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Cadmium assimilation in the terrestrial isopod, *Porcellio dilatatus* – Is trophic transfer important?

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Abstract

Terrestrial isopods have become important tools for the ecotoxicological assessment of metal-contaminated soils. Their value as an invertebrate model is partly because of their extraordinary capacity to bioaccumulate toxic metals from the environment. Replication of this accumulation process in the laboratory has in the past relied on the amendment of organic food substrates through the addition of inorganic metal salts. However, the bioavailability of the metals when presented through doping regimes may differ from the bioavailability of metals in nature, because over time metals become biologically compartmentalised and form complexes with organic molecules. This study examines the differential bioavailability of Cd to the terrestrial isopod, *Porcellio dilatatus*, when presented as either a Cd-amended diet or pre-incorporated biologically into lettuce (*Lactuca sativa*). Isopods were either provided with lettuce contaminated superficially with $Cd(NO_3)_2$ or lettuce grown hydroponically in growth media containing 100 μM $Cd(NO_3)_2$. Assimilation efficiency of Cd was greater among isopods that were fed the amended diet (71%, S.E.=7%), than among isopods feeding on biologically contaminated lettuce (52%, S.E.=5%) and demonstrates that speciation of Cd is likely to influence the rate of Cd assimilation and accumulation in a laboratory test.

Keywords: Heavy metal; Cadmium; Bioavailability; Trophic transfer; Terrestrial ecotoxicology; *Lactuca sativa*

1. Introduction

In recent years, a growing number of researchers have recognised the need to incorporate the principles of trophic transfer within the design of metal toxicity studies with invertebrates (e.g. Devi et al., 1996; Allinson et al., 2000; Merrington et al., 2001; Maryański et al., 2002; Simon and Boudou, 2002; Green et al., 2003; Hendrickx et al., 2003; Wallace and Luoma, 2003; Hansen et al., 2004; Mann et al., 2004). All these authors provided their test species with prey items that had accumulated metallic contaminants while still alive. In this way, they attempted to simulate the movement of metallic contaminants through the food chain, and thereby incorporate within their tests the complexities of metal speciation and bioavailability in biological systems. The results of such studies are not easily predictable, because both the metal-binding properties of the prey species and subsequent bioavailability to the predator are likely to be highly variable. For example, Hendrickx et al. (2003) described extremely high levels of Cd assimilation and a complete lack of depuration of Cd in wolf spiders (*Pirata piraticus*) feeding on Cd-contaminated flies. Conversely, Hopkin and Martin

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(1985) demonstrated that the spider, *Dysdera crocata* (a species that feeds exclusively on isopods), did not assimilate Cd or lead from contaminated isopods collected from a smelting works. In this case the difference appears to be related to the ability of *D. crocata* to eliminate Cd prior to gut absorption and points to an evolutionary adaptation in a species that specialises in eating crustaceans known to bioaccumulate metals (Hopkin and Martin, 1985; Paoletti and Hassall, 1999).

The bioavailability of metals in soil is generally thought to be dictated by the free ion activity model (FIAM), which predicts that only metals existing as free metal ions (Me^+) are available for uptake across membranes (McLaughlin, 2002). The concentration of Me^+ is dictated by physiochemical properties of the soil such as pH, the nature of metal exchange sites within the organic and inorganic matrices (McLaughlin et al., 2000; Peijnenburg, 2002), their binding affinity for soluble anionic ligands within soil pore-water (e.g. chloride Lock and Janssen, 2003b; Weggler et al., 2004), and competition for those by sites with other cations in a solution. These parameters dictate the "environmental availability" of a metal in any matrix. The biotic ligand model (BLM), developed for use with fish, expands on the FIAM by proposing the gill as a biotic ligand that competes with the various environmental exchange sites for Me^+-binding (Paquin et al., 2002). The capacity of the biotic ligand to bind and internalise metal ions (within the limitation of their environmental availability) is determined by physiological mechanisms and thereby dictates the "bioavailability" of metal ions. Bioavailability models like the BLM perform well with regard to predicting metal bioavailability in water-borne exposures (Niyogi and Wood, 2004), and is likely also to be predictive of metal bioavailability to plants and soft-bodied soil organisms where the major routes of exposure are absorption from pore-water directly across roots (Antunes et al., 2006) or body-walls (Peijnenburg, 2002; Lock and Janssen, 2003a).

The digestive tract also acts as a biotic ligand (Hogstrand et al., 2002). However, the FIAM may not hold true with regard to the dietary exposure route because of the likely presence of active transport mechanisms that have the capacity to transport metal-bound organic (or inorganic) complexes across the gut. Such mechanisms have been demonstrated in mammals (Groten et al., 1991; Sugawara and Sugawara, 1991) and trout (Harrison and Curtis, 1992; Kjoss et al., 2006). Indeed, studies on trout indicate that protein-bound Cu or Cd is more readily taken up via the trout gut, than diets amended with inorganic metal salts. Absorption of metal complexes in the gut has also been demonstrated in aquatic crustaceans (Fisher and Hook, 2002; Xu and Wang, 2002), however it remains unclear if the dietary form or speciation of the metal affects the assimilation efficiency in invertebrates.

Because of their capacity to accumulate large body-burdens of toxic metals, terrestrial isopods have been widely adopted as model species for the examination of metal accumulation and toxicity testing (Drobne, 1997; Hornung et al., 1998b). Because terrestrial isopods are hard-bodied soil invertebrates, accumulation of Cd (among other metals) is predominantly through dietary exposure rather than absorption through the body wall (Vijver et al., 2005). Also, isopods are saprophytic detritivores, and if the food they consume is contaminated with a metallic compound, only a limited proportion of that metal is likely to be present as free Me^+. A large proportion of the metal is likely to be present in a form that has resulted from biological sequestration and transformation by either the micro-organisms growing on the decaying organic matter or by the organic matter itself while it was part of a living system (Ledin et al., 1999; Rauser, 1999; Magyarosy et al., 2002). However, virtually all previous laboratory-based examinations of metal accumulation and toxicity in terrestrial isopods have relied exclusively on addition of inorganic metal salts to organic substrates. The degree to which the metals in those studies were transformed into 'species' of greater or lesser bioavailability is dependant on the physiochemical environment and the degree of microbial activity within the experimental systems, and is therefore a source of variability within the experimental systems. The aim of this study was to examine the role of biological metal sequestration in the assimilation efficiency of cadmium in a terrestrial isopod.

Cadmium was chosen because it is a priority pollutant in Europe (Council Directive 76/464/EEC), is readily accumulated by isopods with low to negligible depuration rates (Witzel, 2000), and permits comparisons with other animal models that have examined similar questions (i.e. Harrison and Curtis, 1992; Zalups and Ahmad, 2003; Mann et al., 2006).

We provided terrestrial isopods with lettuce that had been, either:

1. Biologically contaminated via hydroponic culture in contaminated media. Lettuce contaminated in this way will have a high proportion of the Cd in the form of Cd–protein complexes or Cd-*S*-conjugates (e.g. Cd–glutathione, Cd–cysteine) (Maier et al., 2003), or
2. Superficially contaminated with $Cd(NO_3)_2$.

2. Materials and methods

2.1. Food substrate

Lettuce was selected as a suitable food substrate based on previous feeding and contamination trials (Mann et al., 2005). Three treatments (diets) were established to study the influence of metal speciation on the bioavailability of Cd to the terrestrial isopod *Porcellio dilatatus*.

1. Biologically contaminated lettuce (BCL)
2. Superficially contaminated lettuce (SCL)
3. Non-contaminated (control) lettuce (CON)

2.2. Test organisms

Isopods were selected from in-house cultures of *P. dilatatus* derived from individuals collected from a secondary coastal dune system in central Portugal. They were maintained at 20 °C with a 16:8 h (light/dark) photoperiod on a substrate of sand within plastic containers. Alder leaves were provided as food (Caseiro et al., 2000; Kautz et al., 2000).

2.3. Lettuce growth and contamination

Lettuce (*Lactuca sativa* cv. Reine de Mai de Pleine Terre) plants were grown from seed as described in Mann et al. (2005). Briefly, lettuce seeds were germinated on a bed of perlite moistened with distilled water and subsequently grown hydroponically at 25 °C on a ~6 mm column of perlite within polystyrene seedling trays (24 mm; Polisur 2000, Huelva, Spain) floating on aerated nutrient media within plastic boxes. The nutrient media used for growth of lettuce was based on Hoagland's media: macronutrients – KNO_3, 6 mM; $Ca(NO_3)_2$, 4 mM; $NH_4H_2PO_4$, 2 mM; $MgSO_4$, 2 mM; micronutrients – H_3BO_3, 50 μM; $MnCl_2$, 10 μM; $ZnSO_4$, 0.77 μM; $CuSO_4$, 0.36 μM; Na_2MoO_4, 0.37 μM; Fe^{3+}–EDTA, 4.5 μM. For all plants a 16:8 h (light/dark) photoperiod was established with an array of fluorescent tubes (Mazdafluor Prestilflux TFP 36W/CFT) suspended ~30 cm above the seedlings/plants. After 5 weeks of culture the nutrient media was altered to include 100 μM Cd as $Cd(NO_3)_2$ (Mann et al., 2005). The Cd solution included 200 pCi ml^{-1} ^{109}Cd (Perkin-Elmer, Boston, MA, USA). The lettuce plants were grown within the contaminated media for a further 7 days with replacement of growth media every 2 days to avoid depletion of nutrients and changes in Cd concentration as a consequence of evaporation, exclusion from the plants or adsorption to plant roots (Mann et al., 2005). The plants were dried (2 days at 60 °C) and individual leaves cut into sections (midvein excluded) according to desired mass (~10 mg) and Cd content. Cd content varied even within individual leaves (Mann et al., 2005). Therefore, leaf sections that contained between 300 and 600 μg Cd g^{-1} dry wt were selected for use in the experiment.

Uncontaminated dried and sectioned lettuce designated for use as SCL was contaminated by topical addition of 10 μl mg^{-1} of a 360 μM $Cd(NO_3)_2$ stock solution that also contained 660 pCi ml^{-1} ^{109}Cd (Perkin-Elmer, Boston, MA, USA). The leaves were again dried before use. All Cd-amended leaf sections were analysed for Cd by radiospectrometry to ensure that they contained approximately 400 μg Cd g^{-1} dry wt.

2.4. Feeding study

One day before starting the experiment a total of 60 juvenile isopods were selected by weight (mean = 47 mg, range = 35–65 mg) and isolated for 24 h without food to purge their gut. They were placed in individual polyethylene terephthalate (PET) boxes (∅ 85 mm × 43 mm; Termoformagen, Leiria, Portugal). No distinction was made between sexes. The bottom of each box was replaced with a 2-mm nylon screen. Each of these boxes was inserted within a second box containing a thin layer of plaster of Paris mixed with activated charcoal (8:1 vol./vol.) for the retention of added moisture. The distance between the nylon screen and the plaster of Paris was ~5 mm. The screen allowed faecal pellets to drop through to the plaster substrate where they could be collected for weighing and analysis for Cd, and prevented coprophagy.

Twenty individuals were impartially allocated to each treatment. The food was cut into individual portions weighing between 5 and 10 mg (dry wt) and moistened before placing it within each box. Animals were fed for a period of 4 weeks exclusively on lettuce according to treatment. Faecal material was removed from the surface of the plaster of Paris every 2 days and dried (2 days at 60 °C). At the end of each week, the food was replaced with fresh leaves of a known mass, and the remains of the old food were dried (2 days at 60 °C) and weighed. The food was replaced to prevent the consumption of food which had become inoculated with fungi – the growth of fungi may have altered the bioavailability of Cd. At the end of 4 weeks, the isopods were left for 24 h without food to purge their guts, weighed and analysed for Cd by radiospectrometry. Data on isopod, faecal pellet and leaf mass were used to determine indices of isopod growth, food consumption and assimilation efficiency.

2.5. Cadmium analysis

Sections of dry lettuce leaf (before feeding and lettuce remains after feeding), isopods and faecal matter were analysed for Cd by radiospectometry. Samples were counted in a Genesis Gamma-1 bench-top gamma counter (Laboratory Technologies, Maple Park, IL, USA). Data on Cd content of leaves, isopods and faecal material were used to determine indices of Cd consumption and assimilation efficiency. The 360 μM and 100 μM contamination solutions were analysed for Cd by inductively coupled plasma mass spectroscopy (ICP–MS) in a X-series ICP–MS with PolyCon nebuliser. Specific activities of the 360 μM and 100 μM Cd contamination solution were assessed by comparing gamma counts with measurements obtained by ICP–MS.

2.6. Data analysis

Lettuce assimilation efficiency was calculated as:

$$AE_{lett} = (C_{lett} - F)/C_{lett} * 100$$

where C_{lett} is mass of lettuce consumed, and F is the mass of faecal material produced. Cadmium assimilation efficiency, was calculated as either:

$$AE_{Cd} = I_{Cd}/C_{Cd} * 100$$

where I_{Cd} is the amount of Cd within the isopod at the termination of the feeding trial, and C_{Cd} is the amount of Cd consumed, or as:

$$AE_{Cd}(C_{Cd} - F_{Cd})/C_{Cd} * 100$$

where F_{Cd} is the amount of Cd within the faecal pellets.

SigmaStat (version 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests. One-way ANOVA with Student–Newman–Keuls' post hoc test was used to determine differences ($\alpha = 0.05$) in mass gain/loss, lettuce consumption, and lettuce assimilation efficiency among CON, BCL and SCL treatment groups. Student's t-tests were performed to determine differences ($\alpha = 0.05$) in indices of Cd consumption, assimilation and assimilation efficiency. Where data failed to fit a normal distribution, a Mann–Whitney rank sums test was employed ($\alpha = 0.05$).

3. Results

3.1. Analyses of Cd content in BCL and SCL treatment groups

ICP–MS analysis indicated that the nominally 360 and 100 μM contamination solutions were 365 and 102 μM, respectively. Measured concentrations were used for all calculations. Superficially contaminated leaf sections provided to the SCL treatment group contained (mean ± S.D.) 391 ± 31 μg Cd g^{-1} dry wt (range: 338 to 450 μg Cd g^{-1} dry wt). Biologically amended leaf section provided to the BCL treatment group contained (mean ± S.D.) of 482 ± 94 μg Cd g^{-1} dry wt (range: 327 to 604 μg Cd g^{-1} dry wt).

3.2. Isopod growth, lettuce and Cd consumption, assimilation and assimilation efficiency

Isopods in all treatment groups lost weight during the trial (Fig. 1A). Some animal mortality occurred in each treatment group – 5, 7 and 5 animals died in the CON, BCL and SCL treatment groups, respectively.

Lettuce consumption was low. Isopods ate approximately 0.3 mg mg $animal^{-1}$ (wet wt) over 4 weeks

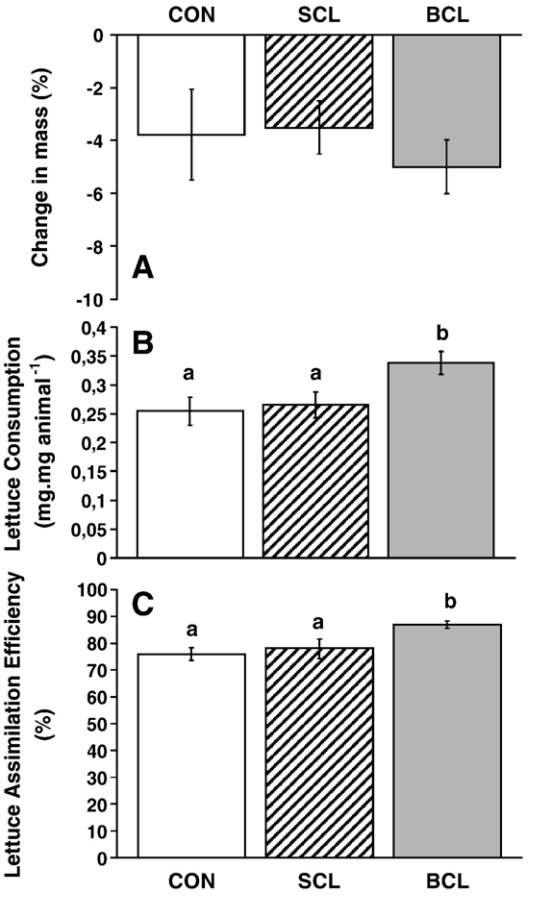

Fig. 1. (A) Change in isopod mass (%). (B) Lettuce consumption by isopods. (C) Lettuce assimilation efficiency by isopods. All error bars represent S.E. ($n = 13$–15). Lower case letters a, b and c denote statistically significant ($P < 0.05$) groupings following an ANOVA with Student–Newman–Keuls' post hoc test. CON – control lettuce; SCL – superficially contaminated lettuce; BCL – biologically contaminated lettuce.

(Fig. 1B). There were statistically significant differences in lettuce consumption between treatment groups (one-way ANOVA, $P=0.029$; Student–Newman–Keuls' test, $P<0.05$).

Lettuce assimilation efficiency (AE_{lett}) was high among all treatment groups (Fig. 1C). There were statistically significant differences in AE_{lett} between biologically contaminated lettuce and control and between biologically contaminated lettuce and artificially contaminated lettuce (one-way ANOVA, $P=0.020$; Student–Newman–Keuls' test, $P<0.05$).

Although a *t*-test indicated a marginally non-significant difference ($P=0.054$), isopods in the BCL treatment group consumed more Cd than the SCL groups (Fig. 2A). The amount of total Cd assimilated by the two treatment groups was the same (Fig. 2B) with no statistically significant differences between them (Mann–Whitney, $P=0.240$). Assimilation efficiencies, when calculated as $AE_{Cd}=(I_{Cd}/C_{Cd})*100$ were (mean±S.E.) 52±5% and 71 x±7% for the BCL and SCL groups, respectively (Fig. 2C) with a statistically significant difference between them (*t*-test, $P=0.047$). Assimilation efficiencies, when calculated as $AE_{Cd}=(C_{Cd}-F_{Cd})/C_{Cd}*100$, were (mean±S.E.) 77±2% and 84±3% for the BCL and SCL groups, respectively (data not shown). The difference was not statistically significant (*t*-test, $P=0.089$).

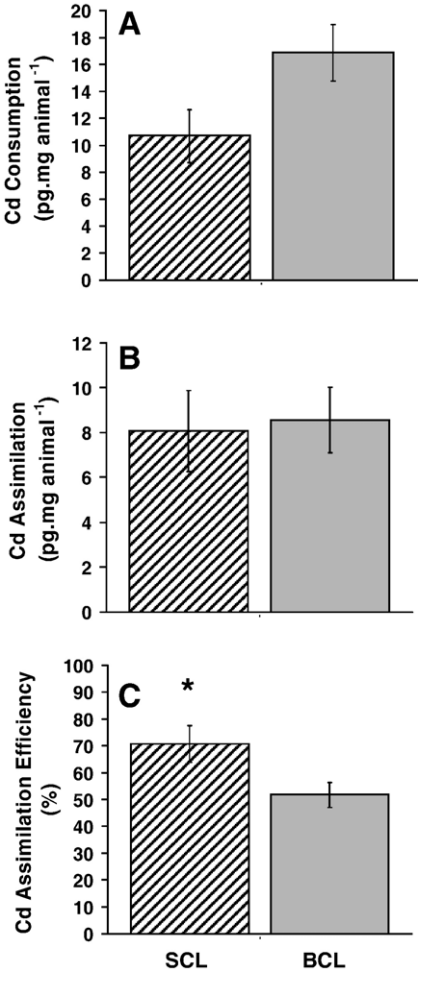

Fig. 2. (A) Cd consumption by isopods. (B) Cd assimilation by isopods. (C) Assimilation efficiency of Cd by isopods. All error bars represent S.E. ($n=14$). The asterisk denotes a statistically significant difference ($P<0.05$) following Student's *t*-test. CON – control lettuce; SCL – superficially contaminated lettuce; BCL – biologically contaminated lettuce.

4. Discussion

This study provides support for the contention that Cd-speciation influences the level of Cd assimilation by terrestrial isopods. Isopods ate more lettuce if it had Cd biologically incorporated within it (BCL), and as a consequence they consumed more Cd than those isopods feeding on lettuce with Cd added superficially (SCL). Despite this, the actual amount of Cd assimilated by each treatment group was similar because the SCL group assimilated Cd more efficiently than those eating biologically contaminated lettuce (BCL). This result is consistent with the results obtained in numerous studies with mammals (Zalups and Ahmad, 2003; Andersen et al., 2004) and reptiles (Mann et al., 2006) but contrary to those described for rainbow trout (Harrison and Curtis, 1992).

Consumption of lettuce was 3 to 4 times lower than observed in a previous study that indicated that lettuce was readily consumed and promoted growth in juvenile isopods (Mann et al., 2005). By contrast, isopods in this study lost weight irrespective of treatment group, with at least 25% mortality which is assumed to be related to inadequate nutrition. The only notable difference between the two studies was the age of the isopods. The earlier study used younger animals (~17 mg), and the imperative to eat is possibly greater among very young animals. Failure to eat at the commencement of this kind of test is a common problem when the food substrate is fresh leaf material. Leaves generally become more palatable to isopods only after the onset of microbial colonisation (Zimmer, 2002), possibly because microbial pre-conditioning tends to lower the C/N ratio (Zimmer et al., 2003). Many dietary studies have overcome this problem by either using alder leaves (Mann et al., 2005), which have inherently low C/N ratios (Kautz et al., 2000), or by augmenting leaf substrates with N-rich pet-foods (e.g. Crommentuijn et al., 1995; Farkas et al., 1996; Hornung et al., 1998a; van

Straalen et al., 2005). In this study, increased consumption would be expected if the leaves were left long enough to allow microbial preconditioning; however, it was important to avoid widespread microbial colonisation which might have changed the speciation of the Cd, particularly in the SCL treatment group, so food could not be left for more than a week. Even during the course of one week, it is conceded that Cd bioavailability might change as a consequence chemical interactions within the moistened leaves.

More intriguing is the fact that there were higher levels of food consumption and assimilation among isopods in the BCL treatment group, than in either the CON or the SCL treatment group. We can assume that the Cd itself did not bestow greater palatability upon the lettuce, because the SCL lettuce was no more (or less) palatable than the control food. However, it is possible that the contamination procedure itself altered the palatability of the lettuce. One of the *a priori* assumptions for these trials was that Cd incorporated biologically into the lettuce must exist predominantly as a Cd-*S*-conjugate or Cd–protein complex (Mann et al., 2005). This is a reasonable assumption because it is known that exposure to Cd^{2+} induces the production of amino acid, glutathione and cysteine-rich phytochelatins in lettuce (Costa and Morel, 1994; Maier et al., 2003). Thus, an overall increase in N in the form of metal-binding organic content may afford the lettuce a greater degree of palatability to isopods.

Food assimilation efficiency was high (>70%) and has been a consistent characteristic of these studies when lettuce is provided as food. High food assimilation efficiency (>80%) was also described by Lirette et al. (1992) in snails provided with lettuce. Note, however, that the high food assimilation rates in this study are likely to be an overestimate because of the difficulty in accounting for all the faecal pellets produced by the isopods (see below).

The isopods in this study assimilated 52% (BCL) and 71% (SCL) of the Cd consumed, which is in stark contrast with assimilation rates found in vertebrates, which generally assimilate less than 10% of the Cd that enters the gut (Zalups and Ahmad, 2003; Mann et al., 2006). The AE values reported in this study are also higher than those reported previously for isopods, which range from 30% up to 50% in *Porcellio scaber* (Donker and Bogert, 1991; Khalil et al., 1995). However, Zidar et al. (2003) reported AE values for Cd ranging from 32% to 100% for *P. scaber* feeding on hazel leaves augmented with 1000 μg Cd g^{-1} to 125 μg Cd g^{-1}, respectively.

It could be argued that the higher rates of Cd assimilation in the SCL group could have resulted as a consequence of direct adsorption of Cd to the outer exoskeleton as isopods moved over the contaminated leaves, which is less likely to occur in the BCL group because the Cd is internalised within the lettuce. If this were the case, then an estimation of AE_{Cd} as $AE_{Cd} = (C_{Cd} - F_{Cd})/C_{Cd}$ is likely to provide an indication of gut AE_{Cd} alone. AE_{Cd} calculated in this manner resulted in AE_{Cd} values of 77% and 84% for the BCL and SCL treatment groups, respectively. As expected these are overestimations of AE_{Cd} because of the difficulty in accounting for all the faecal pellets produced by the isopods. Also, leaching of Cd from the faecal pellets to the plaster of Paris may have occurred (the plaster of Paris was not analysed for Cd). Accordingly, caution should be exercised in interpreting these data, which still indicate higher gut AE_{Cd} in the SCL group, but without a statistically significant difference between them. Perhaps more pertinent are the findings of Vijver et al. (2005) who demonstrated that adsorption of Cd to the isopod exoskeleton does not occur, and that the Cd-burden is due exclusively to ingested Cd.

Numerous factors influence metal AE in terrestrial isopods. Zidar et al. (2003) demonstrated that food-metal concentration will effect AE of Zn, Cu and Cd in *P. scaber*. Abdel-Lateif et al. (1998) also performed a similar analysis of the influence of temperature and Cd concentration on the rate of Cd accumulation in *P. scaber*, while Hopkin (1990) demonstrated that different species have different accumulation capacities for Cd, Zn and Pb. Metal speciation can be added to the list of factors which will influence the level and rate of accumulation of metals.

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