calculated as

$$C_{int}(t) = M_{int}(t)/W \quad (4)$$

$$k_u = k_{int}/W \quad (5)$$

where W (g) is the dry weight of soft tissues of individual mussels.

The values of k_{int} and k_e were obtained by simultaneously fitting eq (3) to the contents of both ^{113}Cd and ^{114}Cd in each mussel measured at the end of the exposure (i.e., time *T*2). The best-fit parameter values were estimated using the quasi-Newton least-squares method included in the R package "FME".[16] The detailed procedures of data analysis and parameter estimation are provided in the Supporting Information as R code (Note S1).

A power equation, or equivalently, a linear equation after log-transformation was employed to describe the effects of body size on Cd concentrations and toxicokinetics in mussels[4,17]

$$Y = aW^b \quad (6)$$

$$\log Y = \log a + b \log W \quad (7)$$

where Y represents either Cd concentrations or Cd toxicokinetic parameters (i.e., k_u and k_e) in organisms and a and b are empirical constants. The variable Y is considered influenced by the body size W if the values of b are significantly

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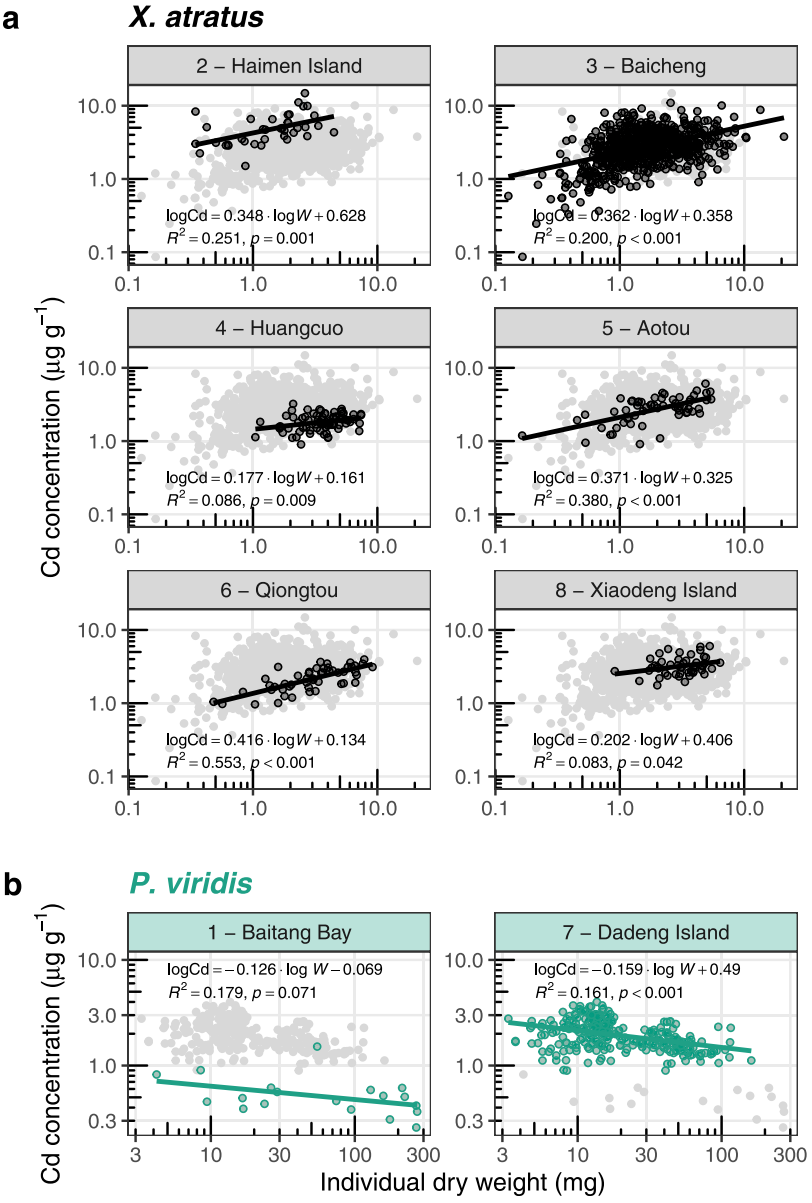

Figure 2. Body size effects on tissue Cd concentrations differ between the two mussel species. (a) Cd concentrations increased with individual dry weight in *X. atratus* collected from six sites. (b) Cd concentrations decreased with individual dry weight in *P. viridis* collected from two sites. Each point represents one individual mussel; the gray points in the background represent individuals collected from all sites. See Figure S1 for the map of sampling sites.

different from 0 (with $p < 0.05$). All statistical analyses, including linear regression and *t*-test, were conducted using R (v4.0.3). The phylogenetic tree was generated using the R packages "rotl" and "ggtree".[18,19]

3. RESULTS

3.1. Body Size Effects on Cd Concentration in Field-Sampled Mussels. The relationship between Cd concentration and body size differed between the two mussel species (Figure 2). In *X. atratus* from all six sites, Cd concentrations ranged from 0.1 to 14.8 $\mu g\ g^{-1}$ ($3.0 \pm 1.6\ \mu g\ g^{-1}$, $n = 1061$) and increased significantly with increasing body size ($b = 0.177-0.416$, Figure 2a). In *P. viridis*, Cd concentrations varied within a much narrower range, i.e., 0.3−4.0 $\mu g\ g^{-1}$ ($1.9 \pm 0.7\ \mu g\ g^{-1}$, $n = 292$). Cd concentrations tended to be lower in larger *P. viridis*, although such trend was only significant at one of the two sampling sites ($b = -0.126$ and -0.159, Figure 2b).

3.2. Cd Toxicokinetics. In the double isotope exposure, the mussels were exposed to ^{113}Cd and ^{114}Cd in the first stage but only to ^{114}Cd in the second stage (Figure 3a). The flow-through system design ensured stable exposure concentrations of Cd isotopes. The measured exposure concentrations were slightly lower than the nominal values, which was partly due to the adsorption loss onto containers and mussel shells. The measured concentrations rather than the nominal values were used in the estimation of Cd toxicokinetics.

As expected, ^{114}Cd content in mussels increased continuously throughout the exposure (from *T*0 to *T*2), whereas ^{113}Cd increased across the first exposure stage (*T*0 to *T*1) and decreased during the second stage (*T*1 to *T*2) (Figure 3b). The overall Cd k_e, estimated from the decrease of median ^{113}Cd from *T*1 to *T*2, were 0.012 and 0.024 d^{-1} in *X. atratus* and *P. viridis*, respectively. These two values were used as the initial values to estimate k_e of individual mussels (see Note S1

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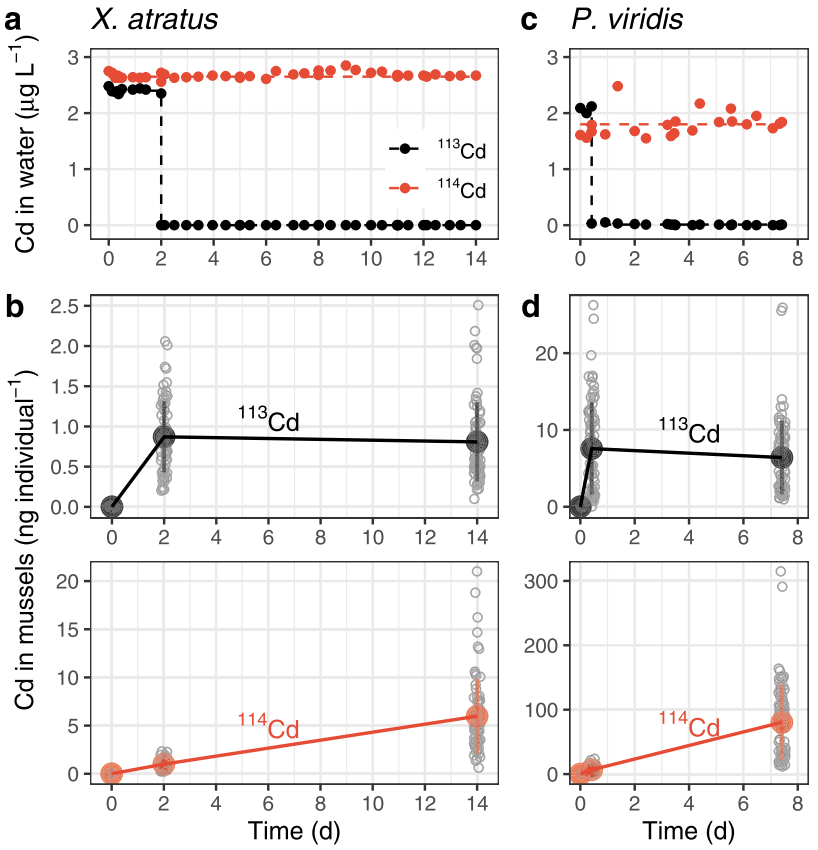

Figure 3. (a, c) Measured concentrations of ^{113}Cd and ^{114}Cd upon exposure to seawater. (b, d) Content of newly accumulated ^{113}Cd and ^{114}Cd in mussels *X. atratus* and *P. viridis* during the exposure; the solid points and error bars are mean values and standard deviations, respectively; the open circles are individual values.

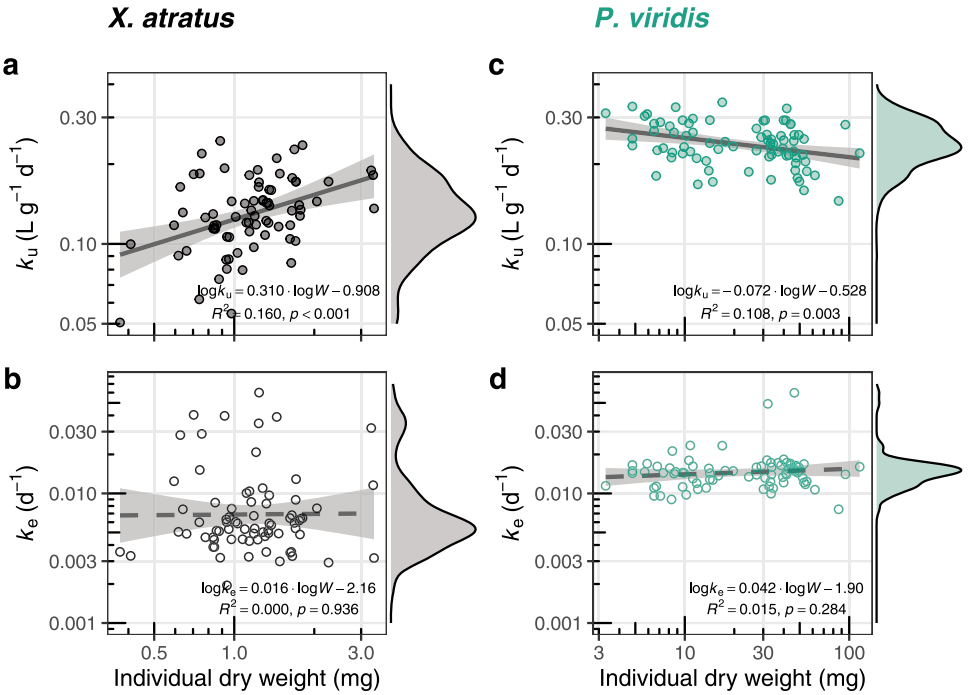

Figure 4. Relationship between Cd toxicokinetics and body size (i.e., dry weight of soft tissue) in the two mussel species. k_u and k_e are the uptake rate constant and elimination rate constant of Cd, respectively. Each point represents one individual mussel; the shaded areas on the right of each panel show the parameter distributions.

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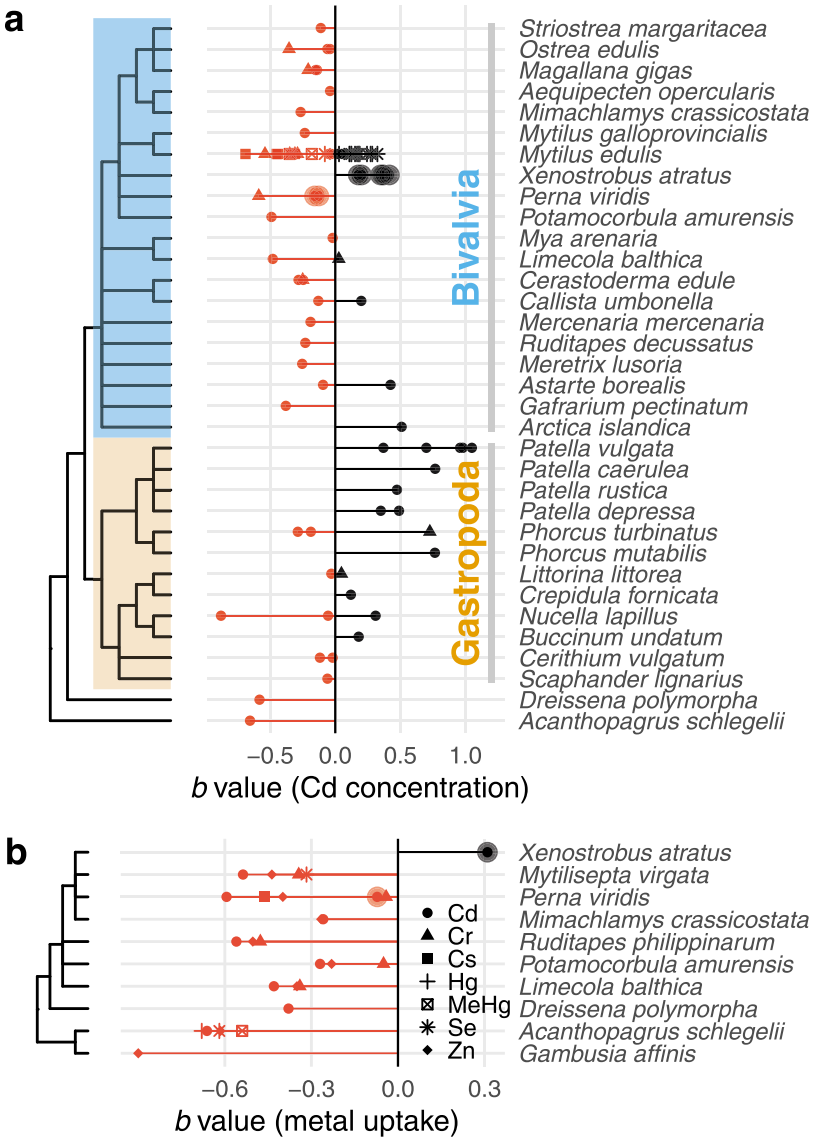

Figure 5. Comparison of b values of different species organized in a phylogenetic tree. (a) Body size effects on tissue Cd concentration; for each species, different symbols represent different studies. (b) Body size effects on the uptake rate of metals. The larger points indicate b values of *X. atratus* and *P. viridis* measured in this study. See eqs (5) and (6) for the definition of b; see Tables S2 and S3 for the references from which b values were compiled.

for R code). The small k_e values are consistent with the almost linear increases of ^{114}Cd in mussels (i.e., without obvious leveling off) throughout the whole exposure period. The growth of the mussels and concentrations of Cd isotopes in the mussels are shown in Figures S5 and S6, respectively.

The uptake and elimination rate constants of Cd (k_u and k_e) in each individual mussel were simultaneously estimated from the double isotope exposure. The one-compartment model (eq 3) well fitted the content of ^{113}Cd and ^{114}Cd newly accumulated in the mussels (Figure S7). The values of k_u were 0.136 ± 0.042 L g^{-1} d^{-1} (mean ± standard deviation) in *X. atratus* (range: 0.051−0.245 L g^{-1} d^{-1}, Figure 4a), significantly lower than that measured in *P. viridis*, which were 0.242 ± 0.044 L g^{-1} d^{-1} (range: 0.145−0.342 L g^{-1} d^{-1}, Figure 4c) (t-test, $p < 0.001$). Values of k_e were also significantly lower in *X. atratus* (0.010 ± 0.011 d^{-1}, range: 0.002−0.060 d^{-1}) than in *P. viridis* (0.015 ± 0.007 d^{-1}, range: 0.008−0.060 d^{-1}) (t-test, $p < 0.001$, Figure 4b,d).

3.3. Body Size Effects on Cd Toxicokinetics. The relationship between Cd uptake rate constant k_u and body size showed opposite trends in the two mussel species. In *X. atratus*, k_u increased significantly with tissue weight ($p < 0.001$, $b = 0.310$, Figure 4a), whereas in *P. viridis*, k_u decreased significantly with tissue weight ($p = 0.003$, $b = -0.072$, Figure 4c), indicating higher Cd uptake rates in larger *X. atratus*, but lower uptake rates in larger *P. viridis*. In contrast, the Cd elimination rate constant k_e remained unaffected by the body size in both mussels ($p > 0.05$, Figure 4b,d).

4. DISCUSSION

Body size effects on organism metal concentrations manifest in multiple toxicokinetic processes, including aqueous uptake, dietary assimilation (feeding rate, assimilation efficiency), elimination, and growth dilution. The observed effects on metal concentration can thus be decomposed into size effects on different toxicokinetic parameters, all of which can be quantified. Measurements of these parameters in individuals

https://doi.org/10.1021/acs.est.1c01582
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(rather than in groups) of different sizes either provide direct explanation or provide clues for further explanations at the physiological and molecular levels.

Measuring toxicokinetics in individual organisms is realized in this study using a double stable isotope method with a special exposure design (Figure 1) and the associated procedures of data analysis (Note S1). It should be mentioned that double (or triple) stable isotope methods had been applied to simultaneously measure metal uptake and elimination (neglecting the interindividual variabilities),[20] and to measure metal uptake from different routes (sediment, food, water);[21,22] however, measuring toxicokinetics in organism individuals had never been resolved.

4.1. Body Size Effects. Multiple mechanisms underlie the observed effects of body size on metal concentration in aquatic organisms. Some mechanisms cause higher concentration in smaller individuals, such as higher surface area (particularly gill surface area) to weight ratio, higher weight-specific filtration rate, and higher metabolic rate, which all may lead to higher uptake rate.[3,8,23] On the other hand, the size is associated with age and thus the length of exposure, which may lead to higher concentrations in larger individuals. Multiple other mechanisms may also lead to higher metal concentrations in either smaller or larger individuals, depending on how these mechanisms are related to the size in each specific case; such mechanisms include feeding preference, synthesis of metal binding molecules (e.g., metallothionein), morphological allometry (e.g., ratio of muscle to viscera), and so on.[3,21] For a given metal and species, the combination of these multiple mechanisms may lead to different directions and magnitudes of size effects.

With the one-compartment toxicokinetic model, we are able to separate, at least partially, the multiple mechanisms (e.g., specific surface area, age), and thus understand and even predict size effects. To simplify the discussion, we assume that the organisms are exposed to a constant concentration of dissolved metal and show an exponential growth in tissue weight. Metal concentration in organisms thus may be expressed as[7]

$$C_{int}(t) = C_0 \cdot e^{-(k_e+g)\cdot t} + \frac{k_u \cdot C_w}{k_e + g}[1-e^{-(k_e+g)\cdot t}] \quad (8)$$

where C_0 is the initial concentration of metal in the organisms and g is the growth rate constant (d^{-1}). At steady state, i.e., $dC_{int}(t)/dt = 0$ (see Figure S4b), metal concentration in organisms would be in an equilibrium with the surrounding environment and can be calculated as

$$C_{ss} = \frac{k_u \cdot C_w}{k_e + g} \quad (9)$$

Combining eqs (8) and (9), we can calculate the exposure time required for the organisms to approach the equilibrium (e.g., reaching 95% of C_{ss}; assuming $C_0 \leq 0.95C_{ss}$)

$$T_{95} = \frac{\ln[20(C_{ss}-C_0)/C_{ss}]}{k_e + g} \quad (10)$$

As discussed above, larger individuals may show slower metal uptake rates (with *X. atratus* as an exception, see the discussion in Section 4.1.2) but may have been exposed to metals for longer periods. These two effects on metal concentration show opposite directions; and their relative importance may vary among species. The species with faster metal efflux (i.e., larger k_e) and faster growth (i.e., larger g) requires less time to reach equilibrium (T_{95}, see eq (10)). Therefore, further exposure would not substantially increase the metal concentration in these species, since the organisms are already close to equilibrium with the environment. In other words, in these species the "slow-uptake effect" offset the "long-exposure effect", leading to a higher probability of seeing negative b (i.e., lower metal concentrations in larger individuals, see eqs (6) and (7)). In contrast, in the species with slower growth and slower metal efflux, reaching equilibrium takes longer time (i.e., larger T_{95}). In these species, "long-exposure effect" may overweigh the "slow-uptake effect" and thus lead to a positive b (i.e., higher metal concentrations in larger individuals).

4.1.1. Effects on Cd Concentration. Both the values and signs of the parameter b (in eq 7), which relates Cd concentrations to body size on a double logarithmic scale, may vary among species and even within a species. We compare the b values measured in our study with those previously reported, with a particular focus on marine mollusks (Figure 5a).

The positive b we observed in the mussel *X. atratus* is uncommon among bivalves (Figure 5a). Interestingly, in another two bivalve species (i.e., *Arctica islandica*, *M. edulis*), positive b was also observed, likely as a result of slow growth of these organisms. Specifically, the mussels *M. edulis* with b greater than 0.1 were collected from the cold waters in Arctic,[24] where mussels grow much slower than those in lower latitudes. The other species with positive b (clam *A. islandica*) was also collected from the high latitude region (Kiel Bay, Germany, 50.52°N), and this clam is known as one of the slowest growing bivalves.[5] However, slow growth may not be the explanation of the positive b in *X. atratus*, although we do not have growth rate information of this species in the field.

The chance to find positive b values in gastropods (10 positive vs 5 negative) is higher than in bivalves (6 positive vs 18 negative, Figure 5a). The differences in feeding strategy between the two groups may explain this. Specifically, bivalves are filter feeders; larger individuals usually have lower weight-specific filtration rate and thus are less efficient in metal uptake from water.[25−27] In contrast, gastropods are not filter feeders, and thus may not be affected by such size-dependent filtration rate. Larger-sized individuals, including bivalves and gastropods, show longer gut retention time and thus may have higher efficiency in assimilating metals from food. Indeed, the assimilation efficiency was found to be positively related to the size for Cd and Zn in the scallop *Chlamys nobilis*,[25] and for Cu in the fish *Oryzias melastigma*.[28] This mechanism of size-dependent dietary assimilation efficiency may have played a more important role in gastropods than in bivalves, and leads to higher probability of positive b values in the gastropod group.

4.1.2. Effects on Cd Uptake. Values of b are predominantly negative in different combinations of species and metals when assessing the relation between the metal uptake rate and the body size (Figure 5b), indicating higher metal uptake rates in smaller organisms. In fact, the positive b value that we observed in *X. atratus* is currently the only record of positive b to our knowledge. The underlying mechanism remains unclear. It contradicts the simplistic predictions based on surface area or filtration rate that all predict negative b. Less perceptible mechanisms, such as physiological and morphological allometry favoring faster uptake of Cd in larger *X. atratus*,

https://doi.org/10.1021/acs.est.1c01582
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may exist; however, this remains speculative and warrants further investigation.

The relations between Cd uptake rate constant (k_u) and body size derived in laboratory exposures (Figure 4) agreed with the relations between tissue Cd concentration and body size in both *P. viridis* and *X. atratus* sampled from the field (Figure 2), suggesting that size-dependent uptake rate was responsible for size effects on tissue Cd concentration. To further examine the role of k_u in size effects in the two mussels, we conducted a multiple linear regression, using both tissue weight and k_u as predictors to predict Cd concentration (Table S4). In both species, only k_u was the significant predictor, while body size was not. It further suggests that size effects on tissue Cd concentration are mainly mediated by Cd uptake rate rather than other size-dependent mechanisms.

4.1.3. Effects on Cd Elimination. The independence of Cd elimination (k_e) on body size that we observed in the two mussels was not uncommon. There is much higher probability to find k_e being unaffected by body size than being affected for various metals including Cd. Below are some of the examples where k_e was unaffected by body size: Cd, Cr, and Zn in the clam *Potamocorbula amurensis*;[23] Cr and Zn in the clam *M. balthica*;[23] Co, Se, and Zn in the mussel *M. edulis*;[8] Cd, Pb, Cu, and Zn in the amphipod *Gammarus zaddachi*;[29] Cd, Se, and Zn in the fish *Acanthopagrus schlegelii*;[30] Hg in the fish *Gambusia affinis*;[31] and ^{137}Cs in seven marine species (crustaceans, mollusks, and fishes).[32]

Nonetheless, size-dependent metal elimination was occasionally observed. Among those cases, usually, slower efflux was observed in larger organisms, for example, Cd in *M. edulis*,[8] Zn in *G. affinis*,[33] and ^{137}Cs in *P. viridis*.[34] In contrast, faster Cd efflux was observed in larger *M. balthica*.[23]

Taken together, metal elimination is a toxicokinetic process less affected by size and may contribute less to size effects on metal concentration in organisms.

4.2. Double Stable Isotope Method. *4.2.1. Comparison to Other Methods.* The values of Cd k_u and k_e in the two mussels measured in this study, using the double stable isotope method, were similar to those previously measured using other methods. For *X. atratus*, we previously investigated their Cd toxicokinetics using a (single) stable isotope method, yet not individually but in groups of mussels.[12] Under similar conditions, k_u was previously estimated to be around 0.1 L g^{-1} d^{-1}, within the range measured here (k_u = 0.136 ± 0.042 L g^{-1} d^{-1}); values of k_e measured in this study (0.011 ± 0.011 d^{-1}, range: 0.002−0.060 d^{-1}) were slightly lower than but still overlapped the previous measurement of 0.029 d^{-1}. For *P. viridis*, the widely used biomonitor, k_u and k_e have been measured in numerous studies, all using the radioactive tracer method.[27,35−40] Under similar conditions (i.e., Cd concentration, salinity), k_u and k_e were measured to be 0.2−0.4 L g^{-1} d^{-1} and 0.01−0.03 d^{-1}, respectively, both close to what we measured in this study (i.e., k_u = 0.242 ± 0.044 L g^{-1} d^{-1} and k_e = 0.015 ± 0.007 d^{-1}).

4.2.2. Method Assumptions, Limitations, and Recommendations. The reliability and meaningfulness of the estimated toxicokinetic parameters using the double stable isotope method rely on the following two major assumptions among others:

(1) Metal elimination can be adequately described by a one-compartment model. The number of compartments can be discerned from the depuration curve on a semi-logarithmic plot, preferably generated using a radioactive tracer method. Two or more (rather than one) compartments are often found for metal loss from marine mollusks.[27,41,42] Despite this, a one-compartment model, as a simplification, can usually provide adequate prediction of metal bioaccumulation.[6,43] Using the double stable isotope method, the measured k_e is a mixture of the elimination rate constants from all compartments; k_e would decrease with the length of exposure since the initial loss is faster. It is thus desirable to have a long enough exposure (e.g., 2−4 weeks) to make the measured k_e better reflect the dominant compartment (i.e., the slow turnover compartment) and thus extrapolatable to the long-term exposures in the field.

(2) Metal uptake follows a first-order kinetics. First-order kinetics is usually a reasonable assumption when metal concentration is low or at environmentally realistic levels.[7] Given this, fluctuating metal concentration in exposure solution is acceptable for the estimation of toxicokinetics since the metal uptake rate is proportional to the dissolved metal concentration with a slope of k_u. Nonetheless, a steady exposure concentration is still favorable to avoid the need of intensive measurement of the aqueous concentration and the associated uncertainties of interpolation. Therefore, a flow-through system similar to that employed in this study is highly recommended. The flow-through system is also helpful to maintain stable water chemistry and to minimize the buildup of metabolites or feces.

With the special design (Figures 1 and S3) and the associated procedures of data analysis (Note S1), it is possible to measure metal toxicokinetics in individual organisms and thus to observe their distribution using the stable isotope method, which previously could only be achieved using radioactive tracers. Nevertheless, the double stable isotope method has several limitations:

(1) Like other stable isotope methods, the method we developed here relies on destructive sample analysis, i.e., the sacrifice of organisms; therefore, metal accumulation cannot be repeatedly monitored in the same individual over time. This method is thus less informative than the radioactive tracer method which can analyze non-destructively.[10]

Parameter estimation depends on the reliable analysis of the two spiked isotopes at $T2$. It thus should be noted that the amount of the isotope which was spiked during only the first stage of exposure (here ^{113}Cd) may be too small to be analyzed reliably if the lengths of $T1$ and $T2$ were not properly set. For example, for a metal with much higher k_e than Cd, it is desirable to increase the ratio of $T1$ to $T2$.

(2) The double stable isotope method requires two isotopes to spike and one more isotope for background subtraction; therefore, this method is only applicable to the elements with three or more stable isotopes, such as Cr, Fe, Ni, Zn, Se, Cd, Hg, and Pb. Several other environmentally important elements, including Mn, Co, Cu, As, and Ag, cannot be investigated using this method as they have only one or two stable isotopes.

(3) The experimental design in the present form considers only uptake of dissolved metals; however, it is possible

https://doi.org/10.1021/acs.est.1c01582
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to measure the dietary uptake rate of metals in individual organisms following a similar experimental design. Using Figure 1 to illustrate, the dissolved ^{113}Cd exposure may be replaced by dietborne ^{113}Cd exposure; meanwhile, ^{114}Cd exposure is started at $T1$ to avoid adsorption of ^{114}Cd onto food particles. The length of $T1$ should be long enough to ensure reliable analysis of ^{113}Cd, but should also be short enough to avoid substantial release of the dietborne ^{113}Cd into the solution.

4.3. Environmental Implications. In marine mollusks, the complex size effects on metal concentrations result from size effects on the multiple processes involved in metal bioaccumulation. For a given species, the variation of their relative importance may lead to opposite directions of size effects in different scenarios (e.g., cold vs warm climate). It is thus unlikely to apply the same size correction formula for different cases; however, a case-specific correction is still possible and is recommended. In comparison, the toxicokinetic parameters, as physiological traits of a species,[44] are more stable in relation to the body size. Therefore, better prediction of size effects can be made based on the knowledge of toxicokinetics and their relation to body size (together with the physicochemical information of the sampling site). For the mollusk species selected as a biomonitor, it is desirable to have thorough investigations of size effects on its metal toxicokinetics.

The double isotope method proposed in this study provides a readily available method for measuring metal toxicokinetics in "individual" aquatic organisms. In addition to the body size effects we investigated, measuring metal toxicokinetics can also be used as a tool to investigate various topics related to metal bioaccumulation, including metal bioavailability, toxicity, biomonitoring, water quality criteria, interspecies differences, and so on. Moreover, such measurements generate the distribution of the toxicokinetic parameters rather than a collective measurement averaged across individuals, providing opportunities to quantitatively define uncertainties in biomonitoring data, and revealing the interindividual variabilities upon which natural selection could act.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c01582.

Map of mussel sampling; flow-through exposure system; comparison of two versions of the toxicokinetic model; weight and shell length of mussels; ^{113}Cd and ^{114}Cd concentrations in mussels; model goodness of fit; natural abundance of stable Cd isotopes; literature b values (Cd concentration vs body size); literature b values (metal uptake rate vs body size); multiple linear regression results; and R code for estimating k_u and k_e from the double isotope experiment (PDF)

Original data of the double isotope experiment for measuring Cd toxicokinetics (XLSX)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Shiyue Zhang and Junlin Huang for their assistance in field sampling and experiments. This study was supported by the National Natural Science Foundation of China (41977347 and 42077372) and the Fundamental Research Funds for the Central Universities (20720190099).

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https://doi.org/10.1021/acs.est.1c01582
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Supporting information to:

Measuring metal uptake and loss in individual organisms: a novel double stable isotope method and its applications in explaining body size effects on cadmium concentration in mussels

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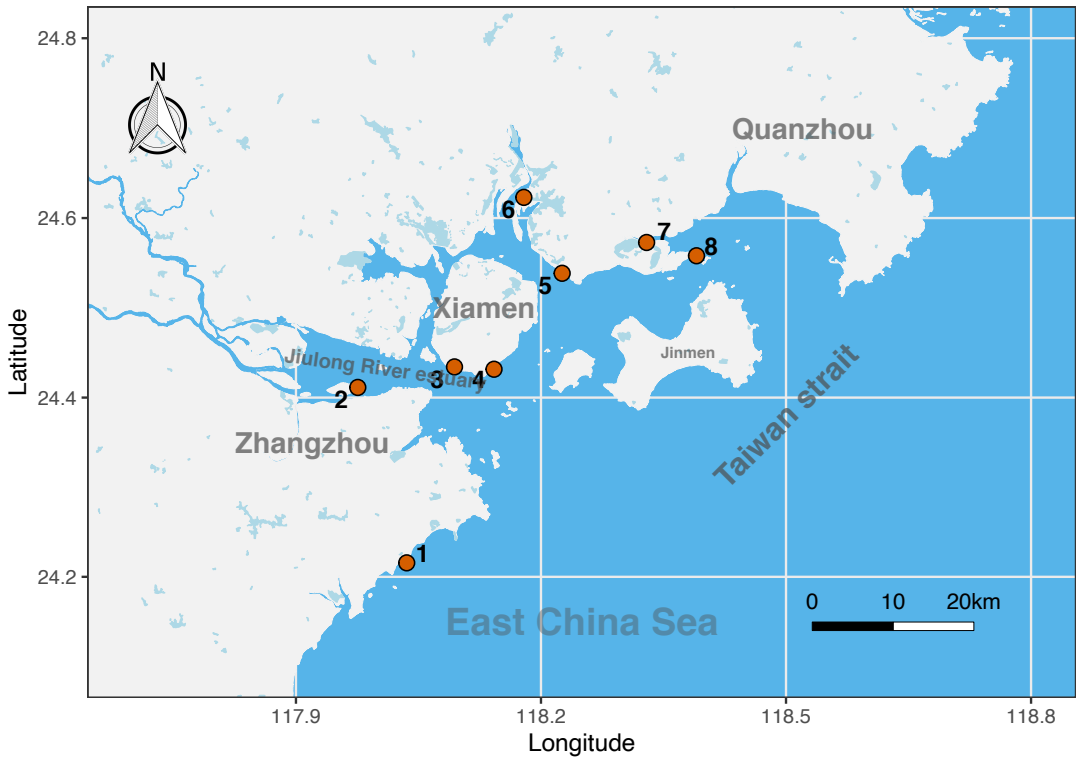


Figure S1. Sites of mussel sampling. The black-pigmy mussel *X. atratus* were sampled from 6 sites, including 2-Haimen Island (117.9757° E, 24.4113° N), 3-Baicheng (118.1058° E, 24.4380° N), 4-Huangcuo (118.1571° E, 24.4447° N), 5-Aotou (118.2269° E, 24.5416° N), 6-Qiongtou (118.1790° E, 24.6231° N), and 8-Xiaodeng Island (118.3904° E, 24.5580° N); the green mussel *P. viridis* were sampled from 2 sites, including 1-Baitang Bay (118.0356° E, 24.2156° N) and 7-Dadeng Island (118.3790° E, 24.5528° N).

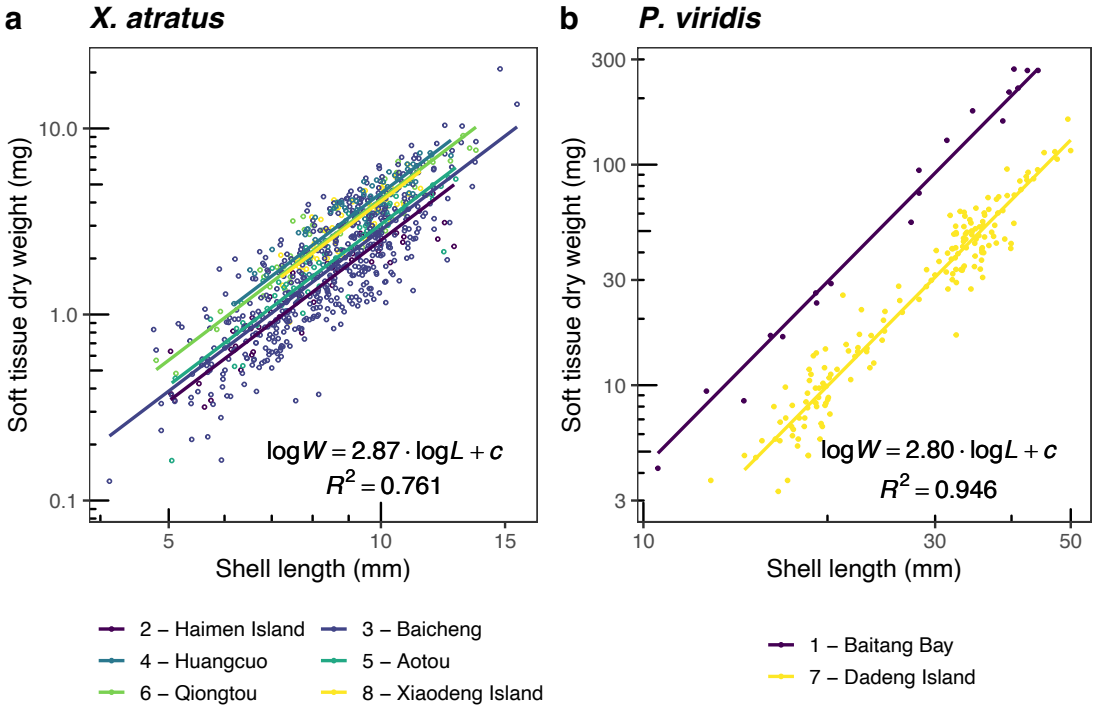


Figure S2. Linear relationship between soft tissue dry weight and shell length in (a) *X. atratus* and (b) *P. viridis* on the double logarithmic scale. The intercept *c* varies among sites: 2-Hiamen Island = − 2.47, 3-Baicheng = − 2.42, 4-Huangcuo = − 2.22, 5-Aotou = − 2.39, 6-Qiongtou = − 2.25, 8-Xiaodeng Island = − 2.26; 1-Baitang Bay = − 5.18, 7-Dadeng Island = − 5.65.

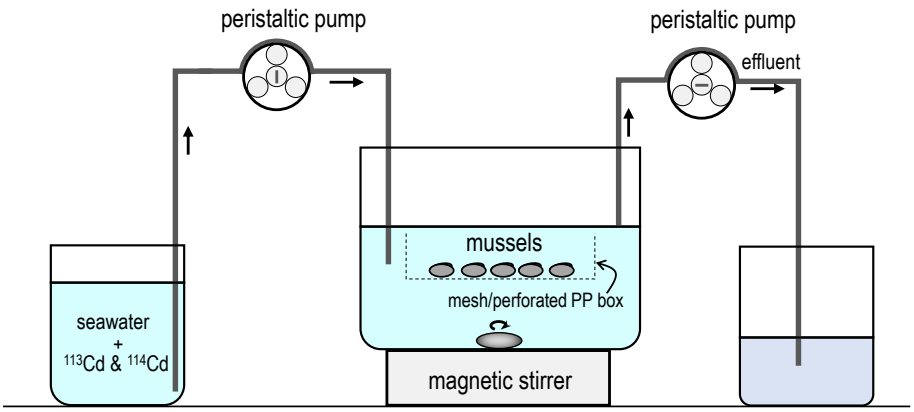


Figure S3. Flow-through exposure system.

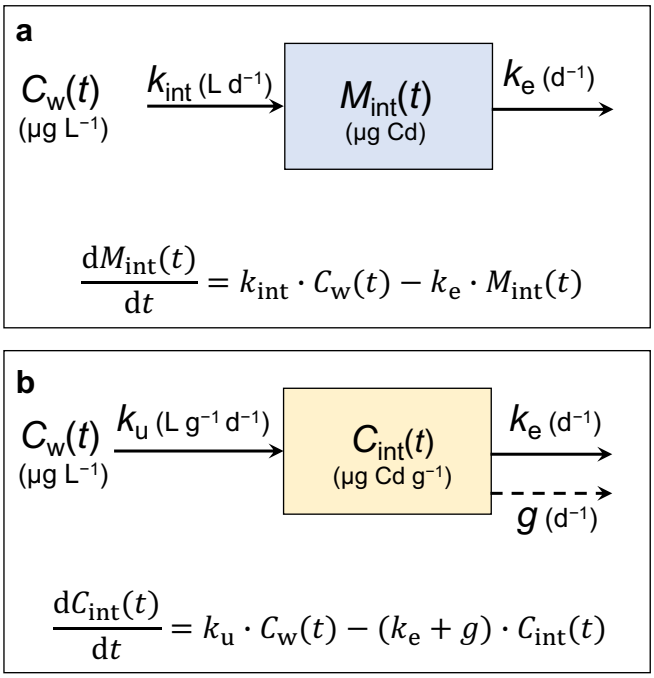


Figure S4. Comparison of the one-compartment toxicokinetic models based on (a) metal mass and (b) metal concentration in organisms. In this study, the model based on Cd mass (i.e., panel **a**) was used in the estimation of toxicokinetic parameters to avoid the need of estimating growth of organisms. The model based on Cd concentration (i.e., panel **b**) is more frequently used in the literature.

The two models can be converted into each other. According to the concept of mass balance, i.e., in a unit of time, mass of Cd accumulated by an organism = mass of Cd entered the organism – mass of Cd eliminated from the organism, we get the mass-based equation (**Figure S4a)**:

$$\frac{dM_{int}(t)}{dt} = k_{int} \cdot C_w(t) - k_e \cdot M_{int}(t) \quad \ldots (S1)$$

$$M_{int}(t) = C_{int}(t) \cdot W(t) \quad \ldots (S2)$$

where M_{int} = mass of Cd in mussels (μg), C_w = concentration of Cd in exposure water (μg L^{-1}), k_{int} = internalization rate constant (L d^{-1}), k_e = elimination rate constant (d^{-1}), C_{int} = concentration of Cd in mussels (μg g^{-1}), and W = dry weight of soft tissues of mussels (g). Substituting eq. S2 into eq. S1, we get:

$$\frac{d[C_{int}(t) \cdot W(t)]}{dt} = k_{int} \cdot C_w(t) - k_e \cdot C_{int}(t) \cdot W(t) \quad \ldots (S3)$$

According to the product rule, eq. S3 transforms to:

$$\frac{\mathrm{d}C_{\mathrm{int}}(t)}{\mathrm{d}t} \cdot W(t) + \frac{\mathrm{d}W(t)}{\mathrm{d}t} \cdot C_{\mathrm{int}}(t) = k_{\mathrm{int}} \cdot C_{\mathrm{w}}(t) - k_{\mathrm{e}} \cdot C_{\mathrm{int}}(t) \cdot W(t) \quad \ldots (S4)$$

Assuming exponential growth of the mussels, we get:

$$\frac{\mathrm{d}W(t)}{\mathrm{d}t} = g \cdot W(t) \quad \ldots (S5)$$

where g = growth rate constant (d^{-1}). Substituting eq. S5 into eq. S4, we get:

$$\frac{\mathrm{d}C_{\mathrm{int}}(t)}{\mathrm{d}t} \cdot W(t) + g \cdot W(t) \cdot C_{\mathrm{int}}(t) = k_{\mathrm{int}} \cdot C_{\mathrm{w}}(t) - k_{\mathrm{e}} \cdot C_{\mathrm{int}}(t) \cdot W(t) \quad \ldots (S6)$$

Rearranging eq. S6, we get the concentration-based model (**Figure S4b**):

$$\frac{\mathrm{d}C_{\mathrm{int}}(t)}{\mathrm{d}t} = k_{\mathrm{u}} \cdot C_{\mathrm{w}}(t) - (k_{\mathrm{e}} + g) \cdot C_{\mathrm{int}}(t) \quad \ldots (S7)$$

where k_{u} is the uptake rate constant (L g^{-1} d^{-1}), and is defined as:

$$k_{\mathrm{u}} = k_{\mathrm{int}}/W(t) \quad \ldots (S8)$$

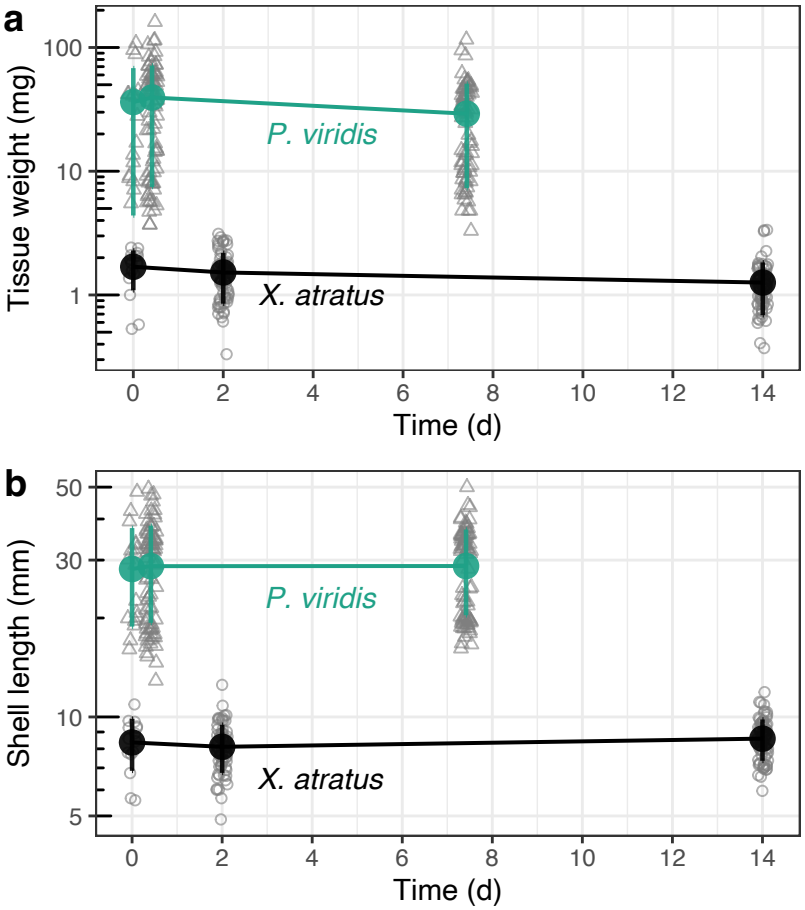


Figure S5. (a) Soft tissue dry weight and (b) shell length of *X. atratus* and *P. viridis* during the exposure. The solid points and error bars are mean values and standard deviations, respectively; the open symbols are individual measurements.

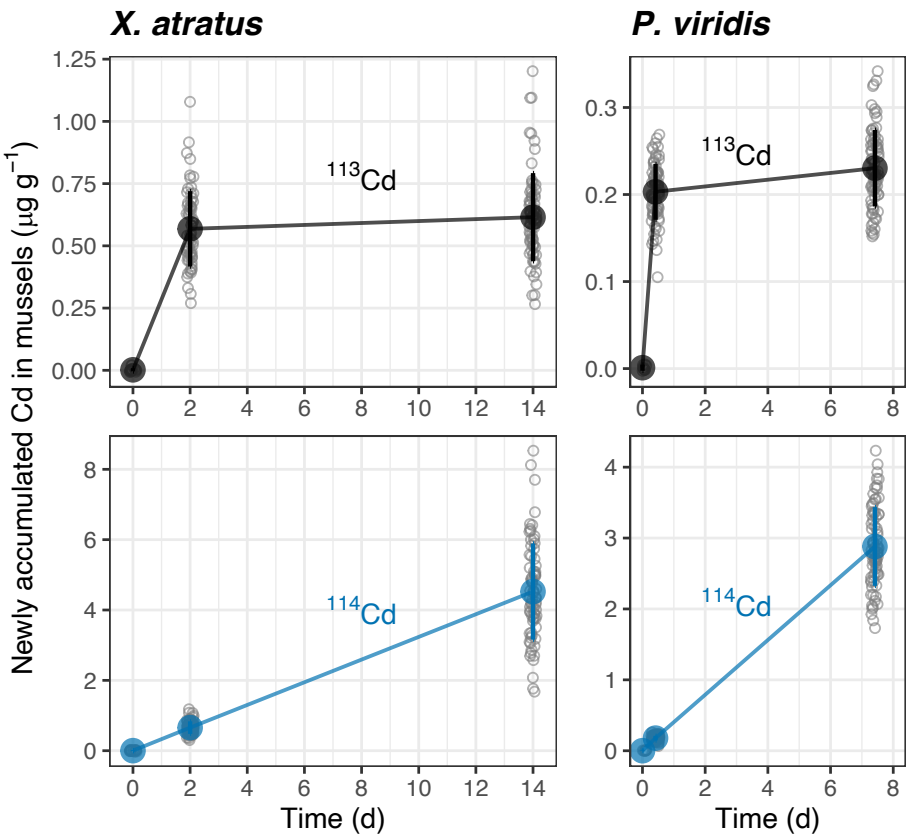


Figure S6. Concentrations of newly accumulated ^{113}Cd and ^{114}Cd in *X. atratus* and *P. viridis* during the exposure. Each open circle represents one individual mussel; the solid points and error bars are mean values and standard deviations, respectively.

It should be noted that concentrations of ^{113}Cd increased slightly, rather than decreased, during the second stage of exposure, although the mussels were not exposed to ^{113}Cd during that period. The slight increase was due to the decrease of tissue weight of the mussels (see **Figure S5a**) and not uptake of ^{113}Cd.

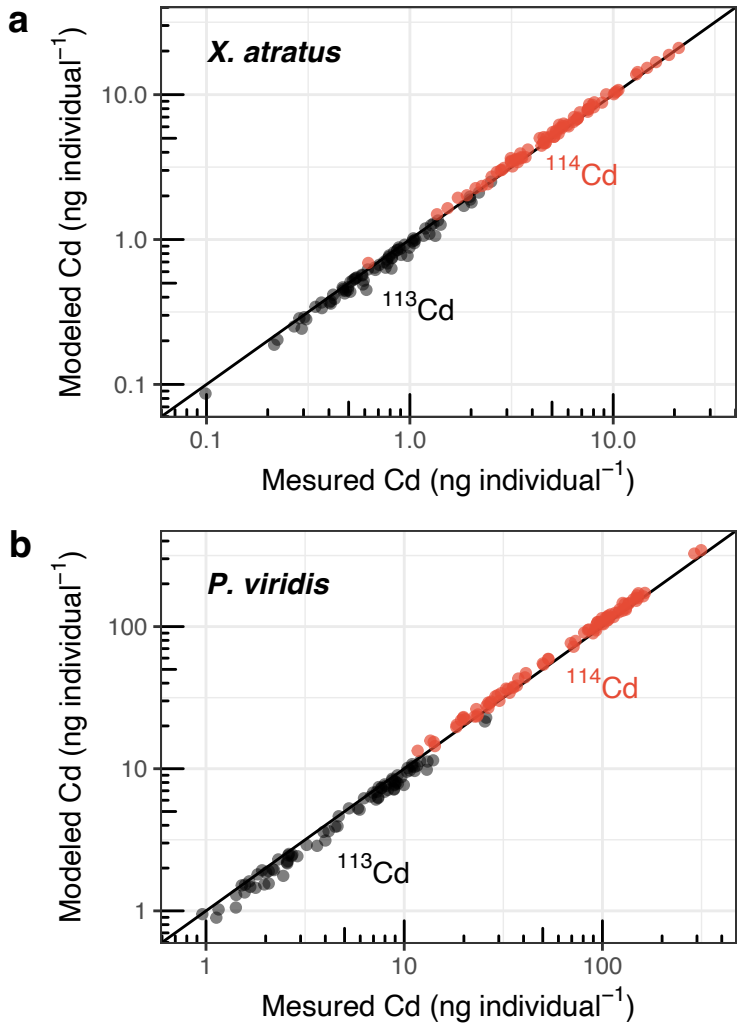


Figure S7. The goodness of fit of the one-compartment first-order toxicokinetic model to the Cd bioaccumulation in (a) *X. atratus* and (b) *P. viridis*. The points represent ^{113}Cd and ^{114}Cd content in mussel individuals; the solid lines are the 1:1 line.

Table S1. Natural abundance of stable Cd isotopes and isotope composition of the enriched Cd isotope materials used in this study.

Isotope	Natural abundance (%)	Enriched ^{113}Cd (%)	Enriched ^{114}Cd (%)
^{106}Cd	1.25	< 0.01	0.005
^{108}Cd	0.89	< 0.01	0.005
^{110}Cd	12.49	0.13	0.005
^{111}Cd	12.8	0.18	0.005
^{112}Cd	24.13	1.63	0.005
^{113}Cd	12.22	**94.8**	0.365
^{114}Cd	28.73	3.03	**99.07**
^{116}Cd	7.49	0.23	0.54

In this study, we selected ^{113}Cd and ^{114}Cd for the double stable isotope measurements. In theory, any two of them can be selected for experiment; nevertheless, there are several scientific and nonscientific factors need to be considered in selecting isotopes:

(1) the isotope is commercially available and economically affordable;

(2) the commercially available isotope has high enrichment level, e.g., 95%, 99%;

(3) the isotope of low relative natural abundance is preferred because it can produce higher sensitivity in chemical analysis;

(4) ICP-MS analysis of the isotope is not subject to substantial isobaric interferences. Although ^{114}Cd and ^{113}Cd are subject to the interference of ^{114}Sn (natural relative abundance 0.43%) and ^{113}In (a rare element), respectively, the interferences are negligible due to the low abundance of ^{114}Sn and ^{113}In. In this respect, ^{116}Cd may not be a good choice due to the interference from ^{116}Sn (natural relative abundance 14.53%).

Table S2. **The *b* values relating Cd concentration in aquatic organisms to body size (expressed as dry tissue weight) on a double logarithmic scale.** See eqs. 6 and 7 for the detailed definition of *b*.

Common name	Species name		*b* value	Reference
	used in reference	currently accepted		
Mussel	*Perna viridis*	*Perna viridis*	-0.126	This study
Mussel	*Perna viridis*	*Perna viridis*	-0.159	This study
Mussel	*Xenostrobus atratus*	*Xenostrobus atratus*	0.348	This study
Mussel	*Xenostrobus atratus*	*Xenostrobus atratus*	0.362	This study
Mussel	*Xenostrobus atratus*	*Xenostrobus atratus*	0.177	This study
Mussel	*Xenostrobus atratus*	*Xenostrobus atratus*	0.371	This study
Mussel	*Xenostrobus atratus*	*Xenostrobus atratus*	0.416	This study
Mussel	*Xenostrobus atratus*	*Xenostrobus atratus*	0.202	This study
Clam	*Meretrix lusoria*	*Meretrix lusoria*	-0.254	Ju et al., 2020 [1]
Clam	*Amiantis umbonella*	*Callista umbonella*	0.20	Tarique et al., 2019 [2]
Clam	*Amiantis umbonella*	*Callista umbonella*	-0.13	Tarique et al., 2019 [2]
Mussel	*Dreissena polymorpha*	*Dreissena polymorpha*	-0.582	Tang et al., 2017 [3]
Mussel	*Perna viridis*	*Perna viridis*	-0.588	Yap et al., 2009 [4]
Scallop	*Chlamys nobilis*	*Mimachlamys crassicostata*	-0.266	Pan & Wang, 2008 [5]
Mussel	*Mytilus galloprovincialis*	*Mytilus galloprovincialis*	-0.234	Conti et al., 2008 [6]
Fish	*Acanthopagrus schlegeli*	*Acanthopagrus schlegelii*	-0.655	Zhang & Wang, 2007 [7]
Clam	*Gafrarium tumidum*	*Gafrarium pectinatum*	-0.38*	Hédouin et al., 2006 [8]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.18	Mubiana, 2006 [9]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.35	Mubiana, 2006 [9]
Snail	*Monodonta turbinata*	*Phorcus turbinatus*	0.727	Cubadda et al., 2001 [10]
Snail	*Monodonta mutabilis*	*Phorcus mutabilis*	0.766	Cubadda et al., 2001 [10]

Common name	Species name		*b* value	Reference
	used in reference	currently accepted		
Limpet	*Patella caerulea*	*Patella caerulea*	0.768	Cubadda et al., 2001 [10]
Limpet	*Patella lusitanica*	*Patella rustica*	0.474	Cubadda et al., 2001 [10]
Snail	*Nucella lapillus*	*Nucella lapillus*	0.31	Leung et al., 2001 [11]
Snail	*Nucella lapillus*	*Nucella lapillus*	-0.878	Leung et al., 2001 [11]
Snail	*Nucella lapillus*	*Nucella lapillus*	-0.055	Leung et al., 2001 [11]
Clam	*Potamocorbula amurensis*	*Potamocorbula amurensis*	-0.49	Lee et al., 1998 [12]
Clam	*Macoma balthica*	*Limecola balthica*	-0.48	Lee et al., 1998 [12]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.272*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.284*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.168*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.152*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.108*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.244*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.18*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.08*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.112*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.324*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.124*	Riget et al., 1996 [13]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.188*	Riget et al., 1996 [13]
Snail	*Monodonta turbinata*	*Phorcus turbinatus*	-0.288*	Catsiki et al., 1994 [14]
Snail	*Monodonta turbinata*	*Phorcus turbinatus*	-0.188*	Catsiki et al., 1994 [14]
Snail	*Cerithium vulgatum*	*Cerithium vulgatum*	-0.118*	Catsiki et al., 1994 [14]
Snail	*Cerithium vulgatum*	*Cerithium vulgatum*	-0.020*	Catsiki et al., 1994 [14]
Clam	*Arctica islandica*	*Arctica islandica*	0.51	Swaileh & Adelung, 1994 [15]

Common name	Species name		*b* value	Reference
	used in reference	currently accepted		
Clam	*Astarte borealis*	*Astarte borealis*	-0.094	Fischer, 1983 [16]
Clam	*Astarte borealis*	*Astarte borealis*	0.425	Fischer, 1983 [16]
Cockle	*Cardium edule*	*Cerastoderma edule*	-0.247	Fischer, 1983 [16]
Cockle	*Cardium edule*	*Cerastoderma edule*	-0.282	Fischer, 1983 [16]
Clam	*Mya arenaria*	*Mya arenaria*	-0.020	Fischer, 1983 [16]
Clam	*Macoma balthica*	*Limecola balthica*	0.028	Fischer, 1983 [16]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.029	Fischer, 1983 [16]
Snail	*Littorina littorea*	*Littorina littorea*	0.047	Fischer, 1983 [16]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.31	Cossa et al., 1980 [17]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.44	Cossa et al., 1980 [17]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.45	Cossa et al., 1980 [17]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.69	Cossa et al., 1980 [17]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.34	Cossa et al., 1980 [17]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.29	Cossa et al., 1979 [18]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.35	Cossa et al., 1979 [18]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.31	Cossa et al., 1979 [18]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.54	Cossa et al., 1979 [18]
Oyster	*Ostrea edulis*	*Ostrea edulis*	-0.06	Boyden, 1977 [19]
Oyster	*Ostrea edulis*	*Ostrea edulis*	-0.04	Boyden, 1977 [19]
Oyster	*Crassostrea gigas*	*Magallana gigas*	-0.15	Boyden, 1977 [19]
Oyster	*Crassostrea gigas*	*Magallana gigas*	-0.14	Boyden, 1977 [19]
Oyster	*Crassostrea gigas*	*Magallana gigas*	-0.15	Boyden, 1977 [19]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.03	Boyden, 1977 [19]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.05	Boyden, 1977 [19]

Common name	Species name		*b* value	Reference
	used in reference	currently accepted		
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.02	Boyden, 1977 [19]
Mussel	*Mytilus edulis*	*Mytilus edulis*	0.08	Boyden, 1977 [19]
Mussel	*Mytilus edulis*	*Mytilus edulis*	-0.05	Boyden, 1977 [19]
Clam	*Mercenaria mercenaria*	*Mercenaria mercenaria*	-0.19	Boyden, 1977 [19]
Clam	*Venerupis decussat*	*Ruditapes decussatus*	-0.23	Boyden, 1977 [19]
Scallop	*Chlamys opercular*	*Aequipecten opercularis*	-0.04	Boyden, 1977 [19]
Snail	*Littorina littorea*	*Littorina littorea*	-0.03	Boyden, 1977 [19]
Snail	*Buccinum undatum*	*Buccinum undatum*	0.18	Boyden, 1977 [19]
Snail	*Scaphander lignarius*	*Scaphander lignarius*	-0.06	Boyden, 1977 [19]
Limpet	*Crepidula fornicata*	*Crepidula fornicata*	0.12	Boyden, 1977 [19]
Limpet	*Patella intermedia*	*Patella depressa*	0.35	Boyden, 1977 [19]
Limpet	*Patella intermedia*	*Patella depressa*	0.49	Boyden, 1977 [19]
Limpet	*Patella vulgata*	*Patella vulgata*	1.05	Boyden, 1977 [19]
Limpet	*Patella vulgata*	*Patella vulgata*	0.98	Boyden, 1977 [19]
Limpet	*Patella vulgata*	*Patella vulgata*	0.96	Boyden, 1977 [19]
Limpet	*Patella vulgata*	*Patella vulgata*	0.70	Boyden, 1977 [19]
Limpet	*Patella vulgata*	*Patella vulgata*	0.37	Boyden, 1977 [19]
Oyster	*Crassostrea gigas*	*Magallana gigas*	-0.208	Watling & Watling, 1976 [20]
Oyster	*Crassostrea margaritacea*	*Striostrea margaritacea*	-0.111	Watling & Watling, 1976 [20]
Oyster	*Ostrea edulis*	*Ostrea edulis*	-0.355	Watling & Watling, 1976 [20]
Cockle	*Cerastoderma edule*	*Cerastoderma edule*	-0.25	Boyden, 1974 [21]

* Body size was expressed as shell length (or width); the listed *b* values are the original *b* values divided by a factor of 2.5, assuming that body weight is proportional to shell length (or width) with an exponent of 2.5.

Table S3. **The *b* values relating metal uptake rate in aquatic organisms to body size (expressed as dry tissue weight) on a double logarithmic scale.** See eqs. 6 and 7 for the detailed definition of *b*.

Common name	Species name		Metal	*b* value	Reference
	used in reference	currently accepted			
Mussel	*Perna viridis*	*Perna viridis*	Cd	-0.072	This study
Mussel	*Xenostrobus atratus*	*Xenostrobus atratus*	Cd	0.310	This study
Mussel	*Dreissena polymorpha*	*Dreissena polymorpha*	Cd	-0.379	Tang et al., 2017 [3]
Fish	*Acanthopagrus schlegeli schlegeli*	*Acanthopagrus schlegelii*	Hg	-0.68	Dang & Wang, 2012 [22]
Fish	*Acanthopagrus schlegeli schlegeli*	*Acanthopagrus schlegelii*	MeHg	-0.54	Dang & Wang, 2012 [22]
Scallop	*Chlamys nobilis*	*Mimachlamys crassicostata*	Cd	-0.259	Pan & Wang, 2008 [5]
Scallop	*Chlamys nobilis*	*Mimachlamys crassicostata*	Zn	-0.266	Pan & Wang, 2008 [5]
Fish	*Acanthopagrus schlegeli*	*Acanthopagrus schlegelii*	Cd	-0.662	Zhang & Wang, 2007 [7]
Fish	*Acanthopagrus schlegeli*	*Acanthopagrus schlegelii*	Se	-0.619	Zhang & Wang, 2007 [7]
Fish	*Acanthopagrus schlegeli*	*Acanthopagrus schlegelii*	Zn	-0.615	Zhang & Wang, 2007 [7]
Mussel	*Perna viridis*	*Perna viridis*	Cd	-0.594	Chong & Wang, 2001 [23]
Clam	*Ruditapes philippinarum*	*Ruditapes philippinarum*	Cd	-0.56	Chong & Wang, 2001 [23]
Mussel	*Perna viridis*	*Perna viridis*	Cr	-0.041	Chong & Wang, 2001 [23]
Clam	*Ruditapes philippinarum*	*Ruditapes philippinarum*	Cr	-0.477	Chong & Wang, 2001 [23]
Mussel	*Perna viridis*	*Perna viridis*	Zn	-0.399	Chong & Wang, 2001 [23]
Clam	*Ruditapes philippinarum*	*Ruditapes philippinarum*	Zn	-0.503	Chong & Wang, 2001 [23]
Mussel	*Perna viridis*	*Perna viridis*	Cs	-0.464	Ke et al., 2000 [24]

Common name	Species name		Metal	*b* value	Reference
	used in reference	currently accepted			
Mussel	*Septifer virgatus*	*Mytilisepta virgata*	Cd	-0.537	Wang & Dei, 1999 [25]
Mussel	*Septifer virgatus*	*Mytilisepta virgata*	Cr	-0.344	Wang & Dei, 1999 [25]
Mussel	*Septifer virgatus*	*Mytilisepta virgata*	Se	-0.317	Wang & Dei, 1999 [25]
Mussel	*Septifer virgatus*	*Mytilisepta virgata*	Zn	-0.437	Wang & Dei, 1999 [25]
Clam	*Potamocorbula amurensis*	*Potamocorbula amurensis*	Cd	-0.27	Lee et al., 1998 [12]
Clam	*Macoma balthica*	*Limecola balthica*	Cd	-0.43	Lee et al., 1998 [12]
Clam	*Potamocorbula amurensis*	*Potamocorbula amurensis*	Cr	-0.05	Lee et al., 1998 [12]
Clam	*Macoma balthica*	*Limecola balthica*	Cr	-0.34	Lee et al., 1998 [12]
Clam	*Potamocorbula amurensis*	*Potamocorbula amurensis*	Zn	-0.23	Lee et al., 1998 [12]
Clam	*Macoma balthica*	*Limecola balthica*	Zn	-0.35	Lee et al., 1998 [12]
Fish	*Gambusia affinis*	*Gambusia affinis*	Zn	-0.90	Newman & Mitz, 1988 [26]

Table S4. Output of the multiple linear regression indicates that k_u was the significant predictor of Cd concentrations in *X. atratus* and *P. viridis* while organism weight was not. The regression model is

$$\log Cd = \beta_0 + \beta_1 \cdot \log k_u + \beta_2 \cdot \log W$$

where Cd is the background Cd concentration in the mussels at the end of the exposure (i.e., T2), k_u is the uptake rate constant of Cd, and W is the dry weight of soft tissues.

(**a**) Mussel *X. atratus*:

	Estimate	Standard error	*t* value	*p* value
Intercept	1.47621	0.25270	5.842	1.38e-07
log(k_u)	0.32856	0.12078	2.720	**0.00817**
log(W)	0.18574	0.09365	1.983	0.05115

(**b**) Mussel *P. viridis*:

	Estimate	Standard error	*t* value	*p* value
Intercept	0.70280	0.07929	8.864	2.19e-13
log(k_u)	0.76497	0.09602	7.967	**1.18e-11**
log(W)	-0.03409	0.02115	-1.612	0.111

Note S1. R code for estimating the toxicokinetic parameters (k_u and k_e)

R code in text format is also available at: https://github.com/tan-qiao-guo/double_stable_isotope

```
library(deSolve) # for using the function "ode" to integrate
library(FME) # for using the function "modFit" to fit
library(readxl) # for importing data from Excel files
library(ggplot2) # for plotting
library(dplyr) # for manipulating data frames
```

1. Cd isotope concentrations in exposure solution

The data on Cd isotope concentrations in exposure solution are imported from the Excel file provided as another Supporting Information document. The *.xlsx file name in the code below should be revised to be the same as the that of the Excel file.

```
d_water <-
  read_excel("SI_data.xlsx", sheet = 1) %>%
  filter(species == "X. atratus")
```

2. Cd isotope concentrations in mussels

Similarly, the data on the Cd isotope concentrations in the mussels during the double isotope exposure are imported from the same Excel file mentioned above but from a different sheet.

```
d_organism <- read_excel("SI_data.xlsx", sheet = 2) %>%
  filter(species == "X. atratus")
```

3. Time points

In the double Cd isotope exposure of *X. atratus*, T1 and T2 were 48 h and 336 h, respectively.

```
T1 <- 48 # T1 = 48 h
T2 <- 336 # T2 = 336 h
```

The total exposure length (i.e., T2 = 336 h) is divided into intervals of 1/48 d (i.e., 0.5 h). The time sequence is used below to interpolate the exposure concentration and to calculate the bioaccumulation of Cd isotopes.

```
times <- seq(from = 0,
             to = T2 / 24,
             by = 1 / 48)
```

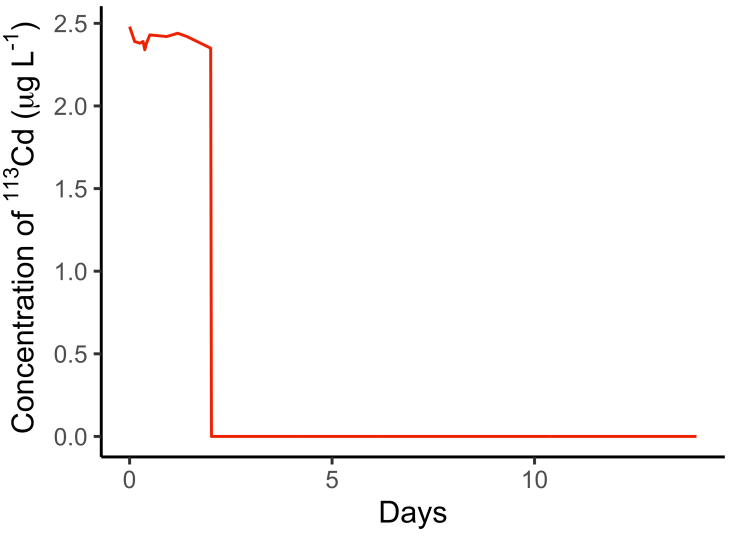
4. Interpolation of Cd isotope concentrations in solutions

Cd isotope concentrations in the exposure solutions during the whole experiment period was interpolated from the measured concentrations using the function `approxfun`.

```
Cw_Cd113 <- approxfun(x = d_water$day,
                      y = d_water$Cw_Cd113_ppb,
                      rule = 2)
```

The interpolated concentrations of ^{113}Cd are shown below:

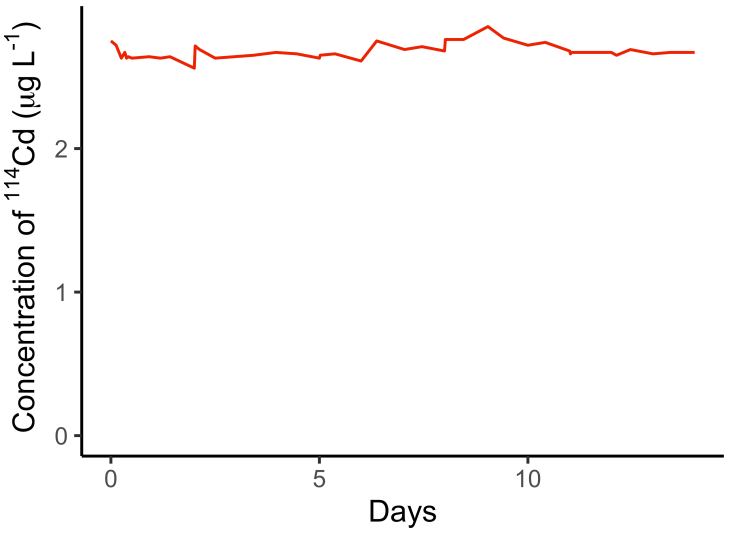
```
data.frame(Day = times, Cd113 = Cw_Cd113(times)) %>%
  ggplot(aes(Day, Cd113)) +
  theme_classic() +
  geom_line(color = "red2") +
  labs(x = "Days",
       y = ~ "Concentration of " * "" ^ "113" * "Cd (" * mu * g ~ "L" ^
         "-1" * ")")
```



The interpolated concentrations of ^{114}Cd are shown below:

```
Cw_Cd114 <- approxfun(x = d_water$day,
                      y = d_water$Cw_Cd114_ppb,
                      rule = 2)

data.frame(Day = times, Cd114 = Cw_Cd114(times)) %>%
  ggplot(aes(Day, Cd114)) +
  theme_classic() +
  geom_line(color = "red2") +
  scale_y_continuous(limits = c(0, NA)) +
  labs(x = "Days",
       y = ~ "Concentration of " * "" ^ "114" * "Cd (" * mu * g ~ "L" ^
         "-1" * ")")
```



5. Toxicokinetic (TK) model

The toxicokinetic model is defined below. The variation of Cd isotope content in a mussel is the balance between uptake ($k_{in} \cdot C_w$) and elimination ($k_e \cdot M_{int}$) of the isotope. See eqs. 3–5 in the main text for detailed descriptions of the model.

```
TK <- function (t, y, parameters) {
  kin <- parameters[1]
  ke <- parameters[2]
  Mint_Cd113 <- y[1]
  Mint_Cd114 <- y[2]
  dMint_Cd113 <- kin * Cw_Cd113(t) - ke * Mint_Cd113
  dMint_Cd114 <- kin * Cw_Cd114(t) - ke * Mint_Cd114

  list(c(dMint_Cd113, dMint_Cd114))
}
```

6. Estimation of the initial parameter values

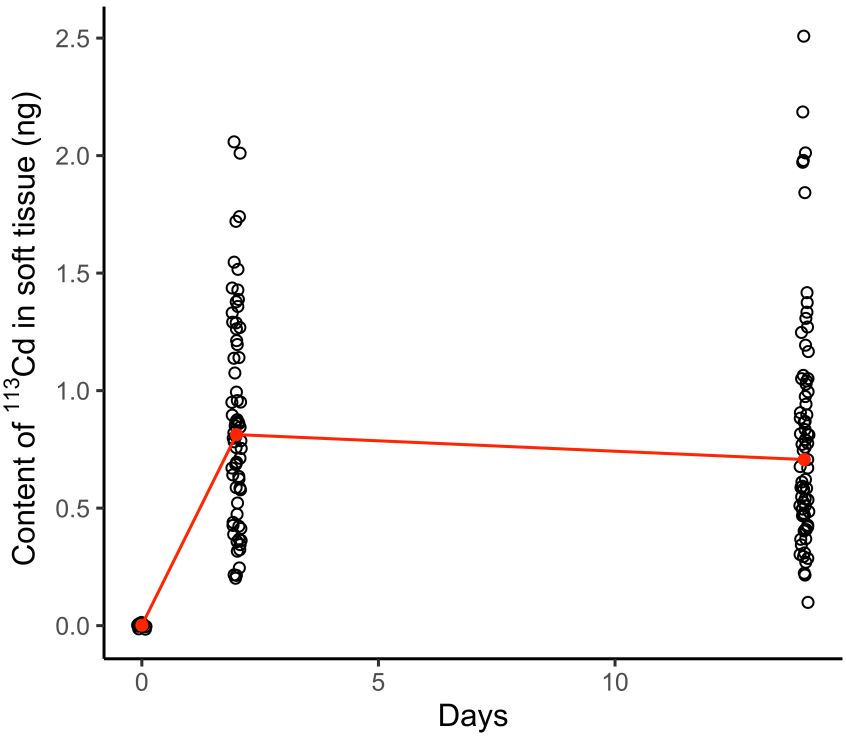
An overall rate of Cd elimination from the mussels can be estimated based on the decrease of ^{113}Cd in mussels during the period between T1 and T2 (see the figure below):

$$k_e = -\frac{\Delta \ln(\text{median of new } ^{113}\text{Cd})}{\Delta t}$$

where the numerator is the decrease of the median natural-log transformed ^{113}Cd content, and the denominator Δt is the duration of the second stage (i.e., T2 – T1).

The overall k_e was used as the initial value to estimate k_e of individual mussels.

```
ggplot(d_organism, aes(day, Cd113_new_ng)) +
  theme_classic() +
  geom_jitter(width = 0.1, height = 0, shape = 1) +
  stat_summary(geom = "point", fun = "median", color = "red") +
  stat_summary(geom = "line", fun = "median", color = "red") +
  labs(
    x = "Days",
     y = ~ "Content of " * "" ^ "113" * "Cd in soft tissue (ng)"
  )
```




```
d_organism_median <- d_organism %>%
  group_by(day) %>%
  summarise(median_Cd113 = median(Cd113_new_ng))

ke_initial <- with(d_organism_median,
                   (log(median_Cd113[2]) - log(median_Cd113[3])) / (da
y[3] - day[2]))
```

7. Objective function for fitting the toxicokinetic parameters

The objective function returns a vector of the weighted residuals, which are the difference between the modeled content of Cd isotopes and the measured content of Cd isotopes. The reciprocal of the measured content of Cd isotopes are used as the weight.

```
fn_resid <- function(parameters_to_fit) {
  kin <- parameters_to_fit[1]
  ke <- parameters_to_fit[2]
  out.Mint <- ode(
    func = TK,
    times = times,
    y = c(Mint_Cd113 = 0, Mint_Cd114 = 0),
    parms = parameters_to_fit
  )
  n <- length(times)

# residuals = (modeled – measured)/ measured
  resid.Mint <- c((out.Mint[n, 2] / organism_i$Cd113_new_ng - 1),
                  (out.Mint[n, 3] / organism_i$Cd114_new_ng - 1))
    return(resid.Mint)
}
```

8. Estimating k_{in} and k_e

Cd isotope contents measured at time T2 are used for estimating k_{in} and k_e.

```
# selecting data of time T2
organism_T2 <- subset(d_organism, hour == T2)

# the number of organisms analyzed at T2
n_organism <- dim(organism_T2)[1]

# empty vectors for storing kin and ke values
kin <- numeric(n_organism)
ke  <- numeric(n_organism)

# create a folder to store multiple figures for checking the goodness
 of fit
fit_SN <- gsub("[: -]", "" , Sys.time())
path <- paste0("fit_check_", fit_SN)
dir.create(path)
```

Fitting the values of k_{in} and k_e for each individual. This step may take **5 to 10 min** to finish.

```
for (i in 1:n_organism) {
  # select data for the i^th individual
  organism_i <- organism_T2[i, ]
  # estimation of initial k_in: content of 114Cd/exposure time/exposure
concentration
  kin_initial <-
    organism_i$Cd114_new_ng / (T2 / 24) / mean(d_water$Cw_Cd114_ppb)
print(system.time(
  # use the optimization function 'modFit' to estimate the values of
k_in and k_e
  fit.TK <- modFit(
    f = fn_resid, # objective function defined above
    p = c(kin_initial, ke_initial), # initial values estimated above
    method = "BFGS", # the quasi-Newton method
    # the lower and upper bound of k_in and k_e
    lower = c(kin_initial * 0.1, ke_initial * 0.1),
    upper = c(kin_initial * 10, ke_initial * 10)
  )
))
# extract and store parameter values
kin[i] <- fit.TK$par[1]
ke[i]  <- fit.TK$par[2]
# extract parameter values for the integration below
kin_i <- fit.TK$par[1]
ke_i  <- fit.TK$par[2]
# integrate the TK model to calculate Cd isotope contents at the end
of exposure
fit_i <- ode(
  func = TK,
  times = times,
  y = c(Cint_113 = 0, Cint_114 = 0),
  parms = c(kin_i, ke_i)
)
d_fit_i <- as.data.frame(fit_i)
# plot the modeled content vs. the measured content to check the
goodness of fit
ggplot(d_fit_i, aes(x = time, Cint_113)) +
  geom_line() +
  geom_line(aes(x = time, y = Cint_114), color = "red2") +
  geom_point(aes(x = organism_i$day, organism_i$Cd113_new_ng)) +
  geom_point(aes(x = organism_i$day, organism_i$Cd114_new_ng), color
= "red2")
ggsave(paste("fit", i, ".png"), path = path)
}
```

9. Saving k_u and k_e

The values of k_u and k_e are saved as a *.csv file.

```
organism_T2$kin <- kin
# ku is calculated from kin: ku = kin / weight of organism
organism_T2$ku <- kin / 1000 / organism_T2$dw_g
organism_T2$ke  <- ke
# save the values as a csv file
write.csv(organism_T2, file=paste0("fitted parameters_", fit_SN, ".cs
v"))
```

10. Goodness of fit

The goodness of fit is visualized by plotting the modeled content vs. the measured content of ^{113}Cd and ^{114}Cd in each organism.

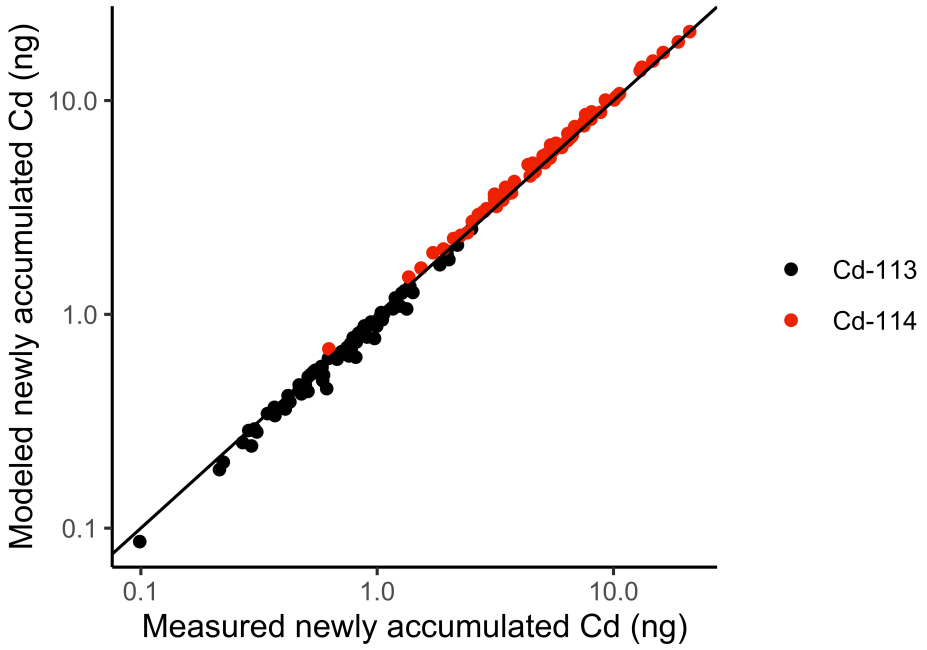
```
# empty vector to store modeled content of Cd isotopes
Cd113_fit <- numeric(n_organism)
Cd114_fit <- numeric(n_organism)

# Cd isotope content calculated by integrating the TK model
for (i in 1:n_organism){
  out.fit <- ode(func = TK,
                 times = times,
                 y = c(Cint_Cd113 = 0, Cint_Cd114 = 0),
                 parms = c(kin = kin[i], ke = ke[i]))
  N_step <- length(times)
  Cd113_fit[i] <- out.fit[N_step, 2]
  Cd114_fit[i] <- out.fit[N_step, 3]
}

organism_T2$Cd113_fit <- Cd113_fit
organism_T2$Cd114_fit <- Cd114_fit

# save the modeled Cd content as a csv file
write.csv(organism_T2,
          file=paste0("fit_Cd_T2_", fit_SN,".csv"))
```

```r
# plot the modeled content vs. measured content
ggplot(organism_T2, aes(Cd113_new_ng, Cd113_fit))+
  theme_classic()+
  geom_point(aes(color = "Cd-113"))+
  geom_point(aes(Cd114_new_ng, Cd114_fit, color = "Cd-114"))+
  geom_abline(slope = 1, intercept = 0)+
  scale_x_log10()+
  scale_y_log10()+
  scale_color_manual(values = c("black", "red2")) +
  labs(x = "Measured newly accumulated Cd (ng)",
       y = "Modeled newly accumulated Cd (ng)",
       color = NULL)
```



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