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DOES SUBCELLULAR DISTRIBUTION IN PLANTS DICTATE THE TROPHIC BIOAVAILABILITY OF CADMIUM TO *PORCELLIO DILATATUS* (CRUSTACEA, ISOPODA)?

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Abstract—The present study examined how subcellular partitioning of Cd in plants with different strategies to store and detoxify Cd may affect trophic transfer of Cd to the isopod *Porcellio dilatatus*. The plant species used were *Lactuca sativa*, a horticultural metal accumulator species; *Thlaspi caerulescens*, a herbaceous hyperaccumulator species; and the nonaccumulator, *T. arvense*. Taking into account that differences in subcellular distribution of Cd in plants might have an important role in the bioavailability of Cd to a consumer, a differential centrifugation technique was adopted to separate plant leaf tissues into four different fractions: cell debris, organelles, heat-denatured proteins, and heat-stable proteins (metallothionein-like proteins). Plants were grown in replicate hydroponic systems and were exposed for 7 d to 100 μM Cd spiked with ^{109}Cd. After a 14-d feeding trial, net assimilation of Cd in isopods following consumption of *T. caerulescens* and *T. arvense* leaves reached 16.0 ± 2.33 and 21.9 ± 1.94 μg/g animal, respectively. Cadmium assimilation efficiencies were significantly lower in isopods fed *T. caerulescens* (10.0 ± 0.92%) than in those fed *T. arvense* (15.0 ± 1.03%). In further experiments, Cd assimilation efficiencies were determined among isopods provided with purified subcellular fractions of the three plants. On the basis of our results, Cd bound to heat-stable proteins was the least bioavailable to isopods (14.4–19.6%), while Cd bound to heat-denatured proteins was the most trophically available to isopods (34.4–52.8%). Assimilation efficiencies were comparable in isopods fed purified subcellular fractions from different plants, further indicating the importance of subcellular Cd distribution in the assimilation. These results point to the ecological relevance of the subcellular Cd distribution in plants, which directly influence the trophic transfer of Cd to the animal consumer.

Keywords—Assimilation efficiency Centrifugal fractionation Dietary metal *Porcellio dilatatus* Trophically available metal

INTRODUCTION

Cadmium (Cd) is a toxic metal that is able to accumulate in soils. It reaches soils mainly from mining (e.g., zinc mining), several industrial activities, and agricultural use of phosphate fertilizers and sewage sludge. Cadmium can be strongly cytotoxic and mutagenic to plants and animals, interfering in a wide variety of metabolic processes in plants and animals [1]. The toxic action of Cd is facilitated through its propensity to bind to the sulfhydryl groups of proteins and substitute for essential metals such as Zn in metalloenzymes and through the inhibition of DNA mismatch repair and production of reactive oxygen species [1,2].

However, some plants are able to accumulate Cd in edible tissues at high concentrations without showing symptoms of toxicity, thereby introducing the metal into the food chain by trophic transfer. Plants use sequestration mechanisms to detoxify metals and prevent interaction with important biomolecules. These mechanisms include binding to proteins and other ligands (e.g., metallothionein [MT]-like proteins) and storage of metals into metabolically inactive cellular sites, such as granules inside vacuoles [1,3]. These two major detoxification pathways in plants may have implications for the trophic transfer to animal consumers.

Extensive studies have been made on Cd bioaccumulation and toxicity to organisms, and models have been developed to predict the bioavailability and toxicity of metals [4,5]. However, they do not yet consider the contribution of the dietary route of metal exposure and the relevance of the complexity of internal metal subcellular partitioning in prey, which may significantly affect the subsequent trophic transfer of metals to predators [4]. In an attempt to develop a predictive model for the dietary accumulation of metals in marine food chains, a subcellular fractionation procedure has gained popularity [6–12]. Wallace and Luoma [8] postulated that Cd associated with the subcellular fractions organelles, heat-denatured proteins (HDP), and heat-stable proteins (HSP) of prey was trophically available metal (TAM) and was assimilated at an efficiency of approximately 100% by the predator, while Cd bound to metal-rich granules was less bioavailable to predators [8,10]. This is considered a simple and pragmatic approach in the prediction of trophic transfer of metals and a first step toward a practical tool that could explain most of the variability observed in metal accumulation and toxicity in organisms [13]. However, there is a need to apply this approach to other food chains in order to verify its utility.

To the best of our knowledge, trophic transfer of metals from plants through terrestrial food chains has not received much attention, despite being of great relevance. However, there have been some studies focusing on the cellular and

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subcellular distribution of metals in plants. Subcellular localization of Cd in plants has been assessed through subcellular fractionation [14,15], and other techniques such as autoradiography [16], energy dispersive X-ray microanalysis [16–18], and electron energy loss spectroscopy [19]. In lettuce 64% of accumulated Cd is partitioned to cell walls [15], and both *Lactuca sativa* L. and *Thlaspi arvense* L. possess detoxification mechanisms in which phytochelatins (PC) play an important role [20,21]. *Thlaspi caerulescens* was found to mainly store Cd in electron-dense granules inside vacuoles by means of complexation with malate [22,23]. Cadmium distribution reflects internal processing that occurs during Cd uptake and accumulation in plants and can be used to interpret metal toxicity and tolerance. In addition, knowledge of how organisms handle their accumulated metal may allow more accurate predictions of the eventual transfer of metals to higher trophic levels [8].

In the present study we tested the hypothesis that subcellular distribution in plants will dictate the trophic bioavailability of Cd to isopods. To achieve this, the transfer of Cd in a food chain comprised of plant leaves and a detritivorous animal, the isopod *Porcellio dilatatus*, was examined with the following specific aims: to investigate the relevance of different subcellular distribution in plants in the assimilation efficiency (AE) of the isopod, and to determine the assimilation of Cd from each subcellular fraction of plants, to directly assess the role of each fraction in the assimilation of Cd by isopods. For this, three plants with different patterns of Cd accumulation were studied: *T. caerulescens* J. & C. Presl is a hyperaccumulator of several metals, including Cd, and is a plant commonly used as a model in metal transport and accumulation studies with a view to use in phytoremediation [24–26]; the related nonaccumulator *T. arvense*; and lettuce (*L. sativa*), which is a Cd-accumulating plant and an important human food crop. The terrestrial isopod *P. dilatatus*, inhabiting the upper layer of the soil and surface leaf litter, is quite abundant in southern Europe and an important representative of the detritivorous soil fauna. Moreover, isopods have an enormous capacity to accumulate large body burdens of toxic metals [27] making them a valuable model for the examination of metal assimilation and accumulation.

MATERIALS AND METHODS

Cadmium trophic transfer from plants to isopods was assessed in three different and complementary experiments: assessment of Cd subcellular distribution in the plants *L. sativa*, *T. caerulescens*, and *T. arvense*; feeding experiment 1, an assessment of isopod (*P. dilatatus*) Cd AEs from plant leaves of *T. caerulescens* and *T. arvense*; feeding experiment 2, an assessment of isopod Cd AEs from individual subcellular fractions of *L. sativa*, *T. caerulescens*, and *T. arvense* plant leaves.

Plant culture and growth conditions

Seeds from *L. sativa* (Reine de Mai de Pleine Terre) (Oxadis, Saint Quentin Fallavier, France), *T. caerulescens* (Saint-Félix-de-Pallières, Ganges, France), and *T. arvense* (Amsterdam, The Netherlands) were germinated under dark conditions on filter paper moistened with distilled water. After germination, seedlings were transferred to perlite support media in polystyrene seedling trays floating on nutrient solution. The trays were maintained in a plant growth chamber (APT.line® KBWF, Binder, Tuttlingen, Germany) with controlled temperature (20 ± 1°C), 16:8 h light:dark photoperiod, 80% humidity, and 200 μmol/($m^2 \cdot s$) light intensity. Lettuce was grown in modified Hoagland's nutrient solution according to Monteiro et al. [28]. *Thlaspi* plants were grown on modified Rorison nutrient solution with the following basic composition (μM): 1,500 KNO_3, 1,000 $Ca(NO_3)_2 \cdot 4H_2O$, 500 $NH_4H_2PO_4$, 500 $MgSO_4 \cdot 7H_2O$, 46.25 H_3BO_3, 0.77 $ZnSO_4 \cdot 7H_2O$, 0.36 $CuSO_4 \cdot 5H_2O$, 0.37 $NaMoO_4 \cdot 2H_2O$, 10.12 $MnCl_2 \cdot 4H_2O$, 17.91 $FeCl_3$. Before Cd exposure, *L. sativa* and *T. arvense* were grown for five weeks. Since *T. caerulescens* grows more slowly, to achieve a similar plant biomass, this plant was grown for nine weeks before Cd exposure. Plants were exposed to Cd for 7 d; the respective nutrient solution was supplemented with $Cd(NO_3)_2$ at 100 μM and 77 nCi/ml ^{109}Cd (Amersham Biosciences, London, England). Control plants were maintained in nutrient solution with no addition of Cd. The nutrient solution was continuously aerated and changed on alternate days to avoid depletion of nutrients and changes in Cd concentration during the course of the exposure to the metal [29]. After 7 d of exposure, plant leaves were frozen in liquid nitrogen and stored at −80°C until subcellular fractionation analysis or dried at 60°C for subsequent use in isopod feeding experiment.

Subcellular Cd distribution in plant leaves

Differences in plant subcellular Cd distribution were investigated by subjecting plant leaves to the following procedure. Replicated (n = 6–10) 1-g samples of leaves from each species were each reduced to powder with liquid nitrogen using a mortar and pestle and homogenized in 4 ml of buffer containing 0.25 M sucrose, 1 mM dithioerythritol, and 50 mM Tris-HCl (pH 7.5) [14]. All steps were performed at 4°C and according to Weigel and Jäger [14] with some modifications based on Wallace et al. [6]. The resulting homogenate was filtered through nylon cloth (50 μm) and washed twice with homogenization buffer. The filtrate was then centrifuged at 500 *g* for 5 min. The resulting pellet combined with the residue of the filtration contained mainly cell walls, tissue fragments, and other cellular debris and was designated as cell debris. The supernatant of the first centrifugation step, containing the cytosol, was then centrifuged at 100,000 *g* for 30 min to sediment organelle components (i.e., chloroplasts, mitochondria). The pellet was designated as the organelle fraction. The 100,000 *g* supernatant containing the cytosol fraction was then heat denatured at 80°C for 10 min and cooled on ice for 15 min. Heat-denatured proteins were separated from the HSPs (MT-like proteins) by centrifugation at 50,000 *g* for 10 min. All fractions were assayed for Cd by radiospectrometry and metal contents were used to calculate distributions of Cd within plant leaves based on summation of Cd content of the four subcellular fractions.

Isopod culture and feeding test conditions

Cadmium AEs in isopods fed *T. arvense* and *T caerulescens* leaves was assessed in this experiment. A similar experiment has already been performed in *L. sativa* leaves by Calhôa et al. [30]. In the current experiment we used *P. dilatatus* from laboratory cultures derived from individuals collected in a secondary coastal dune system in central Portugal. Isopods were maintained in plastic containers with sand substrate and kept at 20°C with a 16:8 h light:dark photoperiod. Alder leaves were provided as a source of food and distilled water was added to maintain moisture.

Twenty juvenile isopods (weight range: 14–19 mg) per

treatment were selected and isolated individually in test boxes for 24 h before the test without food to purge the gut. No distinction was made between sexes. Polyethylene terephthalate boxes (diameter 85 mm × 43 mm; Termoformagen, Leiria, Portugal), containing a thin layer of plaster of Paris mixed with activated charcoal (8:1 v/v) for the retention of added moisture were used as individual test boxes. Food was replaced every week to prevent consumption of food that had become inoculated with fungi; fungi growth may alter Cd bioavailability. Fecal pellets were collected every day to prevent coprophagy.

Feeding experiment 1

Leaves from control and Cd-exposed plants of *T. arvense* and *T. caerulescens* were cut into individual portions weighing approximately 10 mg (range 8.1–10.9 mg dry wt), assayed for ^{109}Cd (438.9 ± 88.76 and 236.2 ± 53.30 μg Cd/g dry weight [mean ± standard deviation] in Cd-exposed *T. arvense* and *T. caerulescens*, respectively) and moistened before being placed in test boxes. Animals were fed for a period of 14 d exclusively on leaves according to treatment. Food was replaced every week with fresh leaves and the remains of food were dried (2 d at 60°C), weighed and analyzed for Cd by radiospectrometry. After 14 d, isopods were left for 24 h without food to purge their guts and were then weighed and analyzed for Cd. Fecal pellets were collected and dried (2 d at 60°C) to be weighed. Data on isopod, fecal pellet, and leaf mass were used to determine indices of isopod growth, food consumption, and AE. Plant AE by isopods was calculated as

$$AE_{plant} = (C_{plant} - F)/C_{plant} \times 100 \quad (1)$$

where C_{plant} is the mass of plant leaf consumed by isopods, and F is the mass of fecal material produced. Radiospectrometry data obtained from the isopods and food were used to determine indices of Cd AE. Cadmium AE was calculated as

$$AE_{Cd} = I_{Cd}/C_{Cd} \times 100 \quad (2)$$

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.

Feeding experiment 2

Leaves of *L. sativa*, *T. arvense*, and *T. caerulescens* exposed to Cd and were subjected to subcellular fractionation as described above. The four different fractions obtained (cell debris, organelles, HDP, and HSP) were mixed (1:2) with a gelatine solution prepared from 2.5 g gelatine powder (VWR Prolabo, Fontenay Sous Bois, France) and 12.5 ml ultra pure water and were then mixed by vortexing [31]. As a supernatant, HSP fraction presented higher volume than the other pellet fractions, therefore these fractions were concentrated by evaporation under a stream of nitrogen before being mixed with gelatine. Aliquots of 7 μl of the mixture (fraction and gelatine) were pipetted onto Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA), forming gelatine discs that were stored frozen at −20°C until required [32]. Additionally, gelatine discs were prepared containing either gelatine alone or a mixture of homogenate of control plant leaves and gelatine solution (1:2) to be used as a control foods (control 1 and control 2, respectively).

Isopods were fed gelatine discs for a period of 28 d. Because some of the fractions were likely to contain very small quantities of Cd, a longer duration was chosen for this feeding experiment to ensure that accumulated Cd was above the detection limits of analysis. This longer period of exposure will not influence Cd AE since elimination of Cd is negligible in isopods [33] and therefore allows for comparisons with the above experiment. Gelatine discs were previously assayed for Cd by radiospectrometry before being fed to isopods and were replaced every week; the remains of food were also assayed for Cd. After 28 d isopods were left in test boxes without food to purge their guts, and after 24 h were weighed and analyzed for Cd. The Cd content and mass data obtained for isopods and the gelatine discs were used to determine isopod growth and Cd AE as described above.

Estimation of AE of Cd from whole plant leaves (AE_{whole}) based on AE for individual fractions was calculated by the following mass balance equation:

$$AE_{whole} = \sum AE_i \times Cd_i \quad (3)$$

where AE_i is the Cd AE of isopod fed a purified fraction i and Cd_i is the percentage of Cd in the subcellular fraction i [11,32].

Cadmium analysis

All samples of subcellular Cd distribution were placed in 10.4-ml polycarbonate tubes (Beckman instruments, Fullerton, CA, USA) and analyzed for ^{109}Cd in a Genesis Gamma-1 bench-top gamma counter (Laboratory Technologies, Maple Park, IL, USA). Sections of dry plant leaf and gelatine discs (before feeding and remains after feeding), isopods, and fecal material were placed in 3.5-ml Röhren tubes (Sarstedt, Newtown, NC, USA) and were analyzed for Cd by radiospectometry. Data on Cd content of leaves, isopods, and fecal material were used to determine indices of Cd consumption and AE.

Cadmium concentration in the hydroponic culture medium was verified by inductively coupled plasma spectroscopy (Horiba Jobin Yvon, 70 Plus, Longjumeau, France) compared with radiospectometry measurements and used as a reference for calculations of total Cd content.

Statistical analysis

Statistical analysis was carried out by *t* tests or one-way analysis of variance and Tukey post hoc tests as appropriate. When necessary, data were transformed to achieve normality and equality of variance. When these criteria were not satisfied even with transformed data, nonparametric tests were performed, namely Kruskal–Wallis one-way analysis of variance followed by Dunn's method post hoc test. SigmaStat® (Ver 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests.

RESULTS

Cadmium subcellular distribution in plant leaves

Cadmium subcellular distribution in *L. sativa*, *T. arvense*, and *T. caerulescens* in relation to the total Cd accumulated in leaves is shown in Figure 1. The cell debris fraction represents an important pool of the total accumulated Cd (28.0–43.8%) in all the plants analyzed. In lettuce leaves the cell debris fraction was the dominant pool for Cd storage, displaying the highest percentage of the total Cd present in leaves, which is significantly different from the other fractions ($p < 0.05$).

The organelle fraction accounted for 10.2 and 19.7% of the Cd in *T. arvense* and *T. caerulescens*, respectively. In *T. arvense* the organelles fraction accounted for the lowest per-

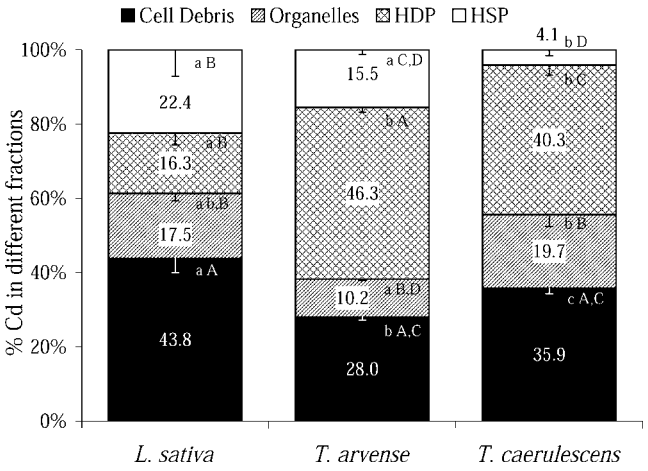

Fig. 1. Subcellular Cd distribution in *Lactuca sativa*, *Thlaspi arvense*, and *Thlaspi caerulescens* in relation to the total accumulated Cd. In each fraction numbers represent mean (%), error bars represent standard error for each fraction (n = 6–10).

centage of Cd in the leaves of this plant, being significantly lower than the percentage of Cd in the cell debris and HDP fractions ($p < 0.05$).

The HDP fraction was the dominant fraction for Cd binding in *T. arvense* (46.3%) and *T. caerulescens* (40.3%), but accounted for a significantly lower percentage of Cd (16.3%) in lettuce leaves ($p < 0.001$).

The HSP fraction contained the lowest percentage of Cd in *T. caerulescens* leaves (4.1%), when compared to the other fractions in the same plant ($p < 0.001$) and also, when compared with the same fraction in the leaves of *L. sativa* and *T. arvense* ($p < 0.05$).

Feeding experiment 1

Isopod growth. During the 14 d of the feeding experiment with *Thlaspi* leaves isopods all increased in weight, except those provided with control leaves of *T. caerulescens* (Fig. 2A). Growth of isopods fed *T. caerulescens* control leaves was significantly different from that of isopods fed *T. arvense* control leaves ($p < 0.05$). No significant difference was found between growth of isopods fed control and treated leaves of both plants. Mortality was below 10% in all treatments.

Plant consumption and assimilation efficiency. Plant consumption by isopods was significantly different between the *Thlaspi* species ($p < 0.01$). Isopods fed *T. caerulescens* consumed less than isopods fed *T. arvense* leaves (Fig. 2B). Treatment with Cd significantly reduced plant consumption for *T. arvense* ($p < 0.001$) but not for *T. caerulescens* ($p > 0.05$). Plant AE was not significantly different between isopods of the different treatments ($p > 0.05$). Isopods fed controls leaves displayed plant AEs of 73 ± 4.6% for *T. arvense* and 77 ± 2.7% for *T. caerulescens* (Fig. 2C).

Cadmium consumption, assimilation, and AE. Cadmium consumption by isopods (Fig. 2D) was similar between the plant species studied. Isopods fed *T. arvense* and *T. caerulescens* consumed (mean ± standard error) 155 ± 9.6 and 162 ± 11.5 μg/g animal, respectively. Assimilation of Cd was lower in isopods fed *T. caerulescens* (Fig. 2E) than in those fed *T. arvense*, however a t test indicated a marginally nonsignificant difference ($p = 0.058$) between treatments. The AE of Cd by isopods fed *T. caerulescens* leaves, 10.0 ± 0.92%, was significantly lower than the AE of Cd by isopods fed *T. arvense* leaves, 15.0 ± 1.03% ($p < 0.001$) (Fig. 2F).

Feeding experiment 2

Growth. The effects of subcellular Cd distribution in plants on Cd AE were further determined in the experiments when isopods were fed pure subcellular fractions (cell debris, organelles, HDP, and HSP) derived from the leaves of *L. sativa*, *T. arvense*, and *T. caerulescens*. Isopods displayed positive growth in all treatments with the three different plant species studied (Fig. 3A). Isopods fed pure gelatine discs (control 1) grew less than isopods fed gelatine added with the respective plant leaf homogenate (control 2) or subcellular fractions. However this difference was not always significant. Growth among isopods in the control 2 treatment was significantly higher than in control 1 for isopods fed *T. caerulescens* leaves. Isopods fed the cell debris fraction of the three plants displayed significantly higher growth ($p < 0.05$) when compared to control 1, as was the case with the organelles fraction of *T. arvense* ($p < 0.05$). Isopods fed the organelle fraction of *T. caerulescens* displayed significantly lower growth when compared to the respective C2 ($p < 0.05$). Furthermore, when comparing growth in isopods fed different subcellular fractions of *L. sativa*, HDP and HSP fractions presented a significant reduction in isopod growth when compared to the cell debris fraction ($p < 0.05$).

Cadmium AE from purified subcellular fractions. As a general pattern across plant species, isopods fed the HSP fraction displayed significantly lower AE compared to the AEs for the other fractions ($p < 0.001$) (Fig. 3B). Isopods fed the cell debris fraction displayed similar AEs to those fed the organelles and HDP fractions for *L. sativa* and *T. caerulescens*, but for *T. arvense*, AE for the cell debris fraction was significantly lower than that for the organelles and HSP fractions ($p < 0.001$). The order of Cd AE from each subcellular fraction was similar across the three species analyzed, HSP (22.8%) < cell debris = organelles = HDP (57.0%) for *L. sativa*; HSP (15.6%) < cell debris < organelles = HDP (47.4%) for *T. arvense*; and HSP (12.1%) < cell debris = HDP = organelles (35.0%) for *T. caerulescens*.

Estimation of Cd AEs

Figure 4 presents a comparison between the predicted and the actual Cd AEs in isopods. Estimation of Cd AEs through mass balance equation (Eqn. 3) using data obtained in feeding experiment 2 indicated that isopods would be expected to assimilate about 44.1, 36.4, and 26.4% of Cd from *L. sativa*, *T. arvense*, and *T. caerulescens*, respectively. The trend displayed by actual AEs (data from feeding experiment 1 and Calhôa et al. [30]) was similar to the estimated AEs; *L. sativa* was the plant species displaying higher Cd AEs in isopods; whereas, Cd in *T. caerulescens* presents the lowest AEs. However, in both *Thlaspi* species AEs seem to be overestimated, whereas in *L. sativa* the estimation of Cd AE is slightly lower than the observed in the study performed by Calhôa et al. [30].

Figure 4 also displays the individual contribution of each subcellular fraction to the total estimated AEs. It is apparent from Figure 4 that Cd sequestered in HSP fraction constitutes only a minor source of the Cd assimilated by isopods from the three plant species studied. In *L. sativa*, the cell debris fraction, which includes cell walls and granules, is the major source of Cd, accounting for about half the estimated Cd AE (21% out of the total estimated 44.1%). In *Thlaspi* species,

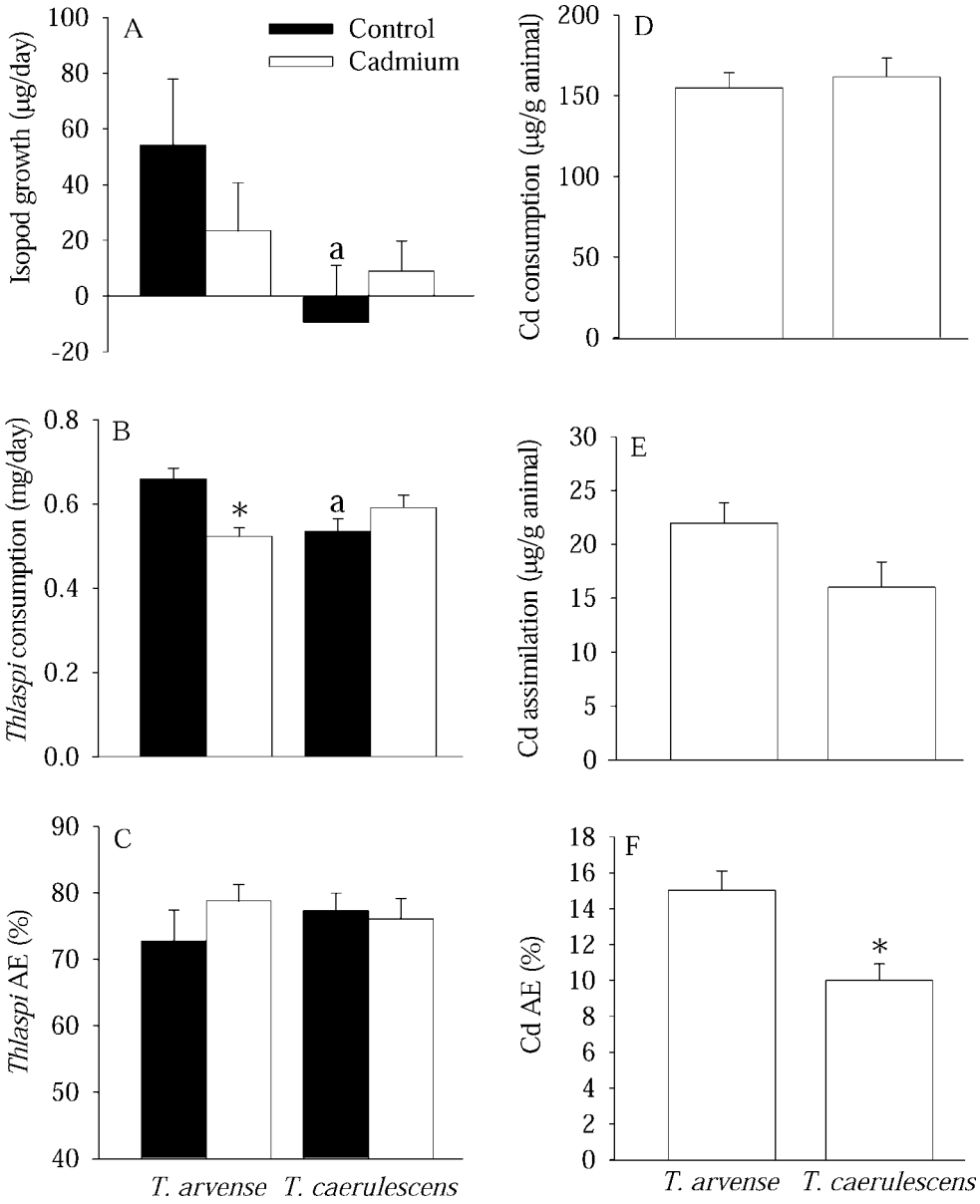

Fig. 2. Growth (**A**), *Thlaspi* plant leaf consumption (**B**), plant assimilation efficiencies (**C**), Cd consumption (**D**), Cd assimilation (**E**), and Cd assimilation efficiencies (AEs) (**F**) of isopods fed exclusively control or Cd-treated *T. arvense* and *T. caerulescens* leaves for 14 d. Bars represent mean ± standard error ($n = 20$). (*) Significantly different from control (B) or between treatments (F), $p < 0.001$. (a) Significant difference between plant controls, $p < 0.05$.

HDP displayed the highest contribution to the estimated AEs, accounting for about half of the estimated Cd AE by isopods (21.9% out of the total 36.4% for *T. arvense* and 13.0% out of the total 26.4% for *T. caerulescens*).

DISCUSSION

Predicting the bioavailability of tissue-bound metals to a consumer or predator is fraught with difficulty, as several important aspects of trophic transfer must be considered. Different prey species will accumulate and partition metals in varying ways depending on the detoxification mechanisms employed. The subsequent bioavailability of those partitioned metals to a consumer will be dictated by digestive and assimilative mechanisms of the digestive tract. Added to this complexity is the varying ability of consumers to discriminate between different foods and contaminants, their nutritional status at the time of consumption, the degree of exposure, and exposure history for the metal in question, all of which can influence the degree of metal assimilation. These various factors are reflected in the wide variety of Cd AEs that have been reported in organisms fed biologically contaminated food, ranging from 1% in rats fed snail viscera [34], to 52% in the isopod *P. dilatatus* fed lettuce [30], and up to 76.2 to 94.2% for whelk *Thais clavigera* fed five different species of prey [35]. In the present study we have shown that the centrifugal fractionation techniques previously advocated by Wallace et al. [6], can be used to explain the pattern of Cd assimilation in isopods fed different plant species contaminated with Cd.

Subcellular distribution of Cd in plants

Latuca sativa. In our work, Cd was found mainly in the cell debris fraction of *L. sativa* leaves, which was obtained from the residue of filtration and the 500 *g* pellet of the filtrate and includes cell walls and cell debris. These results are in

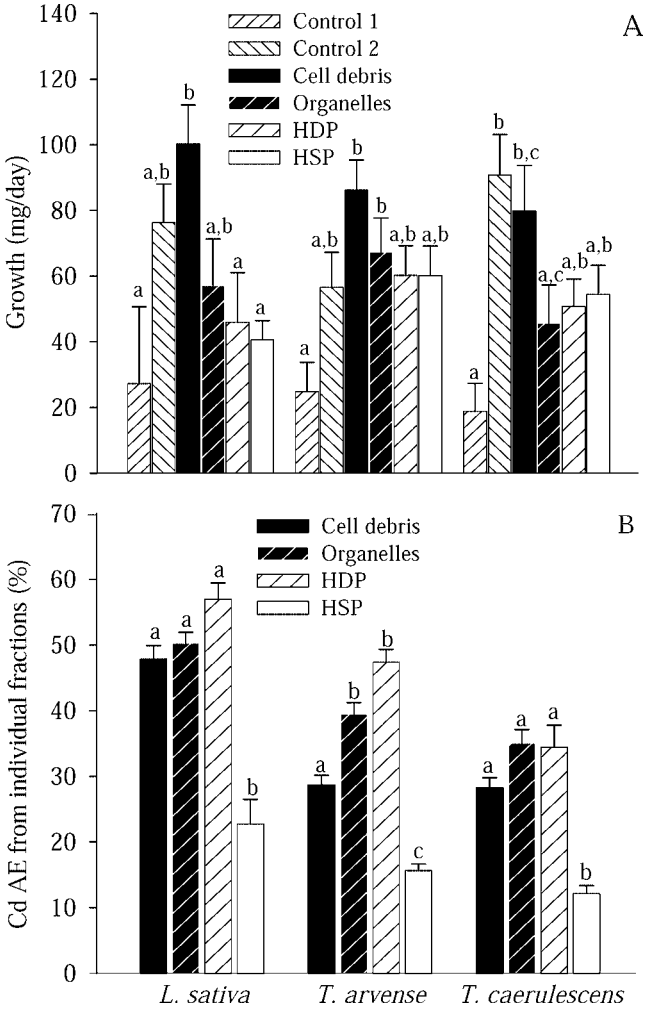

Fig. 3. Isopods fed exclusively separated subcellular fractions (heat-denatured proteins [HDP] or heat-stable proteins [HSP]) of *Lactuca sativa*, *Thlaspi arvense*, and *Thlaspi caerulescens* leaves for 28 d. (**A**) Isopod growth, among control isopods fed pure gelatine discs (Control 1) or gelatine with control leaf homogenate (Control 2), or isopods fed subcellular fractions. Statistical differences: (a) different from Control 1 ($p < 0.03$), (b) different from Control 2 ($p < 0.05$), (c) different from D ($p < 0.05$). (**B**) Isopods Cd assimilation efficiencies (AE), bars with different letters are significantly different ($p < 0.05$) for the same plant. Bars represent mean ± standard error.

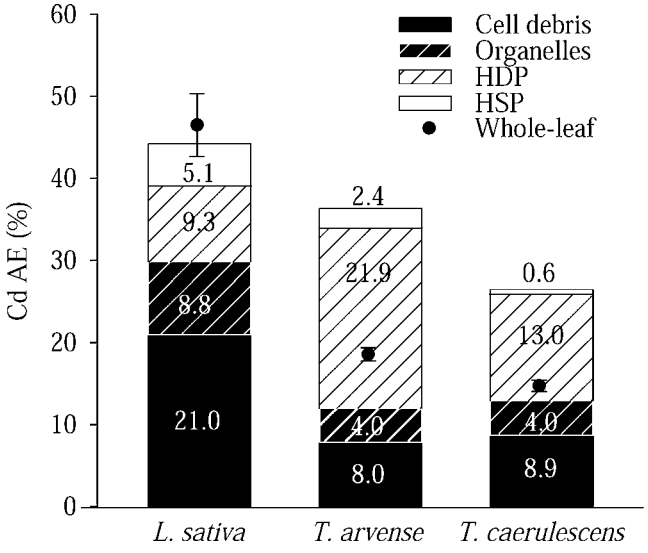

Fig. 4. Estimated and whole-leaf Cd assimilation efficiencies (AEs) in the isopod *Porcellio dilatatus* fed *Lactuca sativa*, *Thlaspi arvense*, and *Thlaspi caerulescens*. Error bars represent standard error (n = 20). The estimated Cd (AEs) were calculated from the isopod Cd AEs from individual fractions (heat-denatured proteins [HDP] or heat-stable proteins [HSP]) and the subcellular distributions of cadmium in the plant (see mass balance equation, Eqn. 3). Whole-leaf Cd AEs from *L. sativa* leaves is from the work of Calhôa et al. [30].

good agreement with those obtained by Ramos et al. [15] for *L. sativa* (cv. Grandes Lagos). Using a subcellular fractionation method similar to the one used in the present study, they found 64% of accumulated Cd associated with the fraction named cell wall fraction, which is equivalent to the cell debris fraction in the present study. Ramos et al. [15] also found approximately 12% of Cd associated with chloroplasts, which also is consistent with the proportion of Cd found in the organelles fraction in the present study. Lettuce also had a substantial proportion of Cd associated with the HSP fraction, and this is consistent with reports that have shown a strong induction of PCs following Cd exposure [20].

Thlaspi caerulescens. In *T. caerulescens* (ecotype Ganges), Ma et al. [22] found that mesophyll was a major storage site of Cd in leaves (65–70% of total Cd) and through the isolation of protoplasts and vacuoles they showed that most of the Cd in the mesophyll cells was localized in the protoplast (91%), and within this partition, 100% was inside the vacuoles. Accordingly, Wójcik et al. [18], using energy dispersive X-ray microanalysis, found Cd only in electron-dense deposits inside vacuoles of *T. caerulescens* leaves, and suggested that vacuoles are the main compartment of Cd storage and detoxification in these plant organs. The form of vacuolar storage was determined to be as a complex with malate [23]. In the present study, only a relatively small proportion of the Cd was partitioned to the organelles fraction; however, it seems likely that large vacuoles would not survive the homogenization procedures used to prepare our fractions. Therefore, any Cd bound to small molecules like malate, is likely to remain suspended within the cytosol and ultimately appear in the HSP fraction, or, if Cd-malate remains as insoluble electron-dense deposits, which seems likely at the pH used in the fractionation buffer (pH 7.5), it will remain in cell debris fraction rather than in HSP. In the present study, Cd bound to HSP, which is presumed to contain predominantly MT-like proteins such as PCs, represents a very low percentage of total accumulated Cd (2.7–4.6%). This result is supported by previous studies that indicated that PCs have no role in the mechanism of *T. caerulescens* tolerance to Cd, since total PC concentration were generally low compared to Cd concentrations [21,36]. Indeed the Cd found within HSP may be at least partially explained by the presence of small Cd bound molecules of vacuolar origin [3,23]. Apart from the cell debris fraction which contained 31.5 to 34.9% of accumulated Cd, HDP seems to be the major site for Cd binding in *T. caerulescens*, suggesting an important role for non–heat-stable proteins in Cd accumulation in this plant. Identification of the Cd-binding components in this fraction might explain the role of HDP in *T. caerulescens* Cd accumulation.

Thlaspi arvense. In contrast to *T. caerulescens*, PCs are known to be an important component in the response to Cd by *T. arvense*. Results obtained by Ebbs et al. [21] for *T. arvense* were consistent with a PC-mediated response; PC concentrations in *T. arvense* (15.5–42.5%) being two- to threefold higher in this species than in *T. caerulescens*. This difference

is reflected in our data that shows a relatively high proportion of Cd within the HSP fraction.

Cadmium assimilation from plants

Other than determining Cd subcellular distribution in plant leaves, this study also demonstrated how this distribution can influence Cd assimilation in isopods. Several studies have investigated the effects of Cd distribution within prey on Cd assimilation by predators in aquatic food chains [4,6,8,9,11,31,32]. A shift in subcellular distribution can have an important impact on the trophic transfer of metals. To the best of our knowledge, this is the first study applying this approach to a plant–animal food chain. It is important to note that metal assimilation is often assessed through pulse-chase techniques that attempt to assess metal assimilation prior to elimination. In both of the feeding experiments of the present study, the assimilation phase continued for an extended period and accumulation of Cd occurred as a consequence of net assimilation. Elimination of Cd in a closely related species, *Porcellio scaber* (and other terrestrial isopods), is known to be negligible, and the error in the calculation of AE is expected to be small [33,37].

Confounding effects. Nutritional quality of the food is known to affect assimilation of contaminants [38]. Although consumption and assimilation of both *Thlaspi* species were similar, isopods fed uncontaminated *T. arvense* displayed significantly higher growth than those fed uncontaminated *T. caerulescens*. However, no significant difference in growth was found among isopods fed contaminated leaves of either species. Therefore, food nutritive quality was unlikely to be an important confounding factor in this study. Results of consumption by isopods of *Thlaspi* plants and the respective AEs are in the range of those obtained for lettuce by Calhôa et al. [30] in a similar feeding study with the same isopod species.

Cadmium assimilation efficiencies. In the present study, plant species with different detoxifying mechanisms and accumulation patterns were studied in order to examine if there were particular relationships between subcellular Cd distributions in plants and Cd AE by isopods. Wallace and Luoma [8] recently introduced the concept of TAM and defined it as a combination of fractions organelles, HDP, and HSP. They deduced a 1:1 relationship between the percentage of Cd in TAM of several invertebrate prey items (bivalve and resistant and nonresistant oligochaetes) and Cd AE by the predator shrimps *Palaemonetes pugio* and *Palaemon macrodactylus*. In further experiments, Seebaugh et al. [39] found a weaker relation between Cd deposition in the proposed TAM fraction of prey *Artemia franciscana* (~63%) and Cd AE by the predator *Palaemonetes pugio* (37%). Cheung et al. [9] have also found a significant positive correlation between the Cd subcellular distribution in HSP and Cd body concentration in the welk *Thais clavigera* fed the snail *Monodonta labio*. However, this type of relationship between the proposed TAM fraction or individual fractions and Cd AE is not universal. In the present study no statistically significant correlation was found between Cd AE by isopods and Cd present in individual or combined subcellular fractions (data not shown). Performing a similar approach for three metals, Zhang and Wang [11] found a positive correlation for Se and Zn from the combination of HDP and HSP fractions, but for Cd no relationship was found between AEs and any of the subcellular fractions or a combination of fractions. These authors suggest that this result for Cd was due to the low AE of Cd in the marine fish *Terapon jarbua* fed different prey types (copepods, barnacles, clams, mussels, and fish viscera).

Assimilation efficiencies were reasonably well predicted on the basis of metal subcellular distribution and from AE of each subcellular fraction in studies with Cd [32] and other metals [11]. Two feeding studies were conducted in the present study; one with whole leaves (*T. arvense* and *T. caerulescens*) contaminated with Cd, and a second with individual fractions (*T. arvense*, *T. caerulescens*, and *L. sativa*). Figure 4 presents for each of the three species both the Cd AE for whole leaves and the estimated Cd AEs obtained through mass balance equation (Eqn. 3), using Cd AE of isopods fed purified fractions and the percentage of Cd in the each subcellular fraction. For the purpose of comparing whole leaf AEs and estimated Cd AEs, we included in Figure 4 data from a previous study by Calhôa et al. [30] that generated AEs for whole lettuce leaf contaminated with Cd. The data from that study are comparable to those collected in the present study because there is a great deal of consistency between the studies, including the nutritional status and source of the isopods, the physical conditions under which the trials were conducted, and the concentrations of Cd in the respective leaves. The concentration of Cd in the food is of particular importance as it is known to affect Cd AE in isopods. Specifically, AE is reduced with increasing Cd concentration in food [40]. The Cd concentration in *T. arvense* and *T. caerulescens* leaves were in the same range (438.9 ± 88.76 and 236.2 ± 53.30 μg Cd/g dry wt [mean ± standard deviation], respectively) as lettuce leaves (300–600 μg Cd/g dry wt [30]).

The Cd AE obtained in the present study by isopods fed *T. arvense* and *T. caerulescens* (whole leaf) were lower than the predicted AEs (Fig. 4) obtained from Cd AE from individual fractions. In contrast, the Cd AE among isopods fed lettuce (whole leaf) reported by Calhôa et al. [30] was very close to the predicted AEs. In order to demonstrate the direct influence of Cd subcellular distribution in plants on Cd assimilation by the isopod, individual subcellular fractions were embedded in gelatine to produce discrete packets of food in feeding experiments. Wallace and Lopez [32] have demonstrated that embedding homogenized preys in gelatine did not alter Cd bioavailability to the predator, since its Cd AE was similar to the AE obtained for predators fed entire preys. Therefore, it was assumed in the present study that this method could be used in feeding experiments with subcellular fractions without affecting Cd bioavailability to the isopod. However Zhang and Wang [11], using the same method, have also obtained overestimation of Cd AE and suggested that the homogenization step for subcellular fractionation may have facilitated the digestion of Cd by breaking the prey tissues into smaller portions, and addition of buffer may have increased the Cd bioavailability. Furthermore, gelatine obviously increases nutritional quality of food to isopods, and this is a biological factor known to directly influence assimilation of contaminants [38]. The inclusion of a homogenate of Cd-contaminated leaves embedded in gelatine in feeding experiments as an additional control treatment would have allowed a direct comparison to the Cd AE of whole leaves and might help to clarify the overestimations of Cd AE observed in the present study.

Figure 4 also displays the individual contribution of each subcellular fraction to the total estimated AEs. It is apparent from Figure 4 that Cd sequestered in the HSP fraction constitutes only a minor source of the Cd assimilated by isopods

from the three plant species studied, in part because only 12 to 23% of this fraction is trophically available (Fig. 3). This result contrasts with those obtained for animals, since HSP fraction is part of the proposed TAM fraction, contributing significantly to the trophic transfer of Cd from several invertebrate animals to shrimp [8,10] and marine fish [11]. It was shown that metal partitioned to a subcellular compartment containing TAM is readily available to predators and may be enhanced by increased binding of metal to HSP [8]. This fraction is considered to be dominated by MT-like proteins, a family of low-molecular-weight, cysteine-rich proteins that can bind to essential metals and sequester toxic metals, and therefore, can also be considered as biologically detoxified metal [6]. As indicated above, HSP may contain other Cd-bound molecules, and a closer examination (or a further fractionation) of the HSP fraction might clarify this apparent difference between the AE of Cd from HSP fraction of prey animals and plants. Beyond differences in HSP content, differences in AE of HSP-bound Cd might also be related to the different capacity of isopods to assimilated Cd bound to HSP, due to digestive physiology, in comparison to other predators, such as marine animals.

In all three species, Cd in the cell debris fraction contributes more to isopod assimilation of Cd than would be expected according to the TAM model proposed by Wallace and Luoma [8]. The composition of the cell debris fraction includes tissue fragments and cell walls and might contain metal-rich granules. This fraction was originally considered as trophically unavailable in marine invertebrate food chains [8]. However, in a previous study, these same authors reported the bioavailability to predators of Cd bound to the cellular debris fraction at 19.0% [32]. Furthermore, direct evidence on the bioavailability of metal-rich granules to a marine predator has been demonstrated [35]. In the present study, among isopods fed the cell debris fraction of lettuce, the AE of Cd was 47.8 ± 2.05%, and this fraction accounts for approximately half the estimated Cd AE (21% out of the total estimated 44.1%); thus, the cell debris fraction can be considered at least partially trophically available. The bioavailability of Cd bound to tissue fragments, cell walls and metal rich granules must to a certain extent be dictated by gut physiology. For example, gut pH may play a role in dissolution of metal-rich granules; the terrestrial isopod *P. scaber* is able to buffer pH in the intestinal tract to approximately 5.5 to 6.0 in the anterior hindgut and approximately 6.0 to 6.5 in the posterior hindgut [41]. Presuming that the *P. dilatatus* has a similar digestive physiology, at these pHs the metal-rich granules, if present, do not seem likely to become available, since dissolution occurs at lower pH. Bioavailability of Cd stored in granules depends on the form and granule elemental composition, but is likely to be higher at lower pH levels [42].

In both *Thlaspi* species, HDP displayed the highest contribution to the estimated AEs, accounting for approximately half of the estimated Cd AE by isopods (21.9% out of the total 36.4% for *T. arvense* and 13.0% out of the total 26.4% for *T. caerulescens*), and this is in closer agreement with the proposed TAM fraction [8]. Similar results were obtained by other authors through direct evidence of bioavailability of Cd bound to HDP [11,35], indicating that Cd-HDP as part of TAM was partially bioavailable to fish [11] and marine snails [35].

CONCLUSIONS

The concept of TAM as defined by Wallace and Luoma [8] is not supported by the data presented here. In contrast with results in marine food chains obtained by other authors, Cd bound to HSP is relatively less available and seems to contribute in lesser extent to the trophic transfer of Cd than other fractions obtained by a centrifugal fractionation procedure. However, the AE of compartment-specific Cd was consistent across the different plant species. These results point to the ecological relevance of the subcellular Cd distribution in plants, which directly influences the trophic transfer of Cd to the animal consumer, and highlight that a shift in Cd subcellular distribution in plants due to different detoxifying mechanisms may have a direct important impact in trophic transfer to the animal consumer. Although predicted Cd AEs from the different plants were overestimated in two of the plants studied, they helped to elucidate the observed Cd AE in isopods, providing the specific contribution of each subcellular fraction on the trophic transfer of Cd.

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