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Abstract :

Gammarids are aquatic amphipods widely used for water quality monitoring. To investigate the copper and cadmium diet-borne metal uptake in Gammarus pulex, we adapted the pulse-chase stable isotopes-based approach to determine the food ingestion rate (IR), the gut retention time (GRT) and the metal assimilation efficiencies (AE). G. pulex were fed with 65Cu-, 106Cd-, and 53Cr-labeled alder leaves for 7.5 h and then with unlabeled leaves for 5 d. The metal stable isotope contents in the gammarids, leaves, filtered water and periodically collected feces were determined. Chromium was poorly assimilated by the gammarids; thus, Cr was used as an unassimilated tracer. The first tracer defecation occurred before the first feces harvest, indicating a gut passage time of less than 9 h. A 24-h GRT and a 0.69 g g−1 d−1 IR were estimated. The Cd AE value was estimated as 5–47%, depending on the assimilation determination method applied. The Cu AE value could not be evaluated regardless of the determination method used, most likely because of the rapid Cu regulation in gammarids in addition to analytical uncertainties when determining the Cu content in leaves. Application of the Cd AE value in the framework of the biodynamic bioaccumulation model shows that the diet-borne uptake of Cd significantly contributes (66–95%) to the metal bioaccumulation in G. pulex fed with alder leaves.

Keywords : Assimilation efficiency, Gammarus pulex, Cadmium, Copper, Biodynamic model, Diet-borne uptake

1 - Introduction

The aquatic amphipod genus *Gammarus* is widely used for water quality monitoring through passive (autochthonous specimens) or active (caged gammarids) approaches (e.g., Besse et al., 2013; Geffard et al., 2010; Khan et al., 2011; Kunz et al., 2010; Sroda and Cossu-Leguille, 2011). Fialkowski et al. (2003) have proven the feasibility of using *Gammarus fossarum* as a metal biomonitor in a river affected by Pb-Zn mining activities. The sensibility of the behavior of gammarids to a metal-polluted environment has also been demonstrated (Dedourge-Geffard O *et al.*, 2009). *Gammarus pulex* is ubiquitous in European freshwaters. The leaf litter serves as a main food source for *G. pulex* that is a key species in metal mobilization in freshwater ecosystems as reviewed by Schaller et al. (2011a). *G. pulex* is one of the rare crustaceans for which the influence of natural and anthropogenic stressors on the ingestion rate has been investigated (Maltby et al., 2002; Coulaud et al., 2011). Gammarids can accumulate waterborne and diet-borne metals. The waterborne uptake route has been intensively studied (e.g., Bourgeault et al., 2013; Lebrun et al., 2012; Vellinger et al., 2012). Although the diet-borne uptake of metals may be a significant pathway for metal accumulation in gammarids (Abel and Bärlocher, 1988), this type of uptake has been poorly documented. It should be mentioned that tolerance mechanisms, including both avoidance (decrease of feeding rate, food selection) and detoxification (Malty et al., 2002; Schaller et al., 2011b), regulate metals uptake. Khan et al. (2011) have shown that copper uptake is lower for historically impacted populations compared to naïve *G. pulex* populations. Schaller et al. (2011b) found that *G. pulex* gut epithelial cells sequester and detoxify metals such as cadmium (Cd) and copper (Cu).

The in situ contributions of diet-borne and waterborne metals to the total bioaccumulation can be estimated using bioaccumulation models, among which is the biodynamic model validated on field data of the metal body-burden in aquatic (freshwater and marine) invertebrates (Ahlf *et al.*, 2009; Golding et al., 2013). Briefly, according to the biodynamic model, the diet-borne influx of a metal is driven by the food ingestion rate (IR), the metal concentration in the food and the assimilation efficiency (AE). The AE value is defined as the fraction of metal ingested via contaminated food that penetrates across the cells of the gut wall, hence incorporating into the tissues. AE values are commonly used to compare bioavailable metal fractions among various conditions characterized by a range of parameters (e.g., food type and species) (Wang and Fisher, 1999). It has been shown that Cd is assimilated more efficiently by the estuarine amphipod *Leptocheirus plumulosus* when feeding with sediments compared with feeding with phytoplankton (Schlekat *et al.*, 2000).

The pulse-chase feeding method (a short dietary exposure to metal-contaminated food followed by a depuration phase) has been proposed to determine AE values in the laboratory (Calow and Fletcher, 1972). The use of tracers (radiotracers or stable isotopes) is necessary to discriminate the added amounts of metals from the background level. After complete excretion of the labeled food, the organisms are sacrificed and the AE values are estimated from the comparison of the amount of tracers accumulated in the organisms and the amount of tracers in the ingested food (or in the feces, providing that a mass balance of the target contaminants can be settled on the system). Alternatively, the AE values can be calculated using relevant tracer ratios in the food and feces only, providing that the food is dually labeled with an unassimilated (or inert) tracer (Roditi and Fisher, 1999). In most cases, gamma-emitting radiotracer methods have been used to determine AE values. Using this technique, Roditi and Fisher (1999) found a Cd AE value in the 19–72% range for the freshwater mussel

87 *Dreissena polymorpha* fed with 8 food types. Recently, the use of stable isotopes instead of
88 radiotracers has been proposed as an improved alternative (Croteau and Luoma, 2005;
89 Croteau *et al.*, 2007). The use of stable instead of radioactive isotopes minimizes the handling
90 and disposal hazards along with the costs, and it increases the range of metals that could be
91 investigated. The AE value of copper could not be measured using radiotracers because the
92 ^{64}Cu half-life (12.7 hours) is too short to perform the pulse-chase experiment. Using the stable
93 isotope tracer ^{65}Cu, Croteau and Luoma (2005) have found a 38% Cu AE value for the
94 freshwater clam *Corbicula fluminea* and Cain et al. (2011) have found that the Cu AE value
95 was higher than 83% for various species of mayflies feeding with periphyton.

96 Although the stable isotope pulse-chase methodology has recently been improved with the use
97 of ^{53}Cr as an unassimilated tracer (Croteau *et al.*, 2007), the application of this promising
98 approach is not straightforward because of the following two issues: (1) the proper labeling of
99 the food that should not alter the natural metal speciation and (2) the discrimination of minute
100 amounts of added tracer from background levels. The precise quantitative determination of a
101 few nanograms of added isotopes remains problematic. Very recently, after the present study
102 was performed, Croteau et al. (2013) have proposed a novel approach of the pulse-chase
103 feeding based on the labeling of the test organisms to circumvent the shortcomings of the
104 method based on food labeling (Croteau et al., 2007) used in this study.

105 Because of the significance of gammarids in freshwater metal biomonitoring, knowledge of
106 the Cd and Cu AE values in *G. pulex* is important. Therefore, the goal of the present study
107 was to determine the AE values of Cd and Cu in *G. pulex*, thus enabling the use of a complete
108 biodynamic model for this species. Waterborne uptake parameters have been previously
109 determined (Pellet *et al.*, 2009; Lebrun *et al.*, 2012). Here, the pulse-chase feeding techniques

110 were associated with the use of stable isotopes to estimate the relative importance of diet-
111 borne exposure.

112112

113 2 - Materials and methods

114 2.1. Study organisms and materials

115 Approximately 100 *G. pulex* specimens were collected from the Mauldre River at Mareil-sur-
116 Mauldre (*Coordinates RGF93* longitude: 01°52'11" E, latitude: 48°53'42" N) on February 19,
117 2008. The mean size of the selected gammarids was 12 ± 2 mm (n=70). The animals were
118 maintained in a temperature-controlled incubator (12.1 ±0.8°C) under a 8:16 light:dark cycle
119 (for acclimatization and the experiment). The gammarids were given alder leaves (*Alnus*
120 *glutinosa*) *ad libitum* for 3 d and starved for the consecutive 3 d prior to exposure to labeled
121 food. Filtered water (sieved through a 200 µm mesh size) from the sampling site was used as
122 the first input in the acclimatization aquaria. This water was progressively renewed by
123 commercial groundwater (Source St Hélène, SE Des Sources Roxane, La Ferrière Bochard,
124 France, Cd, Cr and Cu concentrations were 0.0015, 0.028 and 0.025 µg/L, respectively) over
125 6 d. During the experiment, the physical and chemical characteristics of the water were
126 measured (see the supplemental information). The Cd and Cu concentrations in gammarids
127 prior to exposure were 0.032 and 74.4 mg/kg, respectively. We purchased 10 mg oxides of
128 ^{53}Cr (as Cr_2O_3, 97.7% isotopic purity), ^{65}Cu (Cu_2O, 99.6%) and ^{106}Cd (CdO, 89.8%) from
129 Eurisotop (St Aubin, France). The metallic oxides were partially solubilized for at least 7 d at
130 room temperature in HNO_3 (65% Suprapur, Merck, Darmstadt, Germany) (except for Cr_2O_3
131 for which a stronger acidic solubilization procedure was applied using HF (40% ultrapure,
132 Merck) and heat) and then 1.5 mL of the supernatant was diluted in 10 mL ultrapure water,

133 providing three 100 mg L^{-1} stock solutions of ^{106}Cd, ^{65}Cu and ^{53}Cr. A 1,000-µg L^{-1} Ge
134 solution in 0.5 N HNO_3 was prepared by diluting a 1,000 mg L^{-1} Ge standard solution
135 (PlasmaCal, SCP Science, Courtaboeuf, France).

136 2.2. Labeling of the leaf-discs

137 Alder leaves (*A. glutinosa*) were collected in November 2007 after abscission and before leaf
138 fall and stored in plastic boxes. The labeling protocol was based on the recommendations
139 from Felten (2003) to favor gammarids appetence for the labeled leaves. Prior to the pulse-
140 chase experiment, the alder leaves were placed in an aquarium filled with several liters of
141 stream water for 20 d at 12°C. The water was renewed once (after 2 d) to remove the first
142 exudates from the leaves. Twenty leaf-discs were produced using a 2 cm diameter punch. The
143 discs were exposed for 3 d to mineral water spiked with ^{53}Cr (413 µg L^{-1}), ^{65}Cu (390 µg L^{-1})
144 and ^{106}Cd (793 µg L^{-1}). These solutions were obtained by diluting 200 µL of the ^{53}Cr stock
145 solution, 200 µL of the ^{65}Cu stock solution and 400 µL of the ^{106}Cd stock solution in 50 mL
146 ultrapure water. Because this medium would be too acidic to allow leaf conservation, drops of
147 a 1 M NaOH solution (prepared using NaOH pellets (Acros Organics, SLR pellets extrapure)
148 and deionized water) were added to raise the pH to 7.5 one minute after introduction of the
149 discs. The rapid rise in pH favors the binding of the metallic cations to the ligands on the leaf
150 surface, hence adsorbing the metal onto the disc surfaces. After exposure, the discs were
151 rinsed for 4 d in a 20 L stirred aquarium with ultrapure (Ultra Analytic M2, Elga, Veolia
152 Water, France) water to remove the weakly adsorbed metals.

153 2.3. Pulse-chase feeding method

154 We performed a pulse-chase feeding experiment adapted from previous studies (Croteau *et*
155 *al.*, 2007). The gammarids were given leaf-discs enriched with stable isotopes during a hot-

156 feeding phase (the "pulse" phase) of 7 h and 24 min and were then were given unlabeled
157 leaf-discs during a 7-d depuration phase. The pulse duration must be shorter than the gut time
158 passage (time to defecate the first tracers after the pulse) to avoid any tracer recycling. Based
159 on previous studies (Smokorowski *et al.*, 1998, Felten, 2003), the *G. pulex* gut retention time
160 (GRT), i.e., the time to defecate 90% of the organic matter ingested during the pulse, was
161 assumed to be 3 d. Hence, the gut time passage was expected to be 16 h, applying a 4.5 ratio
162 between the gut time passage and the GRT, as determined by Croteau et al. (2007) for the
163 gasteropod *L. stagnalis*. The duration of the defecation phase was chosen to ensure complete
164 recovery of the tracers. Moreover, Cr was assumed to be an inert tracer of the digestion
165 process in gammarids because it is fully unassimilated, allowing the quantification of the
166 ingested tracers at any time during the pulse-chase experiment.

167 Ten feeding chambers allowing the collection of amphipod feces were designed based on
168 Werner's model (Werner, 2000) (photos are provided in the supplemental information). The
169 fecal production of an individual *G. pulex* within a few hours weighting ~ 10 µg, its metal
170 content was not measurable. Therefore, a group of seven *G. pulex* was used in each chamber.
171 They were placed in the upper portion of a 125 mm^3 chamber constructed of 5 mm mesh
172 cubic bags (immersed) and feces were collected on the bottom of the 600 mL plastic beakers.
173 In two control treatments, labeled C1 and C2 (the eight other treatments were numbered from
174 1 to 8), unlabeled leaf-discs were given to the gammarids during the pulse. Both the labeled
175 and unlabeled leaf-discs were consumed at the same rate (~ one disc per d by seven
176 gammarids, based on visual observations of food residues). At best, to prevent cannibalism,
177 refuges consisting of a 5 mm mesh square (6 cm^2) wedged into a 5 L bottle cap were also
178 immersed in each beaker. In one beaker, one gammarid was consumed by its congeners
179 during the pulse. No other mortality was observed over the course of the experiment.

Exuviated molts were re-ingested by the gammarids (no exuviates were observed during the pulse). Approximately five juveniles, newly born during the experiment, were removed from the beakers when discovered. Feces were harvested at 8.5, 24, 36, 48, 72, 96 and 166 h according to the following method. The feces were concentrated in a clean 5 cm diameter plastic Petri dish using a 10 mL polyethylene pipette. This concentrate was transferred to the top of a filtering system (a polytetrafluoroethylene (PTFE) filter unit on a vacuum system) and filtered through a PTFE membrane filter (Whatman, 0.45 µm pore size, 25 mm). At the end of the experiment, the gammarids were dried for metal analysis. Filtered water samples (Whatman PTFE, 10 mL syringe filters, 0.5 µm) were obtained from each replicate at the beginning of the pulse and before every feces collection. The samples were immediately acidified by the addition of 200 µL ultrapure nitric acid. The leaves remaining at the end of the pulse were also collected for metal analysis.

2.4. Preparation of the samples

The feces on the filters, the reference material ERM-CE 278 mussel tissue (IRMM, Brussels, Belgium) and the pools of gammarids were dried for 48 h at 45°C and handled in individual covered Petri dishes to prevent atmospheric deposition of trace metals. The fecal matter was weighed on pre-weighted PTFE filters on a microbalance (Sartorius SE 2-F, 0.1 µg). Ultra-clean or disposable material was used throughout. The vessels, beakers and the PTFE filtering system were acid washed in a 10% HNO_3 bath for 48 h and rinsed with ultrapure water before use. The mussel tissue reference material (ERM-CE278, 40 and 132 mg) and the gammarids (a pool of seven gammarids of approximately 40 mg), leaves (≈ 10 mg) and feces (pooled feces of the experimental group of gammarids ≈ 4 mg) were digested with HNO_3 (65% Suprapur, Merck, Darmstadt, Germany) (50 µL per mg) for 24 h followed by the addition of 20 µL mg^{-1} H_2O_2 (30% (w/w) in H_2O, Sigma-Aldrich) in closed SCP-Science polyethylene

tubes for DigiPrep. Subsequently, the solution was heated to 95°C for approximately 4 h in a DigiPrep Block (SCP Science) under a laminar flow hood to ensure evaporation to near dryness. The samples were immediately removed from the heating block when dryness was reached (100 µL residue maximum) and volume adjusted to 5 mL with a 0.5 N HNO_3 solution. Finally, 5 µL of a 1,000 µg L^{-1} Ge solution was added to the sample solutions because Ge was used in this study as an internal standard to monitor the ICP-MS signal drifts (see below). All of the volumes were monitored by weighing the tubes at relevant steps during preparation of the samples.

2.5. ICP-(CCT)-MS analysis

Chromium isotopes (of atomic mass 50, 52, 53 and 54), copper (of atomic mass 63 and 65), cadmium (of atomic mass 106, 108, 110, 111, 112, 113, 114 and 116) and germanium (of atomic mass 72 and 74) were analyzed by an ICP-MS equipped with collision-cell technology (CCT) (CCT-X Series, ThermoFisher Scientific, Courtaboeuf, France), as proposed by Nixon et al. (2000) for interference-free measurements of Cr isotopes. The isobaric interferences on the determination of Cr isotope concentrations have been identified by Croteau et al. (2007) as one of the primary analytical issues of the method. The samples were analyzed in the standard mode and in the CCT mode with no mathematical corrections for interferences and Ge was used as an internal standard.

Quality controls of the ICP-MS calibration consisted of analysis of SRM 1640 river water sample (NIST, Gaithersburg, Maryland USA) and mussel tissue ERM-CE 278 in duplicates. In the CCT mode, the limits of detection (LOD), defined as the mean of ten blank signals plus three times the standard deviation (SD) of the blank, were as follows: 0.001 µg L^{-1} for ^{53}Cr, 0.0025 µg L^{-1} for ^{65}Cu and 0.0002 µg L^{-1} for ^{106}Cd.

227 The net tracer concentrations were determined using the equations adapted from Croteau et
228 al. (2004) (see Supporting Information).

229 2.6. Ingestion rate and assimilation efficiencies calculation

230 To calculate the net amounts of the ingested tracers, the mass of the leaf-discs (m_{leaves} in mg)
231 ingested by a pool of gammarids during the pulse was determined as the cumulative net
232 amount of ^{53}Cr defecated during the depuration, $\Sigma\ ^{53}Cr_{feces}$ (ng) divided by the net ^{53}Cr
233 concentration (ng mg^{-1}) in the remaining parts of the leaf-discs sampled at the end of the
234 pulse, as described in the following equation:

$$m_{leaves} = \frac{\sum {}^{53}Cr_{feces}}{[{}^{53}Cr\ ]_{leaves}} \qquad \text{Equation 1}$$

235 The net tracer amounts of the ingested ^{106}Cd and ^{65}Cu ($^{106}Cd_{leaves}$ and $^{65}Cu_{leaves}$) were
236 calculated by multiplying m_{leaves} by the net tracer concentrations of ^{106}Cd and ^{65}Cu in the
237 leaves.

238 The mean ingestion rate IR (g g^{-1} d^{-1}) was calculated as the m_{leaves} divided by the dry mass of
239 the gammarids (m_{org}) at the end of the experiment and the duration of the pulse.

240 The assimilation efficiency (the fraction of ingested metal that remains in the organism) was
241 calculated using the following two methods: the mass balance method (Luoma and al., 1992;
242 Cain *et al.*, 2011) and the ratio method (Wang and Fisher, 1999).

243 Using the mass balance method, the AE values for Cu and Cd were calculated under the
244 assumption that the net amount of ingested metal is equal to the sum of the defecated and
245 internalized tracers. Note that this calculation does not consider the mass of the ingested food
246 or its contamination. For example, for Cd, the AE value was calculated as follows:

248 $$AE = \frac{\Delta^{106}Cd_{org}}{\Delta^{106}Cd_{org} + \sum^{106}Cd_{feces}}$$ Equation 2

249 Using the ratio method, the AE value is derived directly from the ICP-MS signals of the
250 isotopes, including the ^{53}Cr tracer, e.g., for Cd, see the following Eq. 3:

251 $$AE = 1 - \frac{(^{106}Cd/^{53}Cr)_{feces}}{(^{106}Cd/^{53}Cr)_{leaves}}$$ Equation 3

252 where $(^{106}Cd/^{53}Cr)_{feces}$ is the ratio of the ^{106}Cd and ^{53}Cr signal intensities in the feces after
253 depuration and $(^{106}Cd/^{53}Cr)_{leaves}$ is the ratio of the ^{106}Cd and ^{53}Cr signal intensities in the
254 spiked leaf-discs. Using the ratio method, $^{106}Cd_{org}$ is assumed equal to the difference between
255 $^{106}Cd_{leaves}$ and $\Sigma^{106}Cd_{feces}$ and the mass of the ingested tracer is evaluated through the inert
256 tracer ^{53}Cr. The primary advantage of the ratio method over the mass balance method is that
257 the AE value can be calculated even if the defecation is incomplete. Additional
258 methodological considerations on the ratio method have been provided elsewhere (Wang and
259 Fisher, 1999; Croteau *et al.*, 2007). In this study, only the first sample of collected feces was
260 considered. In subsequently produced feces, the ^{53}Cr signal intensities were less than two
261 times that of the control feces, validating the application of the ratio method to the first feces
262 defecated, under the hypothesis that all of the tracers at stake are released simultaneously
263 (Wang and Fisher, 1999). This point was further tested by studying the tracer defecation
264 profiles.

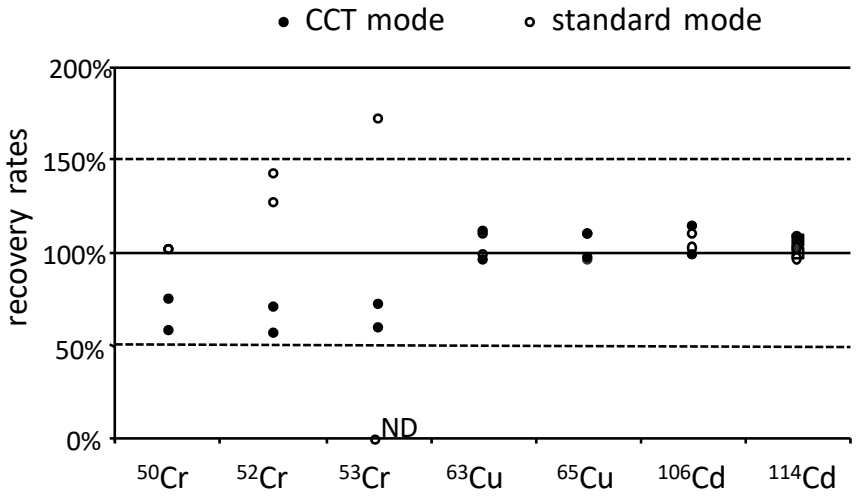
265265

3. Results and discussion

3.1. ICP-(CCT)-MS analytical performances

The observed concentrations of Cd and Cu were within 7% of the reference values in the CCT and standard modes (ERM-CE 278 mussel tissue (Fig. 1) and SRM-1640 water). In opposite, the Cr determined concentrations were significantly different from the certified values for the certified mussel tissue (Fig. 1). Because the reference material is not tracer-enriched, the concentration of one element should be the same regardless of the isotope used to calculate it; this was confirmed for the CCT mode only. Nevertheless, the observed Cr concentrations in the CRM mussel tissue were lower than the certified value with a 36% underestimation. The underestimation value was constant from a replicate, from one isotope to another and when increasing the mass of the digested mussel tissue. The digestion procedure used for certification being potentially more effective for Cr compounds solubilization (Lamberty and Muntau, 2005) than that the one we applied, we attributed the 36% underestimation value on the Cr isotope concentrations observed to a partial but reproducible digestion and assumed an identical underestimation for all of the solid samples (tissue, feces and leaves).

The CCT mode, by decreasing the polyatomic interferences, primarily with argide ions ($^{12}C^{40}Ar$) on ^{52}Cr and ($^{37}Cl^{16}O$) and ($^{13}C^{40}Ar$) (Tanner et al., 2002), significantly improved the isotope content determination in digested invertebrates, feces and leaves, as previously observed by Hammer et al. (2005) for Cr analysis in foodstuffs.



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286 **Figure 1**: The use of collision cell technology (CCT) improves the Cr analysis regularity
287 among its various isotopes. The recovery rates of Cr, Cu and Cd in the reference material
288 mussel tissue (ERM-CE-278) were inferred from various isotopes of Cr, Cu and Cd and are
289 expressed as a percentage of the certified value. The empty marks represent the results in the
290 standard mode, and the filled marks are those in the CCT mode. ND represents "not
291 determined" (the ^{53}Cr-signal in the standard mode for one sample was inferior to the blank
292 value).

293

294 Consequently, the subsequent results were based on the ICP-(CCT)-MS. We verified that the
295 results derived from the net ^{53}Cr measurement (the mass of ingested leaves, IR and AE
296 calculations) remained unchanged regardless of whether we corrected the Cr concentrations of
297 the 36% underestimation.

298 3.2. Tracer analysis in samples of various matrices

299 The stable isotope concentrations in the labeled alder leaf-discs are presented in Fig. 2. The
300 ratios of the tracer concentration in labeled food to that in unlabeled food were 119 for (^{53}Cr),
301 4.7 for (^{65}Cu) and 2914 for (^{106}Cd). The Cu enrichment of the leaves was less than that of
302 other tracers, which is a consequence of the high natural content of copper in alder leaves
303 compared with Cr and Cd and of the high natural abundance of ^{65}Cu. As a result, the total
304 metal concentration in labeled leaves was significantly increased compared to the unlabeled

ones, but did not exceed the metal concentration in litter sampled in a river draining an ancient uranium mining site (Schaller et al., 2010).

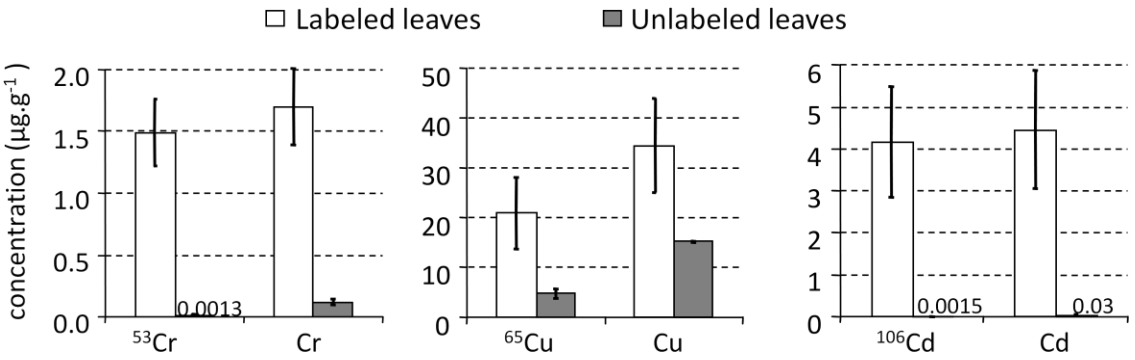

Figure 2: Tracer and elemental composition of the alder leaf-discs. White bars: discs labeled with the three tracers, mean value (*n*=7). Gray bars: unlabeled discs. Mean value ± SD (*n*=10).

The concentrations of the tracers initially occurring in the water a few minutes after introduction of gammarids and leaves were as follows: [^{53}Cr] < LOD, [^{65}Cu] = 275 ± 123 ng L^{-1}, [^{106}Cd] = 2.2 ± 0.7 ng L^{-1} (n=8). At the end of the pulse period, the ^{53}Cr concentration remained undetectable, the ^{65}Cu concentration remained stable ([^{65}Cu] = 352 ± 114 ng L^{-1}, (n=8) and the ^{106}Cd concentration increased significantly to 11 ± 4 ng L^{-1} (n=8) (Student *t*-test, p=6.10^{-6}). During the depuration, the concentrations of the tracers in the water were below the detection limits, except for ^{65}Cu ([^{65}Cu] = 167 ± 41 ng L^{-1}).

The three tracers were nearly completely defecated by the gammarids (>95%) within the first three time steps (Fig. 3), and the initial feces (collected at 8.5 h) contained as much as 82% of the net ^{53}Cr amount recovered during the experiment. Afterwards, a sharp decrease in the fecal tracer content was observed within the first two days. The gut retention time was 24 h,

which is three times less than what was expected based on previous studies (Smokorowski *et al.*, 1998; Felten, 2003). This value is similar to that in the snail *Lymnaea stagnalis* (22.5 h (Croteau *et al.*, 2007)). The high contribution of the first sample of feces to the overall fecal contamination suggests that gammarids began to excrete tracers before 9 h. Thus, the gut time passage could not be precisely determined by the present study; however, it was clearly less than 9 h.

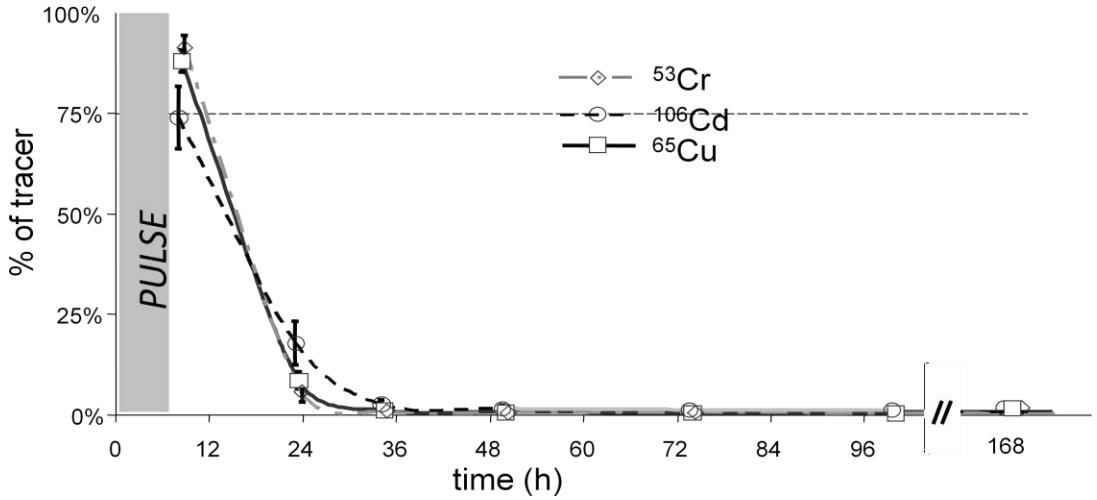

Figure 3: Time series of the fecal tracer release (%). Mean ± SD (*n*=8) of the normalized fecal release (by the sum of the defecated tracer amounts) for each tracer during the depuration phase.

Gammarids did not accumulate detectable amounts of ^{53}Cr. It was verified *a posteriori* that ^{53}Cr was not absorbed through the diet and could be used as the inert tracer for digestion in gammarids. The cumulative amounts of chromium recovered in the feces and the net tracer concentrations in the labeled leaves led to a mean IR of 0.69 ± 0.21 g g^{-1} d^{-1}. During the pulse (7 h 24 min), the *G. pulex* consumed the quantity of food typically consumed in one or two days (see Maltby et al. (2002) for the reference ingestion rate), most likely because of the 3 d starvation period that preceded the experiment. The high IR value also validates the leaves

342 labeling protocol, as Malty et al. (2002) showed that gammarids may decrease their feeding
343 rate in polluted environments.

344 On average, two molts were re-ingested by each group of gammarids during the depuration
345 phase. The mass of an exuviate was approximately 2.5 mg, representing less than 10% of the
346 mass of the food ingested during the depuration phase. Thus, molt ingestion was not
347 considered in further calculations and should not represent a bias of the pulse-chase feeding
348 method.

349 ^{65}Cu and ^{106}Cd were internalized in the gammarids after the 7-d experiment at net tracer
350 concentrations of 378 ± 102 and 161 ± 81 ng g^{-1} (n=8) respectively (vs. 100 and 6 ng g^{-1},
351 respectively, in the control treatments, n=2).

352 To estimate the amount of the assimilated tracer in the gammarids during the pulse, the
353 measurement of the tracers in the gammarids at the end of the depuration phase should be
354 corrected from the possible waterborne influx during the pulse and from the efflux that
355 occurred during the depuration phase. Because of the Cd tracer leakage during the pulse,
356 approximately 0.06 ng of ^{106}Cd may have been taken up by the gammarids by water uptake
357 (considering a 9 ng L^{-1} tracer concentration in the water, an uptake rate from the water of 0.46
358 L g^{-1} d^{-1}(Pellet *et al.*, 2009), a 40 mg dry weight of gammarids and a 0.31 d pulse duration).
359 This value was fifty times lower than the animal body burden at the end of the depuration
360 phase (Table 1). Thus, the contribution of water to the overall tracer accumulation was
361 neglected. Given the 0.032 d^{-1} efflux rate, 19% of Cd assimilated during the pulse should have
362 been excreted during the depuration phase. Thus, to estimate the Cd body burden at the end of
363 the pulse, a 19% correction was applied to the amount of ^{106}Cd measured in the gammarids
364 for the Cd AE calculations.

365365

366366

367 **Table 1**. Net ^{106}Cd amounts (ng) in the various matrices and the mass balance of the net ^{106}Cd
368 assimilation efficiency of Cd. The amount of ingested tracer was estimated from the amount
369 of ^{53}Cr measured in the feces. The tracer content of the gammarids at the end of the pulse was
370 estimated from the amount measured at the end of the experiment, the efflux rate of Cd (Pellet
371 *et al.*, 2009) and the depuration time. The mass balances were evaluated as the ratio of ^{106}Cd
372 outputs (for the gammarids at the end of the pulse and the feces) and inputs (the leaves). The
373 assimilation efficiency was estimated from the mass balance and the ratio methods (see text).

	Ingested Leaves	Leakage during pulse	Feces	Gammarids end of exp.	Gammarids after the pulse	Mass balance	Assimilation efficiency mass balance method	Assimilation efficiency ratio method
1	108	5	68	5.4	6.7	70%	9%	16%
2	87	3	51	5.1	6.3	66%	11%	19%
3	54	3	50	4.0	4.9	101%	9%	29%
4	129	7	31	9.2	11.4	33%	27%	47%
5	93	5	66	5.4	6.6	78%	9%	18%
6	49	3	28	3.0	3.8	65%	12%	42%
7	161	8	111	13.8	17.1	80%	13%	5%
8	128	6	71	6.7	8.2	62%	10%	12%
Mean (n=8)	101	5	60	6.6	8.1	69%	13%	24%
SD	38.2	1.9	26.3	3.4	4.3	19%	6%	15%

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376 Similarly, gammarids exposed to 352 ng L^{-1} of dissolved ^{65}Cu should have taken up 61 ng of
377 ^{65}Cu from waterborne exposure (given a 16.9 L g^{-1} d^{-1} influx rate and a 1.2 d^{-1} efflux rate
378 (Lebrun *et al.*, 2012)). In contrast to Cd, this value is in the same range as the amount
379 remaining in the gammarids at the end of the depuration phase (33 ± 4 ng in the pool of 69
380 gammarids). Given the efflux rates of Cu, more than 99% of the assimilated Cu should have
381 been excreted during the depuration phase (the calculated ^{65}Cu internalized immediately after
382 the pulse is 42 500 ± 11 615 ng). Therefore, the waterborne exposure via potential feces
383 dissolution is not negligible in the amount of assimilated metal during the pulse, but the latter

cannot be determined properly. In our study, the rapid exchange rates of Cu in the gammarids prevent the evaluation of the assimilation of diet-borne copper with the pulse-chase feeding method. The half-time of the substance must be significantly larger than the GRT, which is the case for *Lymnaea* (Croteau *et al.*, 2007) or mayflies (Cain *et al.*, 2011) but clearly not for gammarids.

3.3. Derivation of the assimilation efficiencies

3.3.1 The case of copper

The entire duration of the experiment was too short in regards to copper regulation, preventing the application of the mass balance method; however, the ratio method, limited to the first collected feces sample, still applies. The tracer ratio in the leaves was constant during the experiment, and one can assume than the tracer ratio in the feces is not biased by possible waterborne exposure or tracer released in the water. However, the copper AE value could not be determined by the ratio method because the $^{65}Cu/^{53}Cr$ ratios of the leaf-discs (26 ± 5.7, n=7) were lower than those in the feces (37 ± 6.5, n=7), which hinders the determination of the Cu AE value by this method. The unexpected enhancement of the $^{65}Cu/^{53}Cr$ ratio in the feces compared with that of the leaves was attributed to an analytical issue in the ^{65}Cu determination. Because neither feces from the control experiment nor the blank values of the filtration method were contaminated in ^{65}Cu, it is unlikely that the ratio enhancement is due to ^{65}Cu overestimation in the feces. A ^{65}Cu underestimation in the leaves occurred, which was most likely due to a matrix effect on the copper measurements in the ICP-(CCT)-MS and/or incomplete digestion. A specific and reinforced digestion procedure for the determination of trace level metallic isotopes in the labeled leaf matrix, following the recommendations of Lamberty and Muntau (2005), could improve the ^{65}Cu determination in the leaves.

Additionally, a collision gas mixture containing ammonia would be more efficient than the H_2/He mixture used in this study to reduce the isobaric interferences due to $^{44}Ca^{18}OH^+$ and $^{48}Ca^{16}OH^+$ ions on the ^{63}Cu and ^{65}Cu determinations, respectively, which are interferences that degrade the precision of the isotope content determination (Fialho et al., 2011). Finally, the use of high resolution-ICP-MS may efficiently prevent those analytical issues.

Copper AE values are rare in the literature because of the lack of a suitable radiotracer (Croteau *et al.*, 2004). Hence, one important goal of the present study was to test the feasibility of using ^{65}Cu to trace Cu assimilation in an amphipod. The ability of gammarids to rapidly regulate internalized copper and the relative liability of the element in an organic matrix, which tends to be released in water, added to the analytical issues in the determination of stable isotopes of copper, hindering the determination of the copper AE value for the crustacean *G. pulex*.

3.3.2. Cadmium assimilation efficiency

The ratios of $^{106}Cd/^{53}Cr$ were significantly higher in the leaves compared with the feces. The corresponding AE values calculated with the ratio method are given in Table 1. The mean cadmium AE value was 24% ± 15% (n=8). The assimilation efficiencies of cadmium were also evaluated for each replicate with the mass balance method using the amount of tracer measured in the gammarids and in the feces. The results, displayed in Table 1, are lower than the AE values estimated with the ratio method. This discrepancy is at least partly explained by the unbalanced budget of ^{106}Cd, as displayed in Table 1 in which the sum of ^{106} Cd in the gammarids and feces is lower than the estimated amount of ingested ^{106}Cd. It is possible that a portion of the tracer in the feces is leaked into the water and not measured, which would explain part of the unbalanced budget and the underestimation of the $^{106}Cd/^{53}Cr$ ratio and the

net amount of ^{106}Cd in the feces. Consequently, in both methods, the AE value would have been overestimated. However, Cd leakage from the feces is necessarily limited because no significant net amount of ^{53}Cr was measured in the water at the end of the experiment.

Despite the variation in the estimated AE value according to the calculation method, we infer that the Cd AE value for *G. pulex* is within the range of 5-47%. Although associated with a high uncertainty, all of the estimations of the Cd AE values for *G. pulex* are lower than those measured for the amphipod *Mysis relicta* (the AE value is estimated as 72%, and the tracer defecation kinetics in *Mysis* occur with an approximate GRT of 2 d (Smokorowski et al., 1998)) or to other species, such as gastropods (AE> 73% in *Lymnaea stagnalis*, Croteau et al., 2007), bivalves (AE values in the 19–72% range for *Dreissena polymorpha*, Roditi and Fisher, 1999) or mayflies (AE > 71% for all of the tested species (Cain et al., 2011)). Higher assimilation efficiencies of metals are generally associated with longer food processing times (Decho and Luoma, 1996; Croteau and Luoma, 2005). Thus, the AE values in the present study may have been influenced by the rapid food processing in this particular experiment. Gammarids in the field withstand starvation periods of more than 10 d (Felten, 2003) and can store food in their midgut (Correia and al., 2002); hence, their GTP is plausibly variable. Therefore, the influence of the GTP on the metal AE value in gammarids may deserve further investigation for diet-borne uptake. Indeed, the design of the stable isotopes based pulse-chase feeding method brings a number of potential sources of stresses that may affect strongly the GTP whatever our concerns about favoring appetence and limiting stress factors along the experiment.

451 3.4. Waterborne uptake vs. diet-borne uptake of cadmium

452 Based on the estimated Cd assimilation efficiencies, we evaluated the relative contribution of
453 diet-borne exposure in gammarids to Cd contamination. Leaves, gammarids and water
454 samples were obtained in April 2008 in an upper affluent of La Mauldre River at Vicq
455 (*Coordinates RGF93* longitude: 01°49'57" E, latitude: 48°48'49" N). Three types of leaves
456 located in amphipod habitats were sampled in duplicates as follows: leaves in fine gravel
457 habitats, showing signs of an advance bacterial and fungal decomposition process; the same
458 type of leaves in fine sediments; green leaves, immersed and showing fractionation marks.
459 Pools of five adult gammarids of approximately 10 mm were sampled at the sampling site
460 (eight replicates). The organisms and soft parts of the leaves were dried, weighted and then
461 digested using a protocol described elsewhere (Pellet *et al.*, 2009). The metal labile
462 concentrations in the water were estimated at the sampling site using the diffusive gradient in
463 thin-films (DGT) technique (Davison and Zhang, 1994) by measuring the labile metal
464 concentration using six thin films. Deployment and data processing are described elsewhere
465 (Tusseau-Vuillemin *et al.*, 2007). Pellet et al. (2009) have previously shown that the
466 waterborne Cd uptake in gammarids could be better estimated based on the labile Cd
467 concentration rather than the total dissolved concentration. The Cd contents of all of the
468 samples were analyzed by graphite furnace atomic absorption spectrometry (GF-AAS Varian
469 SpectrAA-20, Les Ulis, France).

470 The Cd concentration in the gammarids was estimated following the biodynamic model at
471 steady-state, according to the following Eq. 4 (Luoma and al., 1992):

4724 $$[M]_{org}^{SS} = \frac{AE.IR}{k_e}.[M]_{leaves} + \frac{k_u}{k_e}[M]_w$$ Equation 4
7
2

where $[M]_{org}^{SS}$ is the metal concentration in the gammarids at steady state (µg g^{-1}), $[M]_{leaves}$ is the metal concentration in the leaves (ng g^{-1}), k_u is the waterborne uptake rate (L g^{-1} d^{-1}), and $[M]_w$ is the labile metal concentration in the water (ng L^{-1}). The parameters and the measured concentrations are reported in Table 2. The k_u and k_e values were obtained from Pellet et al. (2009). A 0.1 g g^{-1} d^{-1} value was estimated based on the measurement of the ingestion rate at 14°C by Maltby et al. (2002). This selected IR value is significantly lower than the value measured during the pulse-chase experiment that could be artificially increased by the starvation step. This will minimize the estimation of the diet-borne uptake.

Table 2: Biodynamic forecasts of Cd bioaccumulation in *G. pulex*. $[M]_w$ is the labile metal concentration measured by the diffusive gradient in thin films technique. $[M]_{org}^{SS}$ is the estimation at steady state of the Cd body burden in *G. pulex*. The max and min data correspond to the maximal and minimal contaminations of the food and the subsequent results in terms of bioaccumulation modeling.

Assimilation efficiency	AE	%		18
Ingestion rate	IR	g g^{-1} d^{-1}		0.1
Uptake rate from water	k_u	L g^{-1} d^{-1}		0.40
Elimination rate	k_e	d^{-1}		0.032
Field observations				
Leaves concentration	$[M]_{leaves}$	µg g^{-1}	min	0.04 ± 0.02
		µg g^{-1}	max	0.65 ± 0.29
Water labile concentration	$[M]_w$	ng L^{-1}		1 ± 0.3
G. pulex body-burden	$[M]_{org}$	µg g^{-1}		0.11 ± 0.03
Model outputs				
Body-burden	$[M]_{org}^{SS}$	µg g^{-1}	min	0.035
		µg g^{-1}	max	0.387
Diet-borne contribution		µg g^{-1}	min	0.023
		µg g^{-1}	max	0.366
waterborne contribution		µg g^{-1}		0.013

Using the range of all of the estimations from both methods, the value of 18% was set for the AE. Green leaves (minimal hypothesis) were less Cd-contaminated than the decomposed leaves sampled on gravels and sediments (maximal hypothesis), leading to two sets of estimations for the diet-borne contribution to the overall Cd bioaccumulation (Table 2). Simulations showed that Cd was bioaccumulated via both uptake pathways by the gammarids and that the waterborne contribution (0.013 µg g^{-1}) was low compared with the Cd diet-borne contribution (0.023-0.366 µg g^{-1}). The Cd diet-borne contribution was 66-94% of the observed Cd body-burden even though we minimized the ingestion rate. As previously hypothesized by Abel and Barlöcher (1988), the diet-borne uptake pathway significantly contributed to the Cd bioaccumulation in freshwater gammarids.

The field-measured contamination of gammarids (0.11 ± 0.03 µg g^{-1}) is between the minimal and maximal estimated values, indicating that *G. pulex* did not feed on only fresh or decomposed leaves. This result highlights the importance of the behavior of gammarids in metal diet-borne transfer. Moreover, the type of leaf may influence not only the contamination of the food and the bioavailability in the gut (AE) but also the feeding strategy. All of these parameters must be addressed to properly evaluate the importance of food quality on the diet-borne contribution to metal transfer in gammarids, and they must be considered in the definition of bioavailability.

4. Conclusion

A methodological adaptation of the pulse-chase feeding method using stable isotope tracers was investigated to evaluate the Cd and Cu assimilation efficiencies of *G. pulex* feeding on alder leaves. The use of collision cell technology in ICP-MS analysis significantly improved the quantification of the chromium isotopes because it allows reliable net tracer

512 ^{53}Cr determinations in unlabeled and labeled samples. The pulse-chase feeding protocol
513 using metal stable isotopes is able to address the large modifications/adaptations that the
514 physiological characteristics of each species require. For the amphipod *G. pulex* feeding on
515 conditioned alder leaves, the Cd assimilation efficiency was evaluated within the range of 5 –
516 47%. In contrast, the approach was not adapted to the copper AE estimation because of the
517 rapid regulation of Cu in gammarids. We also suspect analytical shortcomings with the ^{65}Cu
518 determination in leaves, which might be overcome by adapting the digestion procedure to the
519 various matrices involved in the experiment and/or by using a higher mass resolution
520 analytical instrument.

521 Comparisons between the observed Cd body-burden in gammarids and the estimated diet-
522 borne contributions in field conditions provides evidence that the diet-borne uptake in situ
523 significantly contributes to the overall bioaccumulation of Cd. *G. pulex* is a promising study
524 organism for basic research on diet-borne availability of trace metals in an aquatic ecosystem
525 because it has a large food spectrum and adopts various types of feeding behavior within
526 different physical and ecological conditions.

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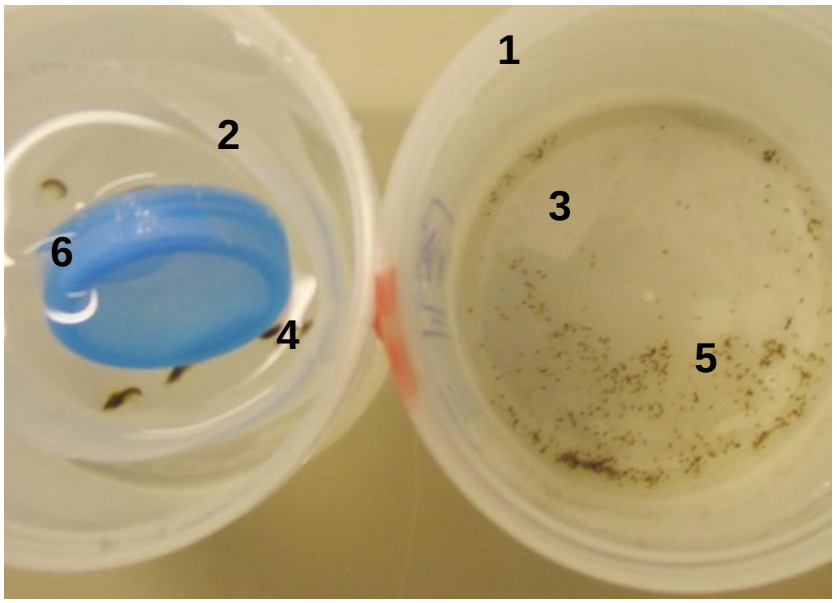
Supplemental information

SI 1: The double-deck defecation chamber. 1. defecation chamber, 2. upper compartment (set aside in a beaker), 3. lower compartment, 4. Gammarids, 5.f eces, 6. toy (so they can hide)



SI 2: Calculation of the net tracer concentrations

Net tracer concentrations in all samples were determined using the equations adapted from Croteau et al. (2004).

First, the p^m ratio was determined for each tracer of atomic mass m (m=53, 65 and 106). p^m is the relative ICP-MS signal intensity over the cumulative total intensities of all analyzed isotopes for the same element in calibration standards. This quantity is assumed to be constant in all non-spiked natural matrixes. For example for Cd, p^{106} was defined as:

$$p^{106} = mean\left(\frac{I(^{106}Cd)}{I(^{106}Cd)+I(^{108}Cd)+I(^{110}Cd)+I(^{111}Cd)+I(^{112}Cd)+I(^{113}Cd)+I(^{114}Cd)+I(^{116}Cd)}\right)_{STANDARDS}$$

Equation 1

where $I(^{106}Cd)$ is the ICP-MS signal intensity (counts) of ^{106}Cd in the calibration standards. The isotopes of atomic masses 50, 52, 53, 54 were used for Cr and 63 and 65 for Cu. We found $p^{106} = 0.011$, $p^{53} = 0.083$ and $p^{65} = 0.33$.

Second, the total tracer concentration ($[^{106}Cd]$) and the original tracer concentration ($[^{106}Cd]^0$) were calculated in the samples.

The total tracer concentration was calculated as:

$$[^{106}Cd] = p^{106} \cdot [T^{106}Cd] \qquad \text{Equation 2}$$

where $[T^{106}Cd]$ is the elemental Cd concentration inferred by the ICP-MS software from the ^{106}Cd signal intensity, in µg L^{-1}. The original tracer concentration is the fraction of the concentration of the tracer expected in absence of a spike, given its relative abundance p^n. It was determined according to:

$$[^{106}Cd]^0 = p^{106} \cdot [T^{114}Cd] \qquad \text{Equation 3}$$

Lastly, the net tracer concentration ($\Delta[^{106}Cd]$) was assessed by subtracting the contribution of the original tracer concentration from the total tracer concentration of the sample, which leads to:

$$\Delta[^{106}Cd] = p^{106} \cdot ([T^{106}Cd] - [T^{114}Cd]) \qquad \text{Equation 4}$$

where all quantities are expressed in µg L^{-1} (in the sample).

Same calculations were performed to calculate the net ^{65}Cu and ^{53}Cr concentration. The isotopes 52 and 63 were used to determine the original tracer concentration of Cr and Cu, respectively.

SI 3: Table of mean values (± standard deviation) of water chemistry parameters.

conductivity(μσ cm^{-1})	1755 ± 64
pH	7.7 ± 0.1
temperature (°C)	12.1 ± 0.8
dissolved organic carbon (mg L^{-1})	2.63
Anions and cations	mg L^{-1}
HCO^{3-}	263
Cl^{-}	23.5
NO^{3-}	0.95
SO_4^{2-}	10.6
Na^{+}	16.1
K^{+}	5.49
Ca^{2+}	93.4
Mg^{2+}	6.9
NH^{4+}	0.46