Subcellular compartmentalization of Cd and Zn in two bivalves. II. Significance of trophically available metal (TAM)

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ABSTRACT: This paper examines how the subcellular partitioning of Cd and Zn in the bivalves *Macoma balthica* and *Potamocorbula amurensis* may affect the trophic transfer of metal to predators. Results show that the partitioning of metals to organelles, 'enzymes' and metallothioneins (MT) comprise a subcellular compartment containing trophically available metal (TAM; i.e. metal trophically available to predators), and that because this partitioning varies with species, animal size and metal, TAM is similarly influenced. Clams from San Francisco Bay, California, were exposed for 14 d to 3.5 µg l^{-1} Cd and 20.5 µg l^{-1} Zn, including ^{109}Cd and ^{65}Zn as radiotracers, and were used in feeding experiments with grass shrimp *Palaemon macrodatylus*, or used to investigate the subcellular partitioning of metal. Grass shrimp fed Cd-contaminated *P. amurensis* absorbed ~60% of ingested Cd, which was in accordance with the partitioning of Cd to the bivalve's TAM compartment (i.e. Cd associated with organelles, 'enzymes' and MT); a similar relationship was found in previous studies with grass shrimp fed Cd-contaminated oligochaetes. Thus, TAM may be used as a tool to predict the trophic transfer of at least Cd. Subcellular fractionation revealed that ~34% of both the Cd and Zn accumulated by *M. balthica* was associated with TAM, while partitioning to TAM in *P. amurensis* was metal-dependent (~60% for TAM-Cd%, ~73% for TAM-Zn%). The greater TAM-Cd% of *P. amurensis* than *M. balthica* is due to preferential binding of Cd to MT and 'enzymes', while enhanced TAM-Zn% of *P. amurensis* results from a greater binding of Zn to organelles. TAM for most species–metal combinations was size-dependent, decreasing with increased clam size. Based on field data, it is estimated that of the 2 bivalves, *P. amurensis* poses the greater threat of Cd exposure to predators because of higher tissue concentrations and greater partitioning as TAM; exposure of Zn to predators would be similar between these species.

KEY WORDS: Subcellular compartmentalization · Cd · Zn · Bivalves · Trophic transfer · Detoxification

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INTRODUCTION

Studies have shown that the use of 'biosentinal species' can provide some degree of insight as to the potential for metal trophic transfer in impacted environments (Cope & Bartsch 1999). Understanding the factors controlling metal trophic transfer in aquatic ecosystems, however, has been enigmatic as some studies show high transfer, with others showing little or none (van Hattum et al. 1989, Connell et al. 1991, Weis & Weis 1993, Wang & Ke 2002). Differences in trophic transfer among metals (Reinfelder & Fisher 1991, 1994a,b) and influences of environmental parameters (Wang & Fisher 1999) also add complexity. Understanding the processes controlling the dietary uptake of metals by predators, however, is important because exposure through food (Wang et al. 1999, Burgos & Rainbow 2001) has been linked to sublethal toxicity (Wallace et al. 2000, Hook & Fisher 2002).

It is well known that bioaccumulation of metals is different among species and metals because of differences in uptake and loss rates, exposure pathways, and influences of environmental parameters (e.g. salinity and temperature) (Howard & Hacker 1990, Wang et al. 1996, Wang & Fisher 1997, Lee et al. 1998). Less is known, however, about how such factors influence the internal storage and detoxification of accumulated metal, and subsequent impacts on trophic transfer (Roesijadi 1980, Jenkins & Mason 1988, Klerks & Bartholomew 1991, Wallace et al. 2000).

Some studies have related dietary uptake of metals by primary consumers, like zooplankton and bivalve larvae, to binding with the cytosol of phytoplankton (Reinfelder & Fisher 1991, 1994a, Wang & Fisher 1996). These relationships, however, do not apply to the dietary bioavailability of all metals (e.g. Cd in some cases) to all consumers (adult bivalves in particular; Decho & Luoma 1991). Trophic transfer from prey to predators has been studied in a few cases, but generalizations are just beginning to emerge (Wallace & Lopez 1996, 1997, Munger & Hare 1997, Wallace et al. 1998). For example, although binding to inducible metal-binding proteins (metallthioneins; MT) and precipitation into insoluble concretions (metal-rich granules; MRG) are 2 major detoxification pathways (Roesijadi 1980, Brown 1982), they may have separate effects on trophic transfer (Wallace & Lopez 1997). For instance, Nott & Nicolaidou (1989, 1990, 1993) demonstrated that metals associated with MRG in herbivorous gastropods pass through carnivorous gastropods undigested. Wallace & Lopez (1997) showed that Cd associated with cytosolic proteins (enzymes and MT) of oligochaetes is absorbed by grass shrimp with an efficiency of ~100%, while that sequestered as MRG was unavailable for transfer. Additionally, although not involved with detoxification, it was also demonstrated that Cd associated with organelles in oligochaete prey is absorbed by grass shrimp with an efficiency of ~70%, and that bound to cellular debris was relatively unavailable to shrimp (Wallace & Lopez 1997).

These studies suggest that metals associated with organelles, enzymes and MT of prey may represent metal that is trophically available for transfer to predators, and may therefore be grouped into a subcellular compartment defined as trophically available metal (TAM). Metal associated with MRG and cellular debris may be unavailable for transfer (Nott & Nicolaidou 1990, Wallace & Lopez 1997). Here we test if the subcellular distribution of metal within the bivalves *Macoma balthica* and *Potamocorbula amurensis* is consistent with this hypothesis, and examine whether subcellular partitioning of Cd and Zn to components of TAM (i.e. organelles, enzymes and MT) differs between the species. A companion paper considers the

subcellular compartmentalization of metal as metal-sensitive fractions (MSF) (i.e. organelles and enzymes) and biologically detoxified metal (BDM) (i.e. MT and MRG) (Wallace et al. 2003).

The bivalves *Macoma balthica* (a facultative deposit feeder) and *Potamocorbula amurensis* (a filter feeder) represent the dominant benthic macrofauna in San Francisco Bay, California. These clams show considerable differences in metal accumulation patterns and, therefore, have different uses as bioindicators (*M. balthica* is a good indicator of Zn pollution and *P. amurensis* is the better indicator of Cd pollution) (Cain & Luoma 1990, Brown & Luoma 1995, Lee & Luoma 1998, Lee et al. 1998). Both clams link benthic, pelagic and terrestrial food webs, supporting a variety of predators (i.e. crabs, fish, water fowl) (Carlton et al. 1990, Ejdung & Bonsdorff 1992, Hiddink et al. 2002). Metal bioaccumulated by these clams is, therefore, available for transfer up food chains.

MATERIALS AND METHODS

Exposure of bivalves to Cd and Zn. Bivalves of various sizes collected from 2 sites within San Francisco Bay (SFB) (*Macoma balthica* from the Palo Alto mud flat, South SFB: David et al. 2002; *Potamocorbula amurensis* from Stn 4.1 in Brown & Luoma 1995) were exposed to metals for 14 d as described by Wallace et al. (2003). Nominal exposure concentrations were 3.5 µg l^{-1} Cd and 20.5 µg l^{-1} Zn, and radioisotopes (^{109}Cd and ^{65}Zn) were used as tracers of stable metals (see Wallace et al. 2003 for further details). Following 14 d of uptake (with renewal of exposure media at 3 d intervals), clams were rinsed with clean 20‰ seawater (0.45 µm) and were stored frozen (–80°C). Some clams were used in feeding experiments with grass shrimp, while others were used for subcellular fractionation.

Feeding experiment. Feeding experiments followed Wallace et al. (1998). Grass shrimp *Palaemon macrodactylus* collected from south SFB were returned to the laboratory and held in aquaria at 20‰ for 7 d prior to use in experimentation. During acclimation, shrimp were fed Tetramin® fish food; however 2 d prior to experimentation, food was withheld. The feeding experiment was initiated by placing the tissue of 1 dissected clam *Potamocorbula amurensis* into a 100 ml glass beaker containing 80 ml of seawater (20‰) and 1 grass shrimp (n = 3). Shrimp promptly ingested all of this tissue (~5 to 10 min).

After ingesting the clam tissue, shrimp were rinsed twice with clean seawater, placed into 20 ml glass scintillation vials containing 5 ml of seawater (20‰) and were assayed for ^{109}Cd. After radioanalysis, shrimp were placed in mesh-bottomed holding chambers

contained within an aquarium (20‰, 10°C), where they were allowed to depurate ingested clam tissue (Wallace & Lopez 1997). During depuration, shrimp were fed once a day on Tetramin® fish food. Over a period of 7 d, shrimp were periodically removed from the aquarium and were radioanalyzed for [109]Cd; fecal strands were also collected and monitored for radioactivity (Wallace & Lopez 1997). The absorption efficiency (AE%) of ingested radioisotope was calculated as: $(S_{fin}/S_{int}) \times 100 = AE\%$, where S_{fin} is radioactivity remaining in shrimp following the production of radiolabelled fecal material (time, $t = 2$ d) and S_{int} is the radioactivity in shrimp following ingestion of the clam tissue ($t = 0$) (Wallace & Lopez 1997).

Subcellular fractionation. The subcellular distribution of metal within clams was determined as described in Wallace et al. (2003). Briefly, clam tissue was homogenized with a Polytron® tissue homogenizer and subjected to subcellular fractionation, as presented in Fig. 1. Fractionation resulted in the isolation of 5 distinct and operationally defined subcellular fractions: MRG (metal-rich granules), cellular debris, organelles (i.e. nuclear, mitochondrial and microsomal fractions), heat-sensitive proteins ('enzymes') and heat-stable proteins (MT and MT-like proteins) (Wallace et al. 2003). For purposes of understanding potential trophic transfer of Cd and Zn to bivalve predators, a compartment containing trophically available metal (TAM) was constructed (i.e. TAM = organelles% + 'enzymes'% + MT%) (Fig. 2). In some instances a second method of calculating TAM was used (i.e. TAM = 100% – [MRG% + cellular debris%]). This second calculation is essentially the same as the first, except that losses introduced due to the fractionation process are reduced.

Radioanalysis, metal body-burden calculations and statistical analysis. Sample radioactivity was determined with a Wallac 1480 Wizard gamma counter equipped with a 7.8 cm type NaI crystal detector.
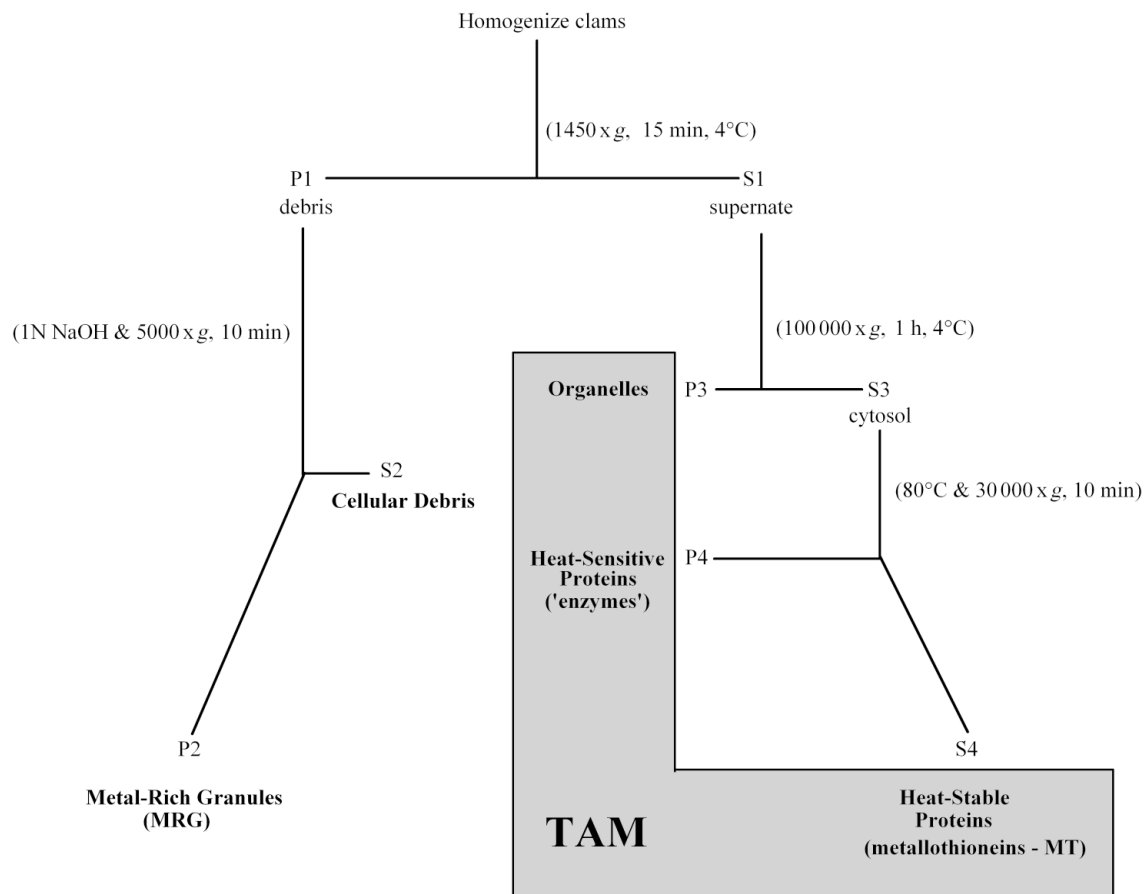

Fig. 1. Procedure for determining the subcellular partitioning of metal within clams. Clams were homogenized, and differential centrifugation and tissue digestion techniques were used to obtain the following subcellular fractions: metal-rich granules (MRG), cellular debris, organelles, heat-sensitive proteins ('enzymes') and heat-stable proteins (metallothioneins—MT). Organelles, 'enzymes' and MT are grouped as trophically available metal (TAM) (i.e. metal that is trophically available to predators). Operationally, all TAM fractions are contained within the first supernate (S1)

Subcellular Compartments

Metal-sensitive fractions (MSF)
(*includes: organelles & "enzymes"*)

Biologically detoxified metal (BDM)
(*includes: MT & MRG*)

Trophically available metal (TAM)
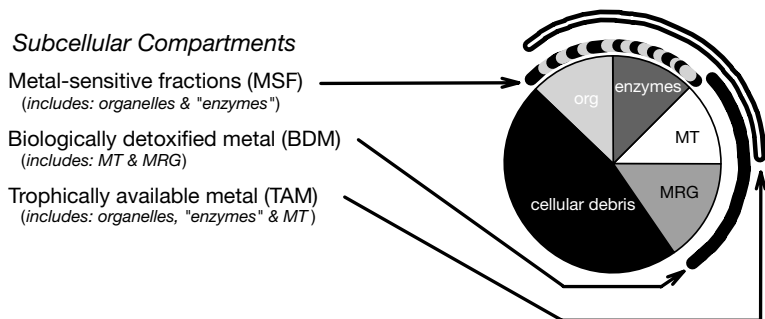(*includes: organelles, "enzymes" & MT*)

Fig. 2. Generalized ecotoxicological pie chart depicting subcellular compartments based on the biological significance of the various subcellular fractions. Subcellular fractions containing metal that is trophically available to predators (i.e. organelles [org], 'enzymes' and metallothioneins [MT]) constitute trophically available metal (TAM: double arc). Subcellular fractions that are potentially vulnerable to metal exposure (i.e. organelles and 'enzymes') are grouped as metal-sensitive fractions (MSF: dashed arc), while those involved with metal detoxification (i.e. MT and metal-rich granules [MRG]) are grouped as biologically detoxified metal (BDM: solid arc). From Wallace et al. (2003)

Photon emissions of ^{109}Cd were determined at 88 keV and ^{65}Zn at 1115 keV. Counting times were 5 min and propagated counting errors were <5%. The raw data, cpm (counts per minute), were converted to dpm (disintegration per minute) using appropriate standards and half-life corrections. No peak separation was necessary. Concentrations of accumulated metal were calculated based on isotopic specific activities, and are
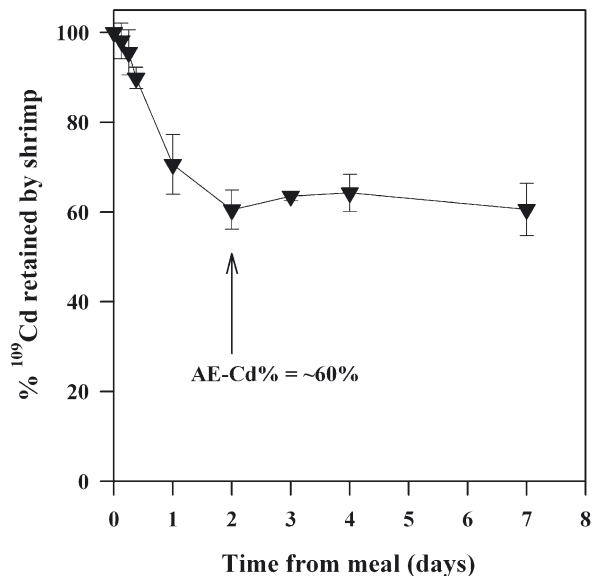
expressed as ng g wet wt^{-1}. Percentage subcellular distributions of ^{109}Cd and ^{65}Zn within clams were calculated based on radioactivity recovered after homogenization. Recovery of homogenate radioactivity subsequent to fractionation was consistently high (>85%; i.e. [summation of radioactivity in each of the 5 subcellular fractions]/[radioactivity following homogenization]).

Data were analyzed using the software Statistica Version 5.1 (1997). Student's *t*-tests (or *t*'-test when variances were not homogeneous, see Sokal & Rohlf 1981) were used to compare tissue concentrations and partitioning (%) to individual subcellular fractions, as well as to the TAM compartment between species. Linear regression analysis was used to understand relationships between clam size (g wet wt) and the partitioning (%) of metal to individual subcellular fractions, as well as to the TAM compartment.

RESULTS

Relationship between TAM% in prey and AE% by predator

In order to test the TAM concept, grass shrimp *Palaemon macrodactylus* were fed *Potamocorbula amurensis* radiolabelled with ^{109}Cd. The shrimp exhibited a 2-component loss of ^{109}Cd, with the initial rapid loss being attributed to the defecation of unassimilated metal (verified by detection of ^{109}Cd within fecal strands; data not shown) (Fig. 3). The plateau in ^{109}Cd retention subsequent to *t* = 2 d results from a cessation in the production of radiolabelled fecal material (Wallace & Lopez 1997). Absorption efficiency of Cd by grass shrimp fed radiolabelled *P. amurensis* (AE-Cd%) was, therefore, calculated as the retention of ^{109}Cd at *t* = 2 d (~60%) (Fig. 3). When considering the subcellular partitioning of Cd in *P. amurensis*, it was found that ~60% of the accumulated metal was associated with fractions (e.g. organelles, 'enzymes' and MT) presumed to contain trophically available metal (i.e. TAM) (see below; Figs. 4 & 5a).

Fig. 4 puts the relationship between TAM-Cd% in *Potamocorbula amurensis* and AE-Cd% by *Palaemon macrodactylus* into perspective with previous studies investigating the trophic transfer of Cd from oligochaetes to a similar predator (grass shrimp *Palaemonetes pugio*) (Wallace et al. 1998). These comparisons indicate that there is, in general, a 1:1 correspondence between the TAM-Cd% of prey and AE-Cd% by a predator. Fig. 4 also shows the relative contribution

Fig. 3. *Palaemon macrodactylus*. Time course in the retention of ^{109}Cd by grass shrimp fed Cd-exposed *Potamocorbula amurensis*. The absorption efficiency (AE-Cd%; mean ± SE) was calculated from the retention of ^{109}Cd at *t* = 2 d, which corresponded to the time when shrimp stopped producing radiolabelled fecal material (data not shown)

of each subcellular fraction (i.e. organelles, 'enzymes' and MT) to the entire TAM-Cd% compartment of each prey organism. Because TAM within prey appears to be of first-order significance in determining at least the trophic transfer of Cd, it is important to understand whether subcellular compartmentalization as TAM varies with prey species, metal and other biological factors (e.g. animal size).

Compartmentalization of Cd and Zn as TAM in bivalves

The partitioning of Cd and Zn to TAM fractions within *Macoma balthica* was similar ('enzymes' [~20%] > organelle [~12%] > MT [~4%]) resulting in a TAM of ~34% for each metal (Fig. 5a,b; left bar in each panel). In *Potamocorbula amurensis*, however, subcellular partitioning was metal-dependent following the pattern: 'enzymes' (~30%) > MT (~20%) > organelles (~11%) for Cd; organelles (~55%) > 'enzymes' (~13%) > MT (~4%) for Zn (Fig. 5a,b; right bar in each panel). TAM in *P. amurensis* was, therefore, also metal-dependent (~60% for TAM-Cd% and ~73% for TAM-Zn%; p < 0.001) (Fig. 5a,b). The preferential storage of Cd into MT and 'enzymes' (p < 0.001), and Zn into organelles (p < 0.001) by *P. amurensis*, over that of *M. balthica*, resulted in a TAM-Cd% and a TAM-Zn% that were, respectively, ~1.7× (p < 0.001) and ~2.2× (p < 0.001) greater than that of *M. balthica* (Fig. 5a,b).

Fig. 5 panels c and d compare absolute concentrations of metals in the whole body and the TAM compartment of both bivalves. *Potamocorbula amurensis* accumulated ~21× more Cd (p < 0.001) and ~2× more Zn (p < 0.001) than *Macoma balthica* during the 14 d exposure. Species-dependence in subcellular metal partitioning, however, resulted in an even greater disparity in TAM between the species, with *P. amurensis* having a TAM-[Cd] and a TAM-[Zn] that were, respectively, ~34× and ~4× greater than that of *M. balthica* (Fig. 5c,d).

Size-dependence and field estimates of TAM

The subcellular partitioning of Cd and Zn within *Macoma balthica* and *Potamocorbula amurensis* was size-dependent (Table 1, Fig. 6) (also see Wallace et al. 2003). In *M. balthica*, the partitioning of both metals to MT decreased with clam size (Table 1, Fig. 6a,c). In contrast, the importance of MT for Cd storage increased with size in *P. amurensis* (Fig. 6b). No size-dependence was observed for the binding of Zn to MT in *P. amurensis* (Fig. 6d), although little Zn was bound
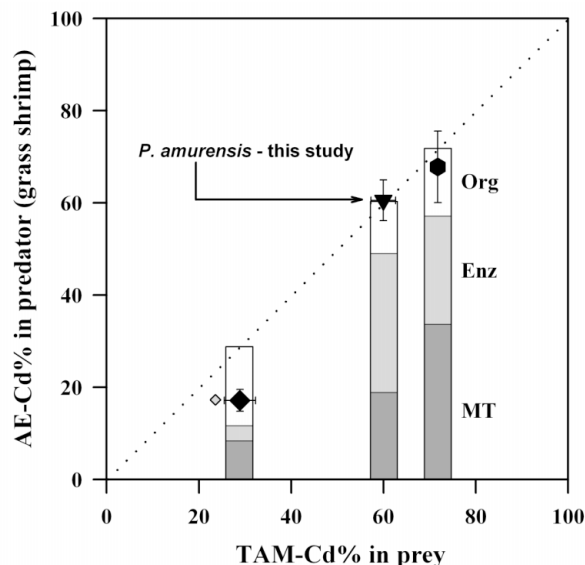

Fig. 4. Relationship between the trophically available metal (Cd; TAM-Cd%) of prey (*Potamocorbula amurensis* [▼], this study; Cd-resistant [◆] and nonresistant [●] oligochaetes, Wallace et al. 1998) and Cd absorbed (absorption efficiency: AE-Cd%) by the predators *Palaemonetes pugio* (Wallace et al. 1998) or *Palaemon macrodactylus* (this study). Symbols (± error bars) intersecting with the dashed line represent a 1:1 relationship between the TAM-Cd% in prey and Cd transferred to shrimp. Stacked bars represent the proportional contribution of each subcellular fraction (organelles [Org] = open; 'enzymes' [Enz] = light gray; metallothioneins [MT] = dark gray) to the entire TAM compartment of each prey item. The half-sized gray diamond at the lower end of TAM-Cd% (~23%) corresponds to a 'recalculated' TAM-Cd% for Cd-resistant oligochaetes. This 'recalculated' TAM-Cd% considers the lower availability of Cd associated with organelles (Wallace & Lopez 1997) and provides a more accurate assessment of the availability to grass shrimp of Cd accumulated by these oligochaetes (see 'Discussion')

to this fraction (~4%). Partitioning of Cd to organelles in *M. balthica* decreased with size (Fig. 6a), but no size-dependence was observed for partitioning of Cd to organelles in *P. amurensis* (Fig. 6b). Finally, in both species, partitioning of Zn to organelles decreased as a function of size (Fig. 6c,d), while binding of metal to 'enzymes' in all species–metal combinations lacked size-dependence (Table 1, Fig. 6).

The composite effect on the TAM compartment of size-dependence in the subcellular partitioning of metals in *Macoma balthica* was a decrease in TAM-Cd% and TAM-Zn% with clam size (Fig. 6a,c). Specifically, TAM in *M. balthica* dropped from ~50% for both Cd and Zn in small clams (~11 mm) to ~42% for Cd and ~36% for Zn in large clams (~28 mm). TAM-Zn% in *Potamocorbula amurensis* dropped from ~81% in small clams (~12 mm) to ~69% in large clams (~25 mm) (Fig. 6d). In spite of the size-dependence in partition-
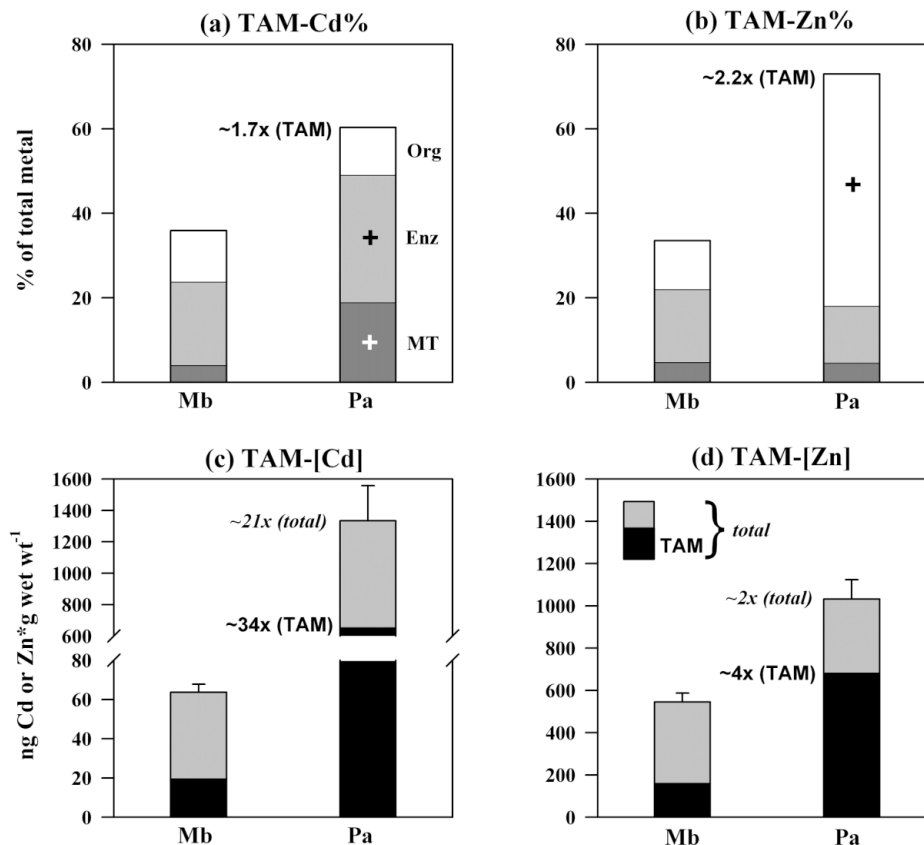
Fig. 5. *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa). Comparisons of the subcellular partitioning and accumulation of (a,c) Cd and (b,d) Zn following 14 d uptake. Partitioning to subcellular fractions constituting trophically available metal (TAM) is reported as mean% (a,b) (TAM-Cd%, TAM-Zn%). Stacked bars represent the proportional contribution of each subcellular fraction (organelles [Org] = open; 'enzymes' [Enz] = light gray; metallothioneins [MT] = dark gray) to the entire TAM compartment. Accumulated metal is reported as ng metal μg wet wt^{-1} (mean ± SE) (c,d). Embedded full shaded bars represent the concentration of accumulated metal associated with the entire TAM compartment (i.e. organelles, 'enzymes' and MT) (TAM-[Cd], TAM-[Zn]). Fold-differences between *M. balthica* and *P. amurensis* in TAM (% and concentration) (bold) (a,b,c,d) and total metal burdens (*italic*) (c,d) are listed to the left of the bar for *P. amurensis* (Fold-difference = [% or concentration of metal in *P. amurensis*]/[percentage or concentration of metal in *M. balthica*]). Placement of (+) within subcellular 'slices' of the stacked bar for *P. amurensis* indicate a greater partitioning ($p < 0.05$) of Cd or Zn to this fraction as compared with *M. balthica* (a,b)

ing of Cd to MT in *P. amurensis* (increasing with clam size) (Table 1, Fig. 6b), there was no size-dependence in TAM-Cd% for this species (~68 % regardless of clam size).

The relationships in Table 1 and Fig. 6 allow for the estimation of size-dependence in the concentration of Cd and Zn associated with the TAM compartment (i.e. TAM-[Cd] and TAM-[Zn]) in *Macoma balthica* and *Potamocorbula amurensis* under natural settings (Fig. 7). Such calculations can provide insight as to how species-, size- and metal-dependence in bioaccumulation, together, might affect trophic transfer in the field. For the purposes of these calculations, tissue concentrations and size-dependence of Cd and Zn in field-collected *M. balthica* and *P. amurensis* were determined by other researchers in concert with these studies (*M. balthica*: Luoma et al. 1998; *P. amurensis*:

C. Brown unpubl.). Cd and Zn in *M. balthica* increased with size (and/or age) (Fig. 7a,c) (Luoma et al. 1998). Large *M. balthica* (~28 mm) had a Cd tissue concentration that was ~7.5× greater than that of small *M. balthica* (~11 mm); there was a ~5.0× difference between small and large *M. balthica* in total Zn tissue concentrations. In *P. amurensis*, tissue concentrations of Cd and Zn were independent of clam size (Fig. 7b,d) (C. Brown unpubl.). Estimates of the concentration of metal associated with the TAM compartment indicate that there is a ~5.9× and ~3.5× difference between small (~11 mm) and large (~28 mm) *M. balthica* with respect to TAM-[Cd] and TAM-[Zn] (Fig. 7a,c). TAM-[Cd] in *M. balthica* is estimated to range from 0.013 to 0.079 μg g dry wt^{-1}, with TAM-[Zn] ranging from 17 to 94 μg g dry wt^{-1} (Fig. 7a,c). Even though TAM-Zn% in *P. amurensis* was size-dependent (Fig. 6d), calculations

reveal no significant size-dependence in the concentration of either metal bound as TAM (i.e. TAM-[Cd] is ~3 µg g dry wt^{-1} and TAM-[Zn] is ~55 µg g dry wt^{-1}) (Fig. 7b,d).

DISCUSSION

The compartmentalization of metal as defined in this and a companion paper is a useful tool to interpret multiple ecotoxicological consequences of the subcellular partitioning of metals within soft-bodied invertebrates (in this case 2 bivalves) (Wallace et al. 2003). Wallace et al. (2003) suggested that because 'enzymes' and organelles may be vulnerable to metal exposure, they comprise a subcellular compartment containing metal-sensitive fractions (MSF), while metal sequestered in MT and MRG can be considered biologically detoxified metal (BDM). Here we build upon this compartmentalization approach by defining metals associated with organelles, 'enzymes' and MT as comprising a subcellular compartment containing trophically available metal (TAM) (i.e. metal that is readily available for transfer to predators) (Wallace & Lopez 1996, 1997, Wallace et al. 1998).

Other researchers defined metal available for trophic transfer from partitioning to 'soft tissues' in prey (Wang et al. 1999, Ni et al. 2000). These methods, however, did not consider the internal or subcellular 'whereabouts' of the accumulated metal, and can therefore not directly address reasons for differences in metal partitioning (i.e. perhaps due to detoxification) and associated impacts on trophic transfer. In this context then TAM may be more informative, as it takes a mechanistic approach and focuses on individual subcellular fractions, which may have differential availabilities to predators (Wallace & Lopez 1997). As such, TAM provides a practical and manageable approach to understand, monitor and even model (Thomann 1981, Reinfelder et al. 1998) population-, species- and metal-specific differences in metal trophic transfer. Additionally, determining subcellular partitioning may help us understand how mechanisms used by prey to avoid or ameliorate the toxic effects of metals may impact subsequent transfer to higher trophic levels (Wallace & Lopez 1997).

Relationship between TAM% in prey and AE% by a predator

In the present study, Cd in *Potamocorbula amurensis* was transferred to grass shrimp with an efficiency of ~60%. When these results were compared with earlier studies (Fig. 4), it became clear that the partitioning of

Cd to TAM in various prey (a bivalve and an oligochaete) was directly related to Cd absorption by predators (2 species of grass shrimp). This further verifies that TAM (i.e. partitioning to organelles, 'enzymes' and MT) does represent bioavailable metal from the diet, and suggests that trophic transfer to some predators may be predictable from an operationally defined TAM compartment of prey. It should be noted, however, that trophic transfer relationships like the one shown in Fig. 4 will probably differ among metals (radioactivities of ^{65}Zn in ingested clam tissue were below detection in the present study, so absorption efficiencies could not be calculated), and that the slope of the relationship between TAM and metal absorption could, due to differences in digestive physiology, vary among predators (Reinfelder et al. 1998).

Table 1. Results of linear regression analysis for size-dependence (g wet wt) in the proportional subcellular metal distributions (organelles [Org]), 'enzymes' [Enz], metallothioneins [MT], metal-rich granules [MRG] and cellular debris [Deb]) of *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa) following 14 d uptake. *p < 0.05; ns: non-significant; avg.: average proportional distributions listed in lieu of regression equations. Due to non-detectable levels of radioactivity within certain subcellular fractions of some small clams, these individuals were excluded from regression analysis. A lack of size-dependence in these instances may be due to the exclusion of these smaller individuals (sz?). The relationships in this table were used to estimate the partitioning of metal to the entire TAM compartment of field collected bivalves (see Fig. 6). Table reprinted from Wallace et al. (2003)

|	r^2	n	Regression equation
Mb-Cd			
Org	0.50*	18	$y = -9.43x + 15.75$
Enz	ns	18	19.75 (avg.)
MT	0.56*	15	$y = -4.93x + 6.06$
MRG	sz?	7	14.57 (avg.)
Deb	0.37*	12	$y = -9.19x + 47.90$
Mb-Zn			
Org	0.40*	18	$y = -7.38x + 14.14$
Enz	ns	18	17.11 (avg.)
MT	0.27*	18	$y = -4.00x + 6.16$
MRG	0.89*	13	$y = 15.41x + -0.93$
Deb	ns	13	51.90 (avg.)
Pa-Cd			
Org	ns	12	11.27 (avg.)
Enz	ns	12	30.10 (avg.)
MT	0.63*	12	$y = 25.44x + 11.40$
MRG	ns	11	1.73 (avg.)
Deb	ns	12	30.67 (avg.)
Pa-Zn			
Org	0.40*	12	$y = -21.41x + 61.27$
Enz	ns	10	13.47 (avg.)
MT	sz?	5	4.58 (avg.)
MRG	0.70*	7	$y = 11.02x + 3.19$
Deb	ns	12	18.91 (avg.)
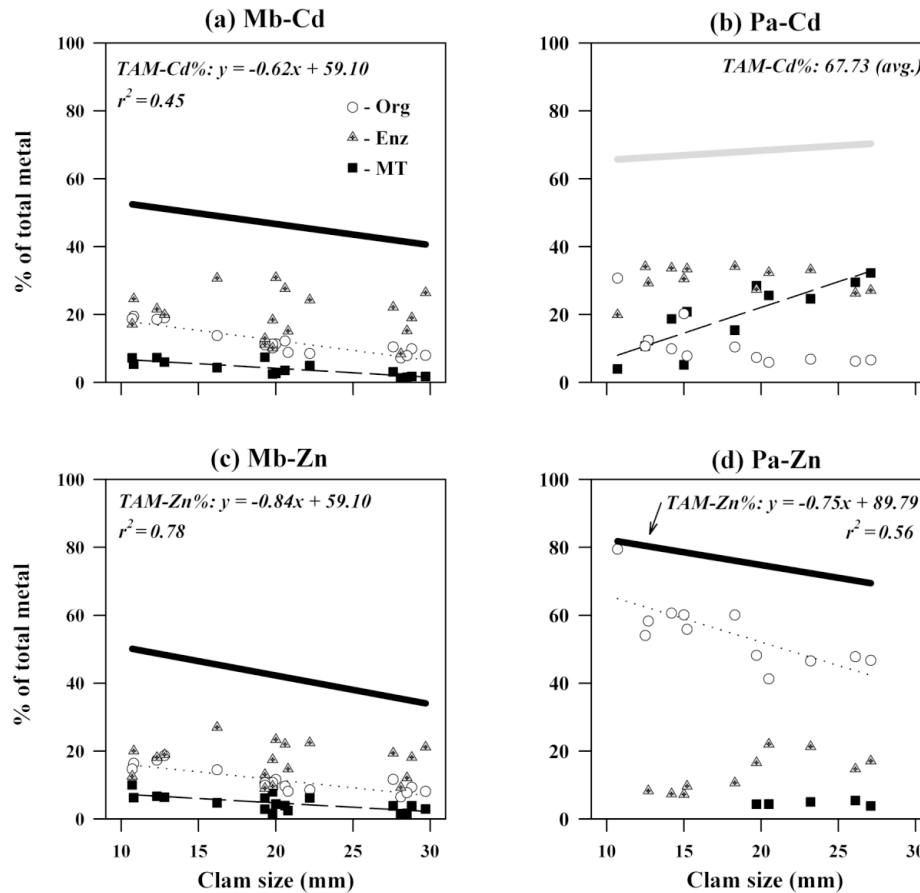
Fig. 6. *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa). Size-dependence (mm) in the subcellular partitioning of [109]Cd (a,b) and [65]Zn (c,d) to fractions comprising trophically available metal (TAM) (i.e. organelles [Org], 'enzymes' [Enz] and metal-lothioneins [MT]) following 14 d uptake. Thin lines represent significant size-dependence ($p < 0.05$; on a g wet wt basis—see Table 1) in the partitioning of metal to organelles (········) or MT (— — —). Bold solid lines as well as the presence of regression equations and coefficients (r^2) indicate significant size-dependence ($p < 0.05$) in the subcellular compartmentalization of metal as TAM (calculated as: TAM = 100 – [metal-rich granules [MRG]% + cellular debris%]; see 'Materials and methods' for a clarification of this calculation). A bold gray line indicates nonsignificance in subcellular compartmentalization as TAM

The relationship in Fig. 4 assumed that 100% of the metal associated with TAM was available for transfer, resulting in a 1:1 relationship between TAM of prey and Cd absorption by grass shrimp. However, if metal associated with organelles has a lower availability than other fractions, as demonstrated by Wallace & Lopez (1997), variable partitioning to this fraction may add variability to the TAM%:AE% relationship. A specific example of the influence of partitioning to organelles on trophic transfer can be seen in Fig. 4, where the largest deviation from the 1:1 (TAM%:AE%) relationship was for grass shrimp having ingested oligochaetes with a majority of accumulated Cd associated with organelles (Cd-resistant oligochaetes; Wallace et al. 1998). A 'recalculation' of the TAM-Cd% for this prey item, using an absorption efficiency of only ~70% for the Cd bound to organelles (Wallace & Lopez 1997) rather than 100%, improved the relationship (i.e. a

predicted Cd transference to shrimp of 23.6% rather than TAM-Cd% of 28.8% as shown in Fig. 4). This difference, however, is negligible, and the simplest approach of using partitioning of metal to the entire TAM compartment is probably adequate for evaluating metal available for trophic transfer.

Compartmentalization of Cd and Zn as TAM in bivalves

The amount of metal bioaccumulated by an organism is determined by the kinetics of uptake (influx rates from all sources and the rate constant of loss; Luoma & Fisher 1997). Studies have shown that predators most strongly bioaccumulate via trophic transfer metals that have rapid influx rates from food and low rate constants of loss (Reinfelder et al. 1998, Wang &

Ke 2002). Subcellular partitioning in prey affects the influx rate to predators because it determines what fraction of the total metal ingested is absorbed or is trophically available for transfer. Determining TAM in prey may, therefore, provide an *a priori* method for predicting this influence. For instance, a prey species that bioaccumulates high concentrations of a metal and fractionates a large proportion to TAM will be a greater threat of metal exposure to predators than one that either bioaccumulates less metal or fractionates less to TAM.

In this study it was found that *Macoma balthica* partitioned ~34% of accumulated Cd and Zn to the TAM compartment, while *Potamocorbula amurensis* partitioned ~60% of accumulated Cd and ~73% of accumulated Zn as TAM. In terms of percentages it would appear that, on average, a predator would absorb more of the Cd and Zn associated with the tissues of *P. amurensis* than with those of *M. balthica.* This, however, does not take into account differences in metal accumulation between species. In this context, based on dissolved uptake alone, it thus appears that *P. amurensis* is, indeed, a much greater threat of Cd transfer to predators (~34× that of *M. balthica*) due to strong bioaccumulation (~24× greater in this study) that stems from a slow loss and preferential storage into TAM fractions (mainly MT) (Lee et al. 1998, Wallace et al. 2003). Although Zn body burdens between the bivalves are, in general, similar (~2-fold difference), *P. amurensis* may also be the greater threat of Zn exposure to predators (~4× greater than *M. balthica*) (averaged across all sizes) because of greater partitioning to TAM (mainly organelles).

Size-dependence and field estimates of TAM

Bioaccumulated metal concentrations are known to vary with animal size (Boyden 1974, Cain & Luoma 1986), as does subcellular metal partitioning (Mouneyrac et al. 2000, Wallace et al. 2003). TAM may therefore also be size-dependent. For example, in this study it was found that in all but 1 of the species–metal combinations (i.e. *Potamocorbula amurensis*-Cd), the compartmentalization of metal as TAM de-

creased with increased clam size. A lower TAM% in large *Macoma balthica*, as compared with small clams, resulted from increased partitioning of both metals to MRG (not included as TAM) with size and presumably age (Wallace et al. 2003). Thus, in terms of potential metal threats to predators, the increase in tissue concentrations of Cd and Zn with size often observed in *M. balthica* in the field (Strong & Luoma 1981) may be offset by a higher proportion of metal in unavailable forms (i.e. MRG) (Wallace et al. 2003). The lack of size-dependence in tissue concentrations, as well as partitioning as TAM (at least for Cd) in *P. amurensis*, results in no size-dependence in the potential threat of Cd and Zn exposure to predators of this bivalve; the dose of Cd (i.e. TAM-[Cd]) to pre-
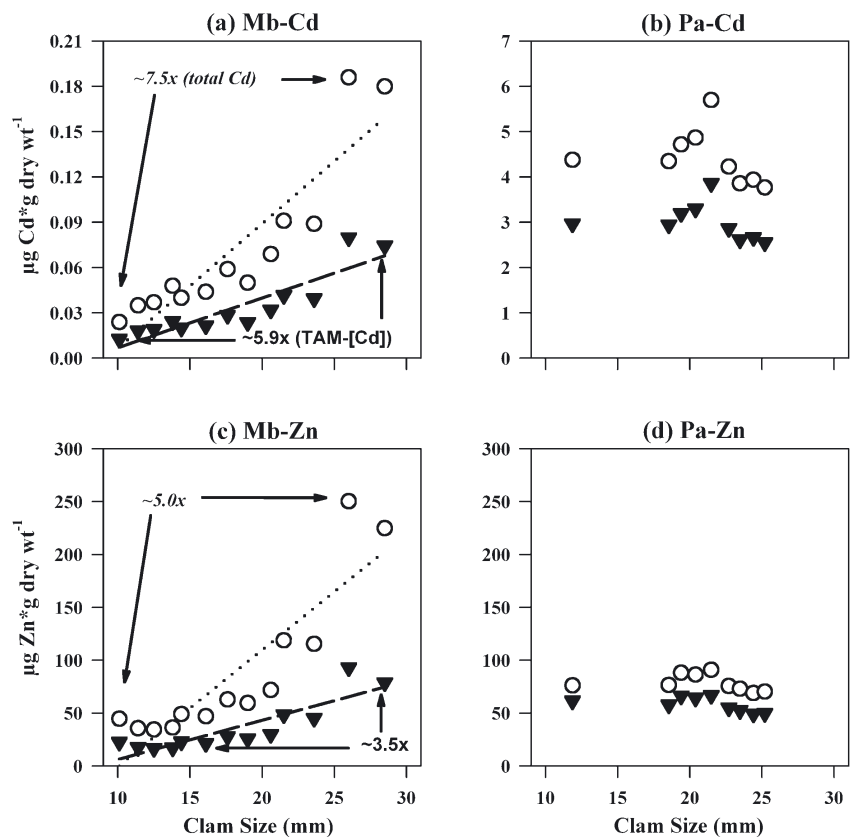

Fig. 7. *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa). Size-dependence in tissue concentrations (○) and estimates of trophically available metal (TAM-[Cd] and TAM-[Zn]) (▼) in clams collected from San Francisco Bay (data source: Mb from Luoma et al. 1998; Pa from C. Brown unpubl.). Tissue concentrations and concentrations of metal associated with the TAM compartment are reported as µg metal g dry wt^{-1}. TAM in field-collected animals was calculated by applying size-dependence in proportional TAM obtained from Fig. 6 to total tissue concentrations. The presence of lines represents significant size-dependence ($p < 0.05$) in tissue concentrations (········) or TAM (— — —). Fold-differences between large (~28 mm) and small (~11 mm) *M. balthica* in tissue concentrations (*italic*) and TAM (**bold**) are also listed (Fold-difference = [concentration of metal in large clam]/[concentration of metal in small clam]). After Wallace et al. (2003)

dators ingesting *P. amurensis* is estimated to be ~3 µg g dry wt^{-1}, which is ~50× that for predators ingesting *M. balthica*. Size-dependent Zn tissue concentrations in *M. balthica*, however, complicates a comparison of the Zn threat to predators of these 2 bivalves. On average, predators ingesting *P. amurensis* (regardless of size) would receive a Zn dose of ~60 µg g dry wt^{-1}, which is similar to that of ingesting large *M. balthica*, but ~2× that of small *M. balthica* (~30 µg g dry wt^{-1}).

Toxicological implications of TAM

The toxicologic significance of dietary exposure to metals is just beginning to be understood (Wallace et al. 2000, Hook & Fisher 2002). Quantifying the fraction of metal associated with the TAM of prey could be used to further understand the link between dietary metal exposure and toxicity, as total metal burdens in the diet may not accurately reflect the 'dose' of metal available for transfer. For example, in studies examining the relationship between dietary Cd exposure (Cd-exposed brine shrimp) and prey capture in grass shrimp, there was a 12-fold difference in the total Cd tissue concentration of prey between the control and highest treatment (3.5 vs 41 µg g wt^{-1}) (Wallace et al. 2000). When partitioning of Cd to the cytosol and organelles of brine shrimp was considered (now defined as the TAM compartment), there was only a 6.5-fold difference between these treatments (2 vs 13 µg g wt^{-1}) (Wallace et al. 2000). The differences in total tissue concentrations therefore did not accurately reflect the true threat to predators of dietary metal. It was further shown that sublethal toxicity in grass shrimp could result from a daily dose of Cd (i.e. TAM-[Cd] of brine shrimp × daily ingested tissue mass) only ~1.8× that of background (0.05 vs 0.09 µg d^{-1}) (Wallace et al. 2000). Interestingly, a grass shrimp ingesting the tissue of a single Cd-contaminated *Potamocorbula amurensis* of ca. 16 mm in length (~0.03 g dry wt) could obtain this minimum toxic dose of Cd (0.09 µg d^{-1}) (Wallace et al. 2000, C. Brown unpubl.). The potential toxicological consequences of TAM-[Zn] in *Macoma balthica* and *P. amurensis* to predators remains unclear.

Influence of metal detoxification (MT vs MRG) on TAM

Detoxification of metal via the induction of MT and precipitation into MRG allows organisms to tolerate, and even thrive, in metal-contaminated environments (Brown 1982, Klerks & Weis 1987, Klerks & Levinton 1989, Roesijadi 1992). The presence of metal-tolerant organisms, however, may influence metal trophic transfer because it increases the chance that a predator will encounter metal-contaminated prey (Roesijadi 1980). It may also influence the total concentration of metal in the predator's diet, particularly if detoxification by prey results in high tissue concentrations (Klerks & Weis 1987, Klerks & Bartholomew 1991). Detoxification strategies (i.e. MT and MRG), however, are population-, species-, metal- and even seasonally dependent (Brown 1982, Klerks & Bartholomew 1991, Bordin et al. 1992, 1996, 1997, Roesijadi 1992, Wallace et al. 1998, 2003). It is, therefore, essential to understand how these dependencies in detoxification impact the food-chain transfer of metals.

Because MT is considered a component of TAM, and MRG is not, the predominant pathway of detoxification by prey may control the bioavailability (or trophic availability) of metals to predators (Nott & Nicolaidou 1990, Wallace & Lopez 1997). For example, if the induction of MT and MT-like proteins is used to detoxify excess metal, it could 'drive up' the percentage of metal trophically available to predators (Roesijadi 1980, Wallace & Lopez 1996, 1997, Wallace et al. 1998). This MT-driven increase in TAM could be viewed as a 'bioenhancement' of metal trophic transfer (i.e. a greater than linear increase, as compared with increases in exposure, in the trophic transfer of metal, simply due to greater proportional partitioning to TAM).

Some evidence already exists for the 'bioenhancement' of Cd trophic transfer due to the induction of MT. Specifically, Wallace et al. (2000) showed that a ~5-fold increase in dietary Cd exposure to grass shrimp (0.05 vs 0.24 µg Cd d^{-1}) resulted in an ~18-fold increase in total Cd tissue concentrations. Because of the induction of MT, however, there was a ~32-fold increase in the storage of Cd in grass shrimp cytosol (i.e. enzymes and MT) (Wallace et al. 2000). Although these grass shrimp were not fed to a predator, this greater than linear increase in the partitioning of Cd to the now defined TAM compartment suggests that a 'bioenhancement' of Cd trophic transfer would have occurred. Recent studies with brine shrimp *Artemia franciscana* exposed to a wide range of Cd concentrations (through solution) has specifically demonstrated MT-driven 'bioenhancement' of Cd trophic transfer along an experimental food chain (brine shrimp → grass shrimp → killifish) (Seebaugh 2002, Seebaugh & Wallace unpubl.). The corollary of MT-driven 'bioenhancement' is MRG-driven 'bioreduction' whereby the storage of metal into metal-rich concretions, which occurs in large *Macoma balthica*, reduces the trophic availability of metal to predators (Nott & Nicolaidou 1993, Wallace et al. 1998).

Conclusions

In this study we have shown that metal-, species- and size-dependence in the accumulation of Cd and Zn can be interpreted by examining not only subcellular partitioning (Wallace et al. 2003), but also subcellular compartmentalization as trophically available metal (TAM). This approach has highlighted a number of important aspects of metal accumulation that should provide avenues for continued research. For instance, although metal tolerance in aquatic species inhabiting contaminated environments has long been reported (Luoma 1977), the impact of detoxification on metal trophic transfer (either 'bioenhancement' or 'bioreduction') is rarely considered (Nott & Nicolaidou 1993, Wallace & Lopez 1997). It is now clear that the influence of detoxification on the trophic transfer of metal needs to be addressed when examining the impacts and cycling of metals in aquatic food chains.

The practical advantages of monitoring metal contamination in organisms occupying lower trophic levels is well recognized (Brown & Luoma 1995). This current study, however, has shown that total tissue burdens in prey may not directly relate to metal transfer to predators. Because there can be toxicological consequences to consumers of metal-contaminated/metal-tolerant organisms (Wallace et al. 2000, Hook & Fisher 2002), the monitoring of TAM in prey could help provide valuable information on the 'dose' of metals available to predators. Such determinations might be an important pre-requisite for studying dietary toxicity in upper trophic-level organisms.

The isolation of TAM in soft-bodied invertebrate bioindicators is a relatively straightforward process, only requiring the homogenization of tissue and centrifugation at ~$1450 \times g$ (or less). Incorporating the analysis of TAM into monitoring and regulatory programs may be a way to screen ecosystems for subtle, and perhaps heretofore underappreciated potential effects on predators. The widespread application of this subcellular compartmentalization approach, focusing on metal associated with TAM (organelles, 'enzymes' and MT), as well as compartmentalization as MSF ('enzymes' and organelles) and BDM (MT and MRG) (see companion paper; Wallace et al. 2003) could provide valuable information on factors controlling the trophic transfer, accumulation and toxicity of metal in aquatic, and perhaps even terrestrial, ecosystems.

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