Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM)

William G. Wallace[1],*, Byeong-Gweon Lee[2], Samuel N. Luoma[3]

[1]Center for Environmental Science, College of Staten Island, 6S-310, City University of New York, 2800 Victory Boulevard, Staten Island, New York 10314, USA

[2]Department of Oceanography, Chonnam National University, 300 Yongbong-Dong, Kwang Ju 500-757, South Korea

[3]United States Geological Survey, 345 Middlefield Road, Mail Stop 465, Menlo Park, California 94025, USA

ABSTRACT: Many aspects of metal accumulation in aquatic invertebrates (i.e. toxicity, tolerance and trophic transfer) can be understood by examining the subcellular partitioning of accumulated metal. In this paper, we use a compartmentalization approach to interpret the significance of metal, species and size dependence in the subcellular partitioning of Cd and Zn in the bivalves *Macoma balthica* and *Potamocorbula amurensis*. Of special interest is the compartmentalization of metal as metal-sensitive fractions (MSF) (i.e. organelles and heat-sensitive proteins, termed 'enzymes' hereafter) and biologically detoxified metal (BDM) (i.e. metallothioneins [MT] and metal-rich granules [MRG]). Clams from San Francisco Bay, CA, were exposed for 14 d to seawater (20‰ salinity) containing 3.5 µg l^{-1} Cd and 20.5 µg l^{-1} Zn, including ^{109}Cd and ^{65}Zn as radiotracers. Uptake was followed by 21 d of depuration. The subcellular partitioning of metal within clams was examined following exposure and loss. *P. amurensis* accumulated ~22× more Cd and ~2× more Zn than *M. balthica*. MT played an important role in the storage of Cd in *P. amurensis*, while organelles were the major site of Zn accumulation. In *M. balthica*, Cd and Zn partitioned similarly, although the pathway of detoxification was metal-specific (MRG for Cd; MRG and MT for Zn). Upon loss, *M. balthica* depurated ~40% of Cd with Zn being retained; *P. amurensis* retained Cd and depurated Zn (~40%). During efflux, Cd and Zn concentrations in the MSF compartment of both clams declined with metal either being lost from the animal or being transferred to the BDM compartment. Subcellular compartmentalization was also size-dependent, with the importance of BDM increasing with clam size; MSF decreased accordingly. We hypothesized that progressive retention of metal as BDM (i.e. MRG) with age may lead to size dependency of metal concentrations often observed in some populations of *M. balthica*.

KEY WORDS: Subcellular compartmentalization · Cd · Zn · Bivalves · Toxicity · Detoxification

INTRODUCTION

Subcellular partitioning of metals within aquatic invertebrates reflects internal processing that occurs during metal accumulation and can provide valuable information about metal toxicity and tolerance (Roesijadi 1980, Jenkins & Mason 1988, Klerks & Bartholomew 1991). Manifestations of sublethal toxicity can coincide with changes in subcellular partitioning, par-

ticularly in cases where there is saturation of certain metal detoxification systems (Brown & Parsons 1978, Sanders et al. 1983). Similarly, partitioning to metal conglomerates or metal-rich granules (MRG) and binding with inducible metal-binding proteins (metallothioneins, MT) can reflect detoxification of metal, which has been linked to metal tolerance and resistance (Roesijadi 1980, Brown 1982, Klerks & Weis 1987, Klerks & Bartholomew 1991). Beyond having

uses in interpreting metal toxicity and tolerance, the subcellular distribution of metal within an organism can be used to understand metal trophic transfer to predators. Specifically, metal in soluble fractions of prey (i.e. cytosol and proteins) has been shown to be more bioavailable to predators than metal bound to non-soluble fractions (i.e. cell walls, exoskeleton and metal concretions) (Reinfelder & Fisher 1994, Wallace & Lopez 1997, Wallace et al. 1998, Wallace & Luoma 2003).

Metal toxicity is often discussed in terms of partitioning to sensitive components of the cell (i.e. organelles and enzymes). For example, the mitochondria of freshwater green algae have been shown to have impaired function upon exposure to Cd, resulting in reduced cellular respiration (Silverberg 1976). Additionally, lysosomes have been shown to become 'leaky' after exposure to metals, releasing hydrolytic enzymes into the cell (Viarengo et al. 1987, Bayne et al. 1988). Other work has linked the onset of metal toxicity in invertebrates to the binding of metal to proteins outside the MT pool (Brown & Parsons 1978, Roesijadi 1980, Jenkins & Mason 1988, Wallace et al. 2000). It therefore seems reasonable that quantifying the binding of metal to all sensitive cellular components (i.e. organelles and enzymes) could provide a more complete understanding of potential mechanisms of toxicity. Metal associated with organelles and enzymes could therefore be viewed together as a subcellular compartment containing metal-sensitive fractions (MSF).

Metal detoxification can take place by way of binding to inducible metal-binding proteins (i.e. MT) or through the precipitation of metal into insoluble concretions (i.e. MRG) (Roesijadi 1980, Brown 1982). Internal storage and detoxification of metal by MT and MRG has been related to increased metal tolerance and even genetic resistance (Brown 1978, Roesijadi 1980, Klerks & Bartholomew 1991). Although these pathways may not be completely independent, as MT may be used to transport metals to sites of MRG formation, they do represent 2 distinct mechanisms of detoxification (George 1983). If metals sequestered in MT and MRG are considered in tandem as a subcellular compartment that confers metal tolerance and perhaps resistance, the metal within this compartment could be defined as biologically detoxified metal (BDM).

It has long been recognized that aquatic organisms can be used to monitor metal pollution. The usefulness of 'biosentinel species', however, is metal- and species-dependent. Factors unique to metals and species include differences in metal uptake and loss rates, routes of exposure and confounding effects of environmental parameters (e.g. salinity and temperature) (Howard & Hacker 1990, Wang & Fisher 1997,

Lee et al. 1998). Additionally, the internal storage and detoxification of metal can also affect bioaccumulation, and differences among metals, species and age classes have not been extensively studied (Klerks & Bartholomew 1991, Mason & Jenkins 1995, Wallace et al. 2000). In this paper, we use the subcellular compartmentalization of metal as MSF and BDM to interpret the significance of metal, species and size dependence in the uptake, loss and subcellular partitioning of Cd and Zn in the bivalves *Macoma balthica* and *Potamocorbula amurensis*. A companion paper considers the subcellular compartmentalization of metal as trophically available metal (TAM) (Wallace & Luoma 2003).

Macoma balthica (a facultative deposit feeder) and *Potamocorbula amurensis* (a filter feeder) represent the dominant benthic macrofuana in San Francisco Bay, CA (SFB). These bivalves co-occur at various locations within the bay, show considerable differences in metal accumulation patterns, and have been successfully used to monitor metal pollution in this estuary (Cain & Luoma 1990, Brown & Luoma 1995, Lee et al. 1998). Although these bivalves bioaccumulate many trace metals, this paper focuses on the bioaccumulation of Cd and Zn.

MATERIALS AND METHODS

Collection and preparation of bivalves. *Macoma balthica* were collected by hand from the Palo Alto mud flat, south SFB, CA, in January 1997, while *Potamocorbula amurensis* were collected subtidally with a Van Veen grab from United States Geological Survey (USGS) Station 4.1, north SFB in January 1998 (Brown & Luoma 1995). Clams were returned to the laboratory, gently scrubbed of adhering sediment, and were acclimated over a 7 d period to experimental conditions (20‰ salinity, 10°C). During this acclimation period, clams were fed the phytoplankton *Rhodomonas salinas*.

Uptake and loss of Cd and Zn. Separate groups of *Macoma balthica* and *Potamocorbula amurensis* (~40 of each species) were exposed to metals for 14 d at 10°C in 4 l polyethylene aquaria containing 2 l of exposure media (Lee et al. 1998). Each group of clams was comprised of a size range (shell length) representative of the natural assemblage: 10.7 to 30.0 mm for *M. balthica* and 10.7 to 26.1 mm for *P. amurensis*. Exposure media, prepared by adding metal standards to 20‰ salinity (0.22 µm filtered) contained nominal concentrations of 3.5 µg l^{-1} (32 nM) Cd and 20.5 µg l^{-1} (320 nM) Zn, which approximated 32× background for each metal of the undiluted seawater (Lee et al. 1998).

Radioisotopes were used as tracers of stable metals, and at the beginning of each experiment (at $t = 0$) aquaria were spiked with appropriate levels of carrier-free [109]Cd and carrier-free [65]Zn (*Macoma balthica*: 6 kBq [109]Cd and 140 kBq [65]Zn; *Potamocorbula amurensis*: 4 kBq [109]Cd and 8 kBq [65]Zn). Both isotopes were diluted in 0.1 N double-distilled HCl. To neutralize the acid added with standards and isotopes, each container received appropriate amounts of 0.1 N double-distilled NaOH. Levels of radioisotopes in exposure media were monitored daily via γ-counting. On Days 3, 6, 9 and 12 of exposure, media within the aquaria were replaced with fresh media containing $t = 0$ concentrations of stable and radioactive metal. In some instances, re-spiking of exposure media was required prior to Day 3 (see 'Results'). Clams were not fed during the 14 d of uptake.

Following 14 d of uptake, clams were removed from the aquaria and were placed in clean seawater (20‰ salinity) for 30 min to allow body cavities to be flushed of retained exposure media. After rinsing, the clams from each experiment were randomly separated into 2 groups of ~20 clams each. One group was stored frozen (–80°C) awaiting subcellular fractionation (see following subsection). The other group was allowed to depurate accumulated metals for 21 d. These clams were transferred to depuration chambers enclosed within a recirculating seawater system (20‰ salinity, 10°C) modified to remove radioisotopes from solution by way of an activated charcoal filter (Wallace et al. 2000). While depurating metals, clams were fed once a day on the phytoplankton *Rhodomonas salinas*. Following 21 d of loss, clams were stored frozen (–80°C) awaiting subcellular fractionation.

Subcellular fractionation. Frozen clams were thawed on ice, measured with a caliper to the nearest 0.1 mm and dissected. Shells were discarded and tissue was dabbed dry with a Kimwipe®. Tissue from individual clams was then transferred into pre-weighed glass vials, weighed (i.e. wet weights were obtained for individual clams) and assayed for radioactivity in a γ-counter. Clam tissue was then homogenized with a Polytron® tissue homogenizer for 30 s on medium speed in 3.3 ml (~1:10 tissue to buffer ratio) of cold 20 mM TRIS buffer (pH 7.6). Homogenized clam tissue was then re-assayed for radioactivity to determine losses due to homogenization (losses were usually <15%). Due to differential sur-

vival during the experiments, there were between 12 and 19 clams homogenized per phase of exposure (uptake vs loss) and per treatment (*Macoma balthica* vs *Potamocorbula amurensis*).

Homogenized tissue was transferred to 50 ml centrifuge tubes and subjected to fractionation modified from that of Wallace et al. (1998) (Fig. 1). Homogenized tissue was first fractionated by centrifugation at $1450 \times g$, producing a pellet (P1) containing tissue fragments and other cellular debris (e.g. membranes, MRG) and a supernatant (S1) containing the cytosol. MRG were isolated from P1 through digestion of tissue fragments with NaOH and centrifugation at $5000 \times g$ (P2) (Silverman et al. 1983). The resulting supernatant (S2) contained metal associated with dissolved tissues (Wallace et al. 1998). It is important to note that even though MRG of various sizes may be membrane-limited and contained within various organelles, this procedure for isolating large concretions is most effective (Brown 1982, Wallace et al. 1998). The $1450 \times g$ supernatants (S1) were centrifuged at $100\,000 \times g$ to produce a pellet (P3) containing various organelles (i.e. nuclear, mitochondria and microsomes); the $100\,000 \times g$ supernatant (S3) contained the cytosol. The cytosol was then heat-denatured (80°C) and centrifuged at
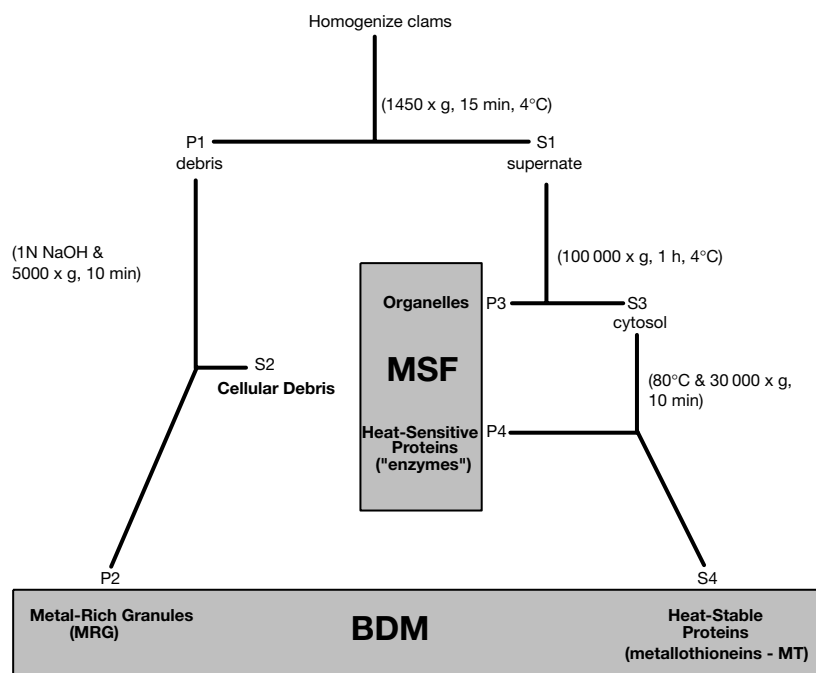

Fig. 1. Procedure for determining the subcellular partitioning of metal within clams. Clams were homogenized, and differential centrifugation and tissue digestion techniques were used to obtain the following subcellular fractions: metal-rich granules (MRG), cellular debris, organelles, heat-sensitive proteins ('enzymes') and heat-stable proteins (metallothionein-MT). Organelles and 'enzymes' are grouped as metal-sensitive fractions (MSF), and MT and MRG are grouped as biologically detoxified metal (BDM)

$30\,000 \times g$ (Bebianno & Langston 1992). This final pellet (P4) contained heat-sensitive proteins ('enzymes'), while the supernatant (S4) contained heat-stable proteins (MT and MT-like proteins). Each fraction was subsequently assayed for radioactivity, and metal contents within the various fractions were used to reconstruct the previously defined subcellular compartments: MSF = organelles + 'enzymes'; BDM = MT + MRG (Fig. 2).

Radioanalysis, metal body burden calculations and statistical analysis. Sample radioactivity was determined with a Wallac 1480 Wizard γ-counter equipped with a 7.8 cm well-type NaI crystal detector. 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 min), were converted to dpm (disintegration per min) using appropriate standards and half-life corrections. 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). Analysis included Student's t-tests (or t'-test when variances were not homogeneous, see Sokal & Rohlf 1981) to compare: (1) tissue concentrations between species after uptake; (2) tissue concentrations within a species after each phase of exposure (uptake vs loss); and (3) metal concentrations in subcellular fractions after each phase of exposure (uptake vs loss). Linear regression analysis was used to understand relationships between clam size (g wet wt) and (1) tissue concentrations after uptake and (2) subcellular partitioning to fractions and compartments after uptake.

RESULTS

Uptake phase (whole body and subcellular partitioning)

Following uptake *Potamocorbula amurensis* attained a tissue concentration of Cd (~1330 ng g wet wt^{-1}) that was ~22× greater (p < 0.001) than that of *Macoma balthica* (~60 ng g wet wt^{-1}) (Fig. 3a, Table 1). Zn accumulation was also species-dependent with *P. amurensis* accumulating ~2× that of *M. balthica* (p < 0.001) (~1030 vs ~550 ng g wet wt^{-1}) (Fig. 3b, Table 1). Daily monitoring of exposure media revealed substantial drops in exposure concentrations for the experiment with *P. amurensis* on Days 1 (for Cd) and 2 (for Cd and Zn) (Fig. 3a,b insets), necessitating the re-spiking of exposure media to re-establish $t = 0$ concentrations. Daily monitoring of radioactivity in exposure media allowed for the reconstruction of the clam's 'exposure history' during uptake. This reconstruction, calculated as a grand mean (gm) of daily average exposures (da) over the 14 d period (gm = [(∑da)/14]; da = [(t_0 activity + t_{24} activity)/2]), revealed that *M. balthica* received an exposure within ~3% of nominal (~100% for Cd and ~97% for Zn). Exposure for *P. amurensis*-however, was somewhat lower, being within ~22% of nominal (~85% for Cd and ~78% for Zn).
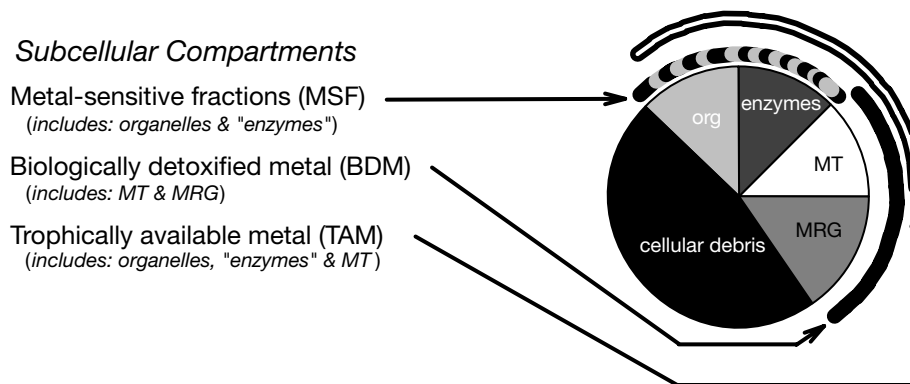

Fig. 2. A generalized ecotoxicological pie chart depicting subcellular compartments based on the biological significance of the various subcellular fractions. Subcellular fractions that are potentially vulnerable to metal exposure (i.e. organelles and 'enzymes') constitute metal-sensitive fractions (MSF: dashed arc). Those fractions that are involved with metal detoxification (i.e. MT and MRG) constitute biologically detoxified metal (BDM: solid arc). Fractions containing metal that is readily available to predators (i.e. organelles, 'enzymes' and MT) constitute trophically available metal (TAM: double arc) (see companion paper: Wallace & Luoma 2003)
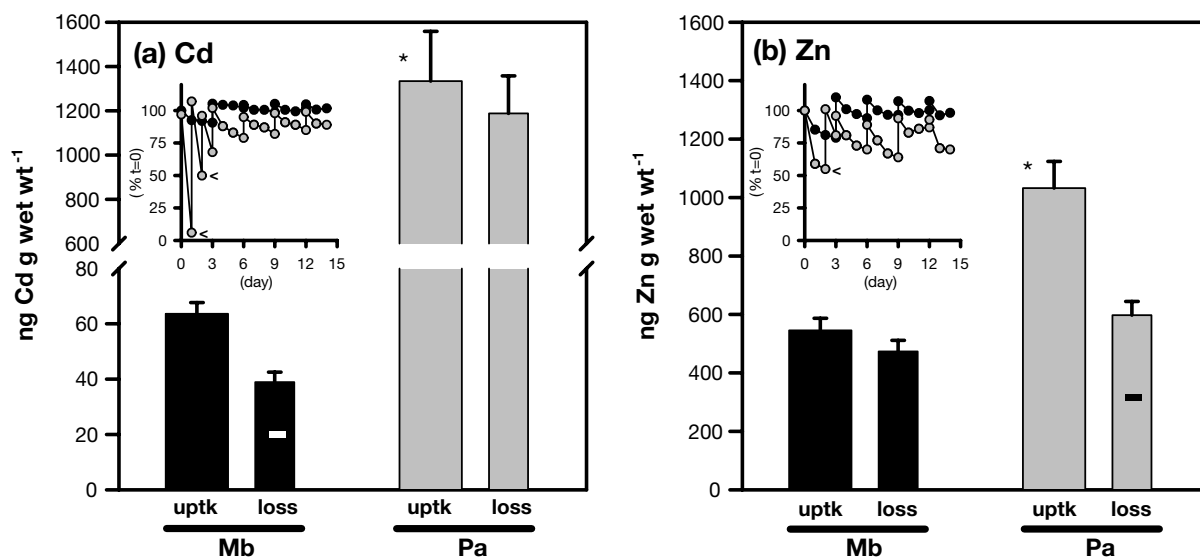
Fig. 3. Comparisons of the (a) Cd and (b) Zn tissue concentrations of *Macoma balthica* (Mb: filled bars) and *Potamocorbula amurensis* (Pa: half-tone bars) following 14 d of uptake (uptk: wide bars) and 21 d of loss (loss: thin bars). Concentrations are reported as ng metal g wet wt^{-1} ± SE. Significant differences ($p < 0.001$) in metal accumulation between clams are indicated by the placement of (⋆) above the uptake bars for *P. amurensis*. Significant losses ($p < 0.001$) of Cd or Zn after 21 d of depuration are indicated by the placement of (–) in the center of the respective loss bar. Inset 3a and 3b: Time course of (a) ^{109}Cd and (b) ^{65}Zn exposure for *M. balthica* (filled circle) and *P. amurensis* (half-tone circle) as a percentage of nominal concentrations ($t = 0$, radioactivity set at 100%). Exposure media was changed on Days 3, 6, 9 and 12, though re-spiking of exposure media prior to Day 3 was required in the experiment with *P. amurensis* as indicated by (<)

Table 1. Concentrations of Cd and Zn associated with whole body or subcellular fractions (organelles, Org; 'enzymes', Enz; metallothioneins, MT; metal-rich granules, MRG; cellular debris, Deb) of *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa). Significant changes upon loss are marked accordingly: decreases, –; increases, +; $p < 0.05$ or greater; no significant change, ns. Values are given as mean ± SD (n) in ng g wet wt^{-1}

			Uptake (n)	Loss (n)	Change
Mb-Cd	Whole body		63.5 ± 18.9 (19)	38.9 ± 15.8 (19)	–
	Subcellular	Org	6.8 ± 4.0 (18)	3.3 ± 2.2 (19)	–
		Enz	10.3 ± 4.3 (18)	4.0 ± 1.6 (18)	–
		MT	2.2 ± 1.6 (15)	1.0 ± 0.6 (3)	ns
		MRG	6.2 ± 2.7 (7)	3.1 ± 2.0 (9)	–
		Deb	23.8 ± 8.8 (12)	20.1 ± 5.9 (16)	ns
Mb-Zn	Whole body		545.4 ± 181.5 (19)	473.5 ± 164.5 (19)	ns
	Subcellular	Org	56.5 ± 33.2 (18)	41.5 ± 22.8 (19)	ns
		Enz	79.2 ± 31.8 (18)	56.8 ± 20.5 (19)	–
		MT	22.8 ± 16.1 (18)	26.3 ± 31.7 (19)	ns
		MRG	18.7 ± 15.5 (12)	71.6 ± 25.1 (16)	+
		Deb	230.8 ± 81.1 (12)	217.3 ± 79.6 (16)	ns
Pa-Cd	Whole body		1333.9 ± 777.7 (12)	1187.8 ± 588.4 (12)	ns
	Subcellular	Org	140.0 ± 146.3 (12)	127.5 ± 138.6 (12)	ns
		Enz	326.0 ± 187.9 (12)	261.8 ± 118.5 (12)	ns
		MT	183.6 ± 111.4 (12)	242.5 ± 191.2 (12)	ns
		MRG	19.9 ± 14.3 (11)	2.9 ± 6.2 (9)	ns
		Deb	344.3 ± 213.0 (12)	287.1 ± 153.5 (12)	ns
Pa-Zn	Whole body		1032.2 ± 317.2 (12)	597.5 ± 162.0 (12)	–
	Subcellular	Org	535.2 ± 236.6 (12)	233.7 ± 74.1 (12)	–
		Enz	113.9 ± 28.4 (10)	107.1 ± 28.1 (8)	ns
		MT	31.0 ± 7.1 (5)	42.2 ± 5.9 (4)	+
		MRG	60.8 ± 26.0 (7)	34.8 ± 18.9 (3)	ns
		Deb	178.4 ± 55.3 (12)	135.5 ± 29.6 (10)	–

Subcellular fractionation of metal within clams revealed that after uptake, Cd and Zn partition similarly within *Macoma balthica*. The distribution of both metals followed the general pattern of (mean%): cellular debris (~50%) ≫ enzymes (~20%) > organelles (~12%) ≈ MT + MRG (~15%) (Fig. 4a,c). Partitioning between MT and MRG in *M. balthica*, however, was metal-dependent; Cd: MT (~4%) < MRG (~14%); Zn: MT (~5%) = MRG (~5%). In *Potamocorbula amurensis*, Cd and Zn distributions were markedly different with the major differences being the preferential storage of Cd in MT (~20% of Cd > ~5% of Zn) and Zn in organelles (~11% of Cd < ~55% of Zn) (Fig. 4b,d).

Grouping the various fractions into the previously defined subcellular compartments (MSF and BDM) reveals that *Macoma balthica* and *Potamocorbula amurensis* partitioned similar proportions of both metals as BDM, with storage of Cd being ~2× that of Zn (~20% Cd > ~11% Zn; $p <$
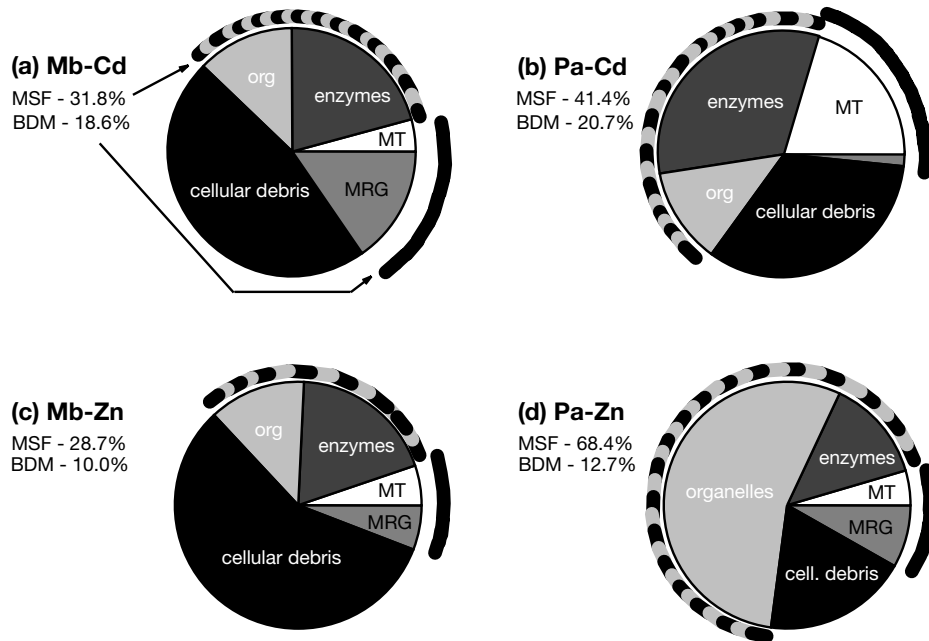
Fig. 4. Ecotoxicological pie charts showing the proportion of accumulated (a, b) ^{109}Cd and (c, d) ^{65}Zn associated with subcellular fractions as well as subcellular compartments, metal-sensitive fractions (MSF: dashed arc) and biologically detoxified metal (BDM: solid arc), of *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa). Distributions are for clams following 14 d of accumulation (average). Subcellular fractions are as follows: organelles (org), heat-sensitive proteins ('enzymes'), heat-stable proteins (metallothionein—MT), metal-rich granules (MRG) and cellular debris
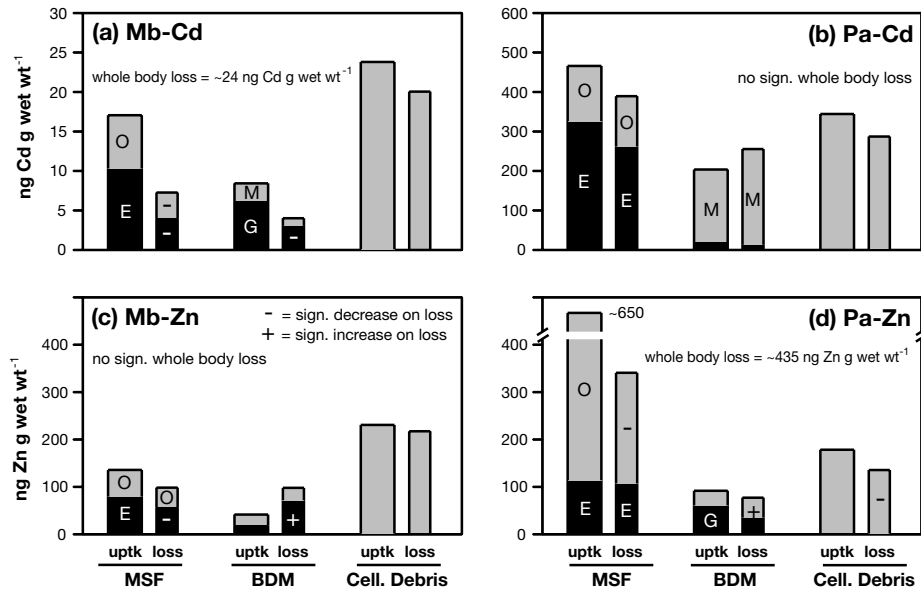

Fig. 5. Comparisons of the subcellular partitioning of (a, b) Cd and (c, d) Zn in *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa) following 14 d of uptake (uptk: wide bars) and 21 d of loss (loss: thin bars). Mean body burdens are reported as ng metal g wet wt^{-1}. Error bars are not included due to the use of a stacked bar presentation (see Table 1 for ±SD). Subcellular fractions are grouped as metal-sensitive fractions (MSF) (i.e. organelles [O: half-tone] and enzymes [E: filled]), biologically detoxified metal (BDM) (i.e. metallothioneins [M: half-tone] and metal-rich granules [G: filled]) or cellular debris (Cell. Debris: half-tone). Fraction labels (i.e. O, E, M or G) are placed in the center of most bars. Significant changes (decrease [–] or increase [+]) (p < 0.05 or greater) in the metal burdens of subcellular fractions following 21 d of loss are indicated by the placement of (–) or (+) within the center of the loss bar. Significant losses in whole body metal burdens following 21 d of depuration are listed within each panel for comparison with individual subcellular fractions

0.05 to 0.01) (Fig. 4a to d). Partitioning of metal to MSF, however, was metal- as well as species-dependent. In *M. balthica*, the MSF contained ~30% of both Cd and Zn (Fig. 4a,c), while the MSF of *P. amurensis* contained ~40% of Cd, compared with ~68% of Zn (Fig. 4b,d).

Loss phase (whole body and subcellular partitioning)

Table 1 lists metal concentrations (whole body and subcellular fractions) for *Macoma balthica* and *Potamocorbula amurensis* following 14 d of uptake and 21 d of loss. Following depuration, *M. balthica* lost a significant (p < 0.001) amount of Cd (~40%; loss of ~24 ng g wet wt^{-1}) dropping to ~40 ng g wet wt^{-1}, while there was no significant loss of Cd from *P. amurensis* (Fig. 3a, Table 1). The pattern in Zn depuration was reversed, with *M. balthica* exhibiting a slow loss and *P. amurensis* loosing ~40% (loss of ~435 ng g wet wt^{-1}) (p < 0.001), retaining a tissue concentration of ~600 ng g wet wt^{-1} (Fig. 3b, Table 1).

In addition to changes in tissue concentrations following loss, there were also changes (decreases as well as increases) in the concentration of metals associated with many subcellular fractions (Table 1). For instance, the concentration of Cd associated with organelles, 'enzymes' and MRG of *Macoma balthica* decreased substantially (p < 0.01 to 0.001) following 21 d of depuration. Like tissue concentrations, however, no subcellular fraction of *Potamocorbula amurensis* exhibited a significant decrease in Cd after loss.

From the standpoint of subcellular compartments, however, a majority of the Cd lost from *Macoma balthica* and the Zn lost from *Potamocorbula amurensis* appears to have originated from fractions within the MSF (i.e. organelles and 'enzymes') (Fig. 5a,d). Specifically, in *M. balthica*, both the organelle and 'enzyme' fractions lost Cd, while ~70% of the total Zn depurated from *P. amurensis* originated from organelles (Fig. 5a,d, Table 1). Additionally, even though *M. balthica* showed no loss of Zn on a whole body basis, Zn was lost from the MSF (i.e. 'enzymes', p < 0.05) by way of redistribution into the BDM (i.e. MRG, p < 0.001) (Fig. 5c). Interestingly, even though *P. amurensis* showed no significant loss or redistribution of Cd following 21 d of depuration, redistribution from MSF (organelles and 'enzymes') into BDM (i.e. MT) is suggested by the subtle, though non-significant, changes in the concentrations of these compartments and fractions (Fig. 5b).

Size dependence in uptake and subcellular partitioning

Macoma balthica and *Potamocorbula amurensis* displayed significant size dependence in the accumulation of both Cd and Zn, with smaller clams accumulating roughly twice as much metal as larger clams (Fig. 6). Although the data are not shown, comparisons of regression equations relating clam size to tissue concentrations after uptake with those following loss reveal no size dependence in metal loss (i.e. slopes after uptake and after loss are the same).

Linear regression analysis was used to analyze size dependence (g wet wt) in the distribution of Cd and Zn among the various subcellular fractions (Table 2) and compartments (Fig. 7) of *Macoma balthica* and *Potamocorbula amurensis*. In all but one of the species-metal combinations (i.e. Pa-Cd; Fig. 7b), the proportion of metal in the organelle fraction of the MSF compartment decreased with size (Table 2). Storage of metal in 'enzymes', however, was independent of size. Interestingly, although neither organelles nor 'enzymes' of *P. amurensis* displayed size dependence in Cd storage (Fig. 7b, Table 2), a grouping of these 2 fractions into the subcellular compartment MSF reveals size dependence in the partitioning of Cd (p < 0.01) (Fig. 7b, MSF). Thus, in all of the species-metal combinations, smaller clams were found to have partitioned more metal to components of the MSF compartment (i.e. organelles and 'enzymes') than larger clams.

In contrast, the importance of the BDM compartment for metal storage increased, in general, with clam size (Fig. 7). This is most dramatic for the storage of Cd in *Potamocorbula amurensis* (Fig. 7b), where binding of Cd to MT (p < 0.01) drives size dependence in the partitioning of Cd to the BDM compartment (p < 0.01). Similarly, even though the detoxification pathway
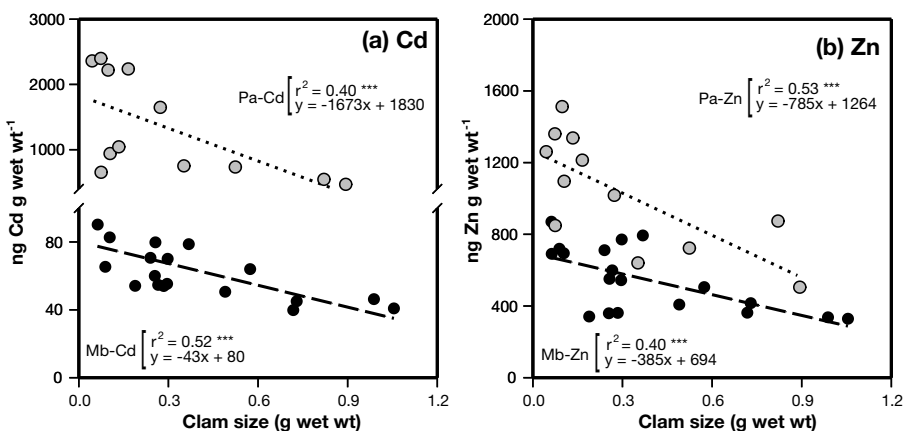

Fig. 6. Size dependence (g wet wt) in the accumulation of (a) Cd and (b) Zn by *Macoma balthica* (Mb: filled circle) and *Potamocorbula amurensis* (Pa: half-tone circle) after 14 d of uptake. Tissue concentrations are reported as ng metal g wet wt^{-1}. Results of regression analysis are listed for each species-metal combination
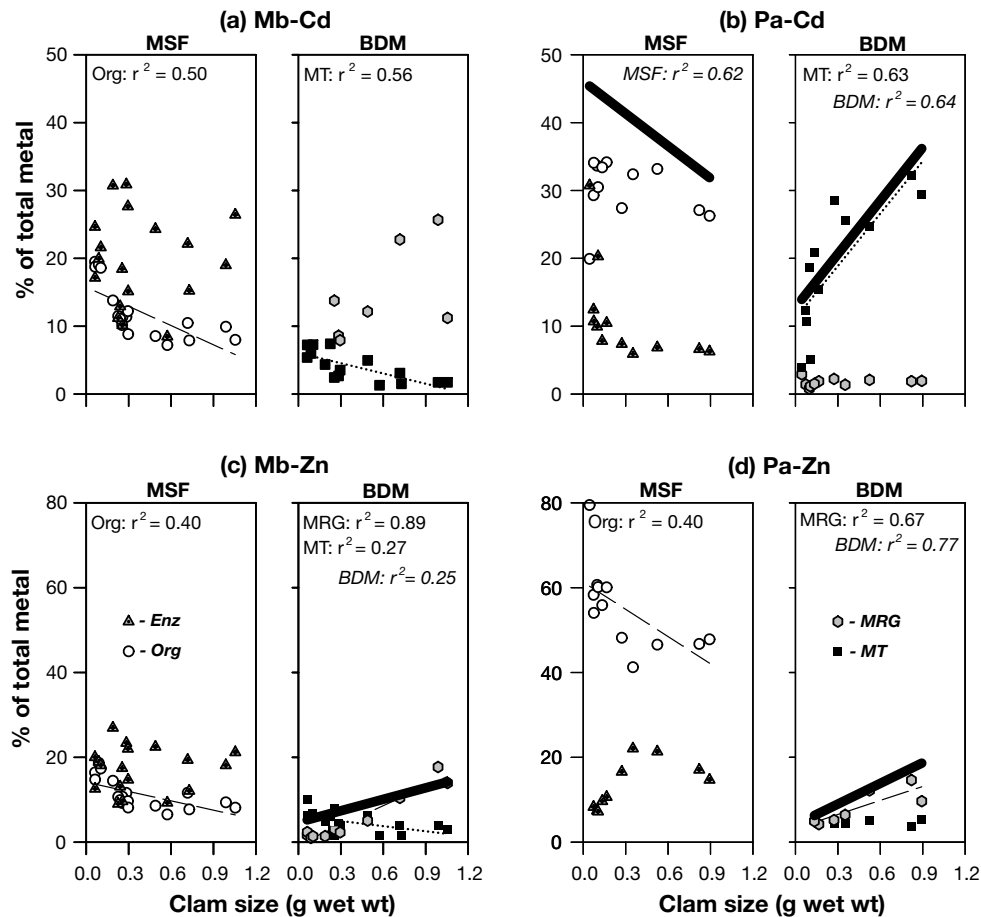
Fig. 7. Size dependence (g wet wt) in the proportional subcellular partitioning of (a, b) [109]Cd and (c, d) [65]Zn in *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa) following 14 d of uptake. Subcellular fractions are grouped as metal-sensitive fractions (MSF) (i.e. organelles [half-tone cross hatched triangle] and 'enzymes' [opened circle]) or biologically detoxified metal (BDM) (i.e. metal-rich granules [half-tone hexagon] and metallothioneins [filled square]). Dashed lines represent significant ($p < 0.05$) size dependence in the subcellular partitioning of metal to 'enzymes' and metallothioneins (······), or organelles and metal-rich granules (— —). See Table 1 for specifics on regressions. Bold solid lines represent significant size dependence ($p < 0.05$) in the subcellular compartmentalization of metal as MSF (calculated as: MSF = %organelle + %'enzyme') or BDM (calculated as: BDM = %metallothionein + %metal-rich granule). Significant ($p < 0.05$) regression coefficients (r^2) are listed for subcellular fractions (arial, left-justified) as well as subcellular compartments (italic, right-justified)

changed as a function of size in *Macoma balthica* (MT for small clams and MRG for large clams; no Cd was detected in the MRG fraction of clams smaller than 0.3 g wet wt), the importance of the entire BDM compartment for the storage of both Cd and Zn increased with clam size (Fig. 7a,c). Interestingly, the importance of MRG for Zn storage in *P. amurensis* also increased with size (Fig. 7d, Table 2).

Estimates of subcellular compartmentalization in field populations

An interesting consequence of metal, species and size dependence in the subcellular partitioning of Cd and Zn in *Macoma balthica* and *Potamocorbula*

amurensis is a suspected impact on total metal concentrations. Work conducted in concert with these studies has demonstrated that Cd and Zn concentrations (µg g dry wt[−1]) of *M. balthica* increase with clam size, with no size dependence being found for *P. amurensis* (Mb: Luoma et al. 1998; Pa: C. Brown unpubl.) (Fig. 8). Specifically, large *M. balthica* (~28 mm) were found to have tissue concentrations of Cd and Zn that were, respectively, ~7.5 and ~5× higher than those of small *M. balthica* (~11 mm) (Fig. 8a,c). Applying generalized size-specific subcellular metal distributions obtained through radioisotope studies (Table 2) to the stable tissue concentrations of 'small' and 'large' *M. balthica* allows for the calculation of fraction-specific metal concentrations within this population of bivalve. Even though these calculations are estimates, the method

Table 2. 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; cellular debris, Deb) of *Macoma balthica* (Mb) and *Potamocorbula amurensis* (Pa) following 14 d of uptake. Regression coefficients marked with $\star$ indicate significance ($p < 0.05$). Non-significant regression coefficients are marked ns and have average proportional distributions (avg.) 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 subcellular distribution of metal within field collected specimens (see Fig. 8)

		r^2	n	Regression equation
Mb-Cd	Org	0.50$\star$	18	$y = -9.43x + 15.75$
	Enz	ns	18	19.75 (avg.)
	MT	0.56$\star$	15	$y = -4.93x + 6.06$
	MRG	sz?	7	14.57 (avg.)
	Deb	0.37$\star$	12	$y = -9.19x + 47.90$
Mb-Zn	Org	0.40$\star$	18	$y = -7.38x + 14.14$
	Enz	ns	18	17.11 (avg.)
	MT	0.27$\star$	18	$y = -4.00x + 6.16$
	MRG	0.89$\star$	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$\star$	12	$y = 25.44x + 11.40$
	MRG	ns	11	1.73 (avg.)
	Deb	ns	12	30.67 (avg.)
Pa-Zn	Org	0.40$\star$	12	$y = -21.41x + 61.27$
	Enz	ns	10	13.47 (avg.)
	MT	sz?	5	4.58 (avg.)
	MRG	0.70$\star$	7	$y = 11.02x + 3.19$
	Deb	ns	12	18.91 (avg.)

provides a rough approximation of the subcellular partitioning of stable metal (Wallace et al. 1998).

These calculations show that the MRG fraction of large *Macoma balthica* contains ~65× more Cd and ~45× more Zn than the same fraction of small *M. balthica* (Fig. 8a,c). The 'enzyme' and cellular debris fractions of large *M. balthica* contain ~5 to 6× the Cd and Zn of the same fractions of small clams (Fig. 8a,c). The final 2 fractions, MT and organelles, of large *M. balthica* are estimated to contain ~3× the metal of small clams. Similar calculations for *Potamocorbula amurensis* indicate that although some subcellular fractions of large clams (~25 mm) exhibit enhanced binding over that of small clams (~12 mm) (i.e. MT for Cd and Zn [~5 to 6×]; enzymes for Zn [~14×]), other fractions display diminished binding in large individuals (i.e. organelles for Cd and Zn [~0.2 to 0.5×]) (Fig. 8b,d).

DISCUSSION

This study evaluated the subcellular partitioning of Cd and Zn in 2 bivalves, *Macoma balthica* and *Potamocorbula amurensis*, that have been used to monitor metal pollution in SFB. Previous work has shown that the 2 species differ in their patterns of Cd and Zn bioaccumulation, and as a result, their optimal uses as biosentinel species may differ (i.e. *M. balthica* for Zn and *P. amurensis* for Cd) (Cain & Luoma 1990, Brown & Luoma 1995). Here, we show that bioaccumulation and subcellular partitioning of these metals also differ between the species, and may help in understanding
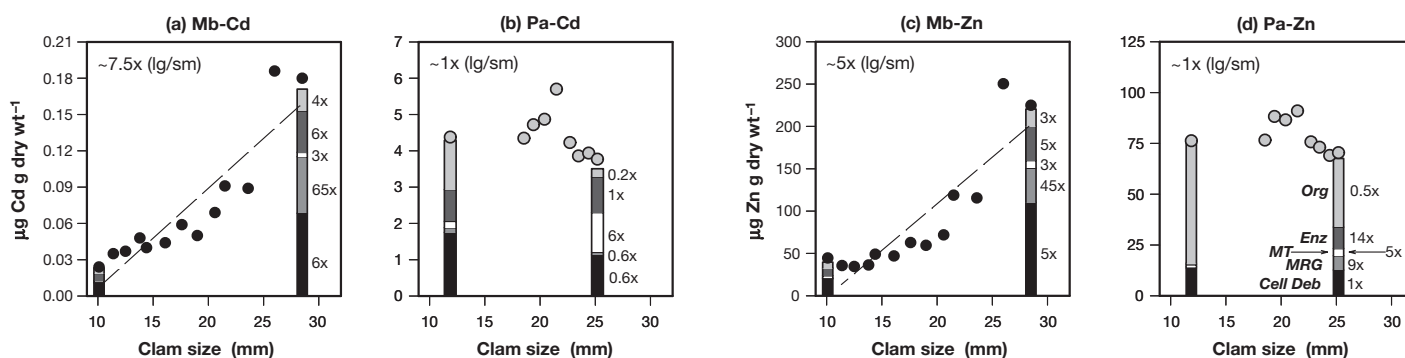

Fig. 8. Size dependence (mm) in the (a, b) Cd and (c, d) Zn concentrations (μg g dry wt^{-1}) of *Macoma balthica* (Mb: filled circle) and *Potamocorbula amurensis* (Pa: half-tone circle) collected from San Francisco Bay, CA (Data source: Mb from Luoma et al. 1998, Pa from C. Brown unpubl.). Significant size dependence ($p < 0.05$) in metal body burdens are indicated by (— —). Fold differences in tissue concentrations between large (Mb: 28 mm; Pa: 25 mm) and small (Mb: 11 mm; Pa: 12 mm) clams for each species are listed in the top left corner of each panel (Fold difference = [concentration of metal in large clam]/[concentration of metal in small clam]). Stacked bars represent estimates of the distribution of total metal among subcellular fractions. Estimates are based on radioisotope data obtained from this work (Table 2). Bars (from top to bottom) are as follows: organelles (Org: 25% tone), 'enzymes' (Enz: 75% tone), metallothioneins (MT: 0% tone), metal-rich granules (MRG: 50% tone), cellular debris (Cell Deb: 100% tone). Numbers adjacent to bar 'slices' for large clams represent fold differences between large and small clams in the concentration of metal associated with the respective subcellular fraction (i.e. the 'enzyme' fraction of large *P. amurensis* contains ~14× more Zn than that of small *P. amurensis*) (Fold difference = [concentration of metal in fraction of large clam]/[concentration of metal in fraction of small clam])

interspecies differences in uptake and, perhaps, differences in vulnerability to contamination. Size dependence in uptake and subcellular storage was also considered. The subcellular partitioning results were particularly useful when individual fractions were grouped into compartments: MSF (i.e. organelles and 'enzymes') and BDM (i.e. MT and MRG).

Accumulation and depuration of Cd and Zn

The enhanced accumulation of Cd and Zn in *Potamocorbula amurensis* over that in *Macoma balthica* observed in this study has been reported by others and is attributed to the higher filtration rates of *P. amurensis* (Lee et al. 1998). This rapid accumulation in *P. amurensis*, however, could have introduced an 'artifact' when comparing the accumulation of metal between these species, as rapid uptake by *P. amurensis* effectively reduced its own exposure. An alternative experimental design would have been a synchronous exposure of both species in the same vessel, so that the drawdown of metal by *P. amurensis* would have also reduced the exposure to *M. balthica*. However, had the experiment been conducted in this manner, the most important conclusions from this aspect of the work (i.e. increased accumulation of both metals by *P. amurensis*) would have only been amplified as *M. balthica* would have also received the reduced exposure.

Differences in whole body depuration were also important in determining metal- and species-dependent accumulation. The rapid loss of Cd by *Macoma balthica* and Zn by *Potamocorbula amurensis* was equivalent to loss rates of ~2% d^{-1} for each species-metal combination. In general, these loss rates are similar to those reported for other bivalves (Wang et al. 1996, Reinfelder et al. 1997, Lee et al. 1998). The slow depuration of metal (Cd by *P. amurensis* and Zn by *M. balthica*; loss rates for these species-metal combinations could not be determined in this study), however, is less frequently observed. Although other factors can certainly affect total accumulation, differences in uptake and loss rates alone suggest that under the same conditions, Cd concentrations would be highest in *P. amurensis*, while Zn concentrations in *M. balthica* would be most responsive to environmental change. Brown & Luoma (1995) made such an observation at a site within SFB where these species co-occur.

Subcellular partitioning after uptake: species- and metal-specific aspects

Although previous studies have investigated the subcellular partitioning of metal within aquatic invertebrates (Johansson et al. 1986, Klerks & Bartholomew 1991, Hylland et al. 1994), the influences of biological, chemical and environmental factors on this partitioning are not fully understood (Howard & Hacker 1990, Bordin et al. 1997, Mouneyrac et al. 2000). The present study shows that biological (size and species), chemical (metal) and temporal (uptake and loss) aspects of exposure must be considered when interpreting an organism's internal metal distribution. Additionally, the use of operationally defined subcellular compartments allowed for a more 'holistic' approach to viewing the subcellular partitioning of metals within these bivalves.

Even though this subcellular compartmentalization approach may have artifacts resulting from possible overlap among subcellular fractions (i.e. lysosomes collected in the organelle fraction may contain metals bound to MT or granules in early stages of deposition) (Brown 1982, Roesijadi & Klerks 1989), the defining of subcellular compartments has merit and has been used by other researchers. For instance, Mouneyrac et al. (1998) and Ettajani et al. (2001) both considered partitioning to heat-stable compounds (including MT), insoluble components (including MRG) and target molecules (including enzymes) when investigating metal accumulation in the oyster *Crassostrea gigas*. The subcellular compartmentalization approach used in the present study, however, is somewhat more detailed as it considers the binding of metals to specific subcellular fractions as well as subcellular compartments (e.g. MSF and BDM). Additionally, because the subcellular partitioning of metal within prey may relate to metal trophic transfer (Reinfelder & Fisher 1994, Wallace & Lopez 1996, 1997), an additional compartment can be defined (trophically available metal, TAM) (Wallace & Luoma 2003).

Biologically detoxified metal (BDM)

Both clams displayed greater partitioning of Cd than Zn to fractions that are likely to represent 'biological detoxification', perhaps reflecting the more toxic nature of Cd (Sadiq 1992). The species, however, differed in the partitioning of metal between MT and MRG, perhaps a reflection of differences in detoxification strategies or isotopic exchange into larger pools of detoxified metal. Both Cd and Zn induce the production of MT in general (Roesijadi 1992), but studies with *Macoma balthica* have shown mixed results with regard to the induction of metal-binding proteins (Johannson et al. 1986, Langston & Zhou 1987). In most laboratory studies, high exposure concentrations (i.e. 10 to 40 µg l^{-1}) have induced the synthesis of MT in *M. balthica* (Bordin et al. 1994, Mouneyrac et al. 2000). However, little

MT was found in field specimens when tissue concentrations of bioaccumulated metal were relatively low (Langston & Zhou 1987, Bordin et al. 1994). The lower-level, short exposures used in the present work yielded results similar to those found by Langston & Zhou (1987) (i.e. ~4% of Cd being bound to MT). To date little is known about the production of MT in *Potamocorbula amurensis*. Our observation that *P. amurensis* sequesters up to 18% of its total Cd in MT or MT-like proteins, with less Zn (~4%) being associated with this fraction, is consistent with other molluscs (Roesijadi 1994).

A second detoxification strategy in invertebrates is storage into MRG (Brown 1982). Earlier work referenced this method of metal storage for *Macoma balthica*, but no specific studies were conducted (Strong & Luoma 1981, Mouneyrac et al. 2000). Storage of metals in granules of *M. balthica* as a byproduct of sulphide detoxification, however, has been observed (Windoffer et al. 1999). In the current work, MRG accounted for ~15% of the Cd in *M. balthica*, and only ~2% of the Cd in *Potamocorbula amurensis*. Partitioning of Zn to MRG for these species was similar (~7%).

Interestingly, if sequestration as MT is considered the only mode of detoxification, it might be concluded that, as compared with *Macoma balthica*, *Potamocorbula amurensis* is more efficient at detoxifying Cd. This conclusion, however, may not be true, as when MT and MRG are considered in tandem as a compartment containing BDM, both species are found to have ~20% of accumulated Cd 'detoxified'. Viewing the partitioning of metals to MT as well as MRG may therefore provide a more accurate estimate of an organism's detoxification capabilities.

Metal-sensitive fractions (MSF)

Approximately 30% of both Cd and Zn in *Macoma balthica* were partitioned to components of the MSF compartment (organelles and 'enzymes'), but in contrast, partitioning as MSF in *Potamocorbula amurensis* differed between the metals. Additionally, a larger proportion of both metals was partitioned to the MSF compartment of *P. amurensis* as compared with *M. balthica*. The enhanced binding of Zn to fractions within the MSF is reasonable as Zn is an essential metal (Rainbow et al. 1990, Sadiq 1992). Preferential binding of Zn to organelles and 'enzymes' (i.e. the MSF compartment) for use with cellular functions would be expected; however, the differences between the species was not. Interestingly, even though the MSF of *P. amurensis* contained a large proportion of accumulated Zn (~70%), with most Zn being associated with organelles (~52%), a majority of the Cd in the MSF of

P. amurensis was associated with 'enzymes' (MSF-Cd ≈40%; 'enzymes'-Cd ≈30%).

Studies have shown that the binding of metals to high and low molecular weight proteins can result in toxicity (Brown & Parsons 1978, Sanders et al. 1983, Wallace et al. 2000). The binding of Cd to the 'enzyme' fraction of *Potamocorbula amurensis* may therefore have important toxicologic implications, as *P. amurensis* in some locations in SFB attain high tissue concentrations of Cd (Brown & Luoma 1995, Lee et al. 1998) and may exhibit Cd-associated stress (Clark et al. 2000, C. Brown unpubl.).

Cellular debris

The only subcellular fraction that was not included in the compartmental analysis of MSF and BDM was the cellular debris. In this operational scheme, the cellular debris is the most 'operational' as it contains tissue fragments, cell membranes and other cellular components of unknown consequence in terms of function. For instance, the binding of metal to cell membranes could result in toxicity; however, binding to less sensitive fractions within the cellular debris could prevent binding to sensitive components of the cell (Lucu & Obersnel 1996).

The cellular debris is therefore not easily defined as MSF or BDM. However, a majority of Zn (~60%) and ~50% of the Cd in *Macoma balthica* were associated with this fraction. The high partitioning of Cd and Zn to both the MSF and BDM compartments in *Potamocorbula amurensis* reduced the partitioning to the cellular debris; only ~30% of Cd and less than 20% of Zn in *P. amurensis* were operationally unclassified.

Changes in subcellular partitioning during loss

Many studies have investigated temporal changes in the subcellular partitioning of metal during accumulation, but few have investigated these processes during the loss (Viarengo et al. 1987, Roesijadi 1994). Estuarine organisms are continuously exposed to fluctuating metal concentrations (e.g. Luoma et al. 1985). This continuum in exposure may result in periods of uptake, followed by periods of loss. If an organism is to survive these exposures, there must be systems in place that protect sensitive components of the cell. During uptake, storage into MT and MRG may act in this capacity, serving as 'primary' mechanisms of detoxification. However, during periods of extreme exposure, these systems may become overwhelmed and metals may bind to more sensitive cellular components (i.e. organelles and 'enzymes'), resulting in toxicity (Brown

& Parsons 1978, Sanders et al. 1983, Wallace et al. 2000). Organisms therefore need 'secondary' mechanisms of cellular protection to facilitate recovery. These 'secondary' mechanisms of protection may take the form of internal redistribution of metal in conjunction with whole body depuration.

Such redistribution and associated reductions in toxicity have been observed. For instance, Viarengo et al. (1987) showed that following a recovery period of 28 d, subsequent to a 9 d exposure to Cd, the amount of Cd bound to MT in *Mytilus edulis* increased to 250% of pre-depuration levels, and the stability of lysosomes increased accordingly. Langston & Zhou (1987) found that the concentration of Cd bound to low molecular weight proteins in *Macoma balthica* decreased substantially upon loss, although no direct toxicological consequences were addressed.

In the present study, metal was redistributed from the MSF of both species by way of (1) depuration from the animal (i.e. Cd in *Macoma balthica*); (2) depuration from the animal in conjunction with redistribution into less toxic forms (i.e. Zn in *Potamocorbula amurensis*); or (3) redistribution into less toxic forms without whole body loss (i.e. Zn in *M. balthica* and perhaps Cd in *P. amurensis*). The combination of these processes could help explain interactions between metal loss and recovery from exposure. Specifically, the rapid loss of Zn from *P. amurensis* is the composite of loss from the MSF (i.e. organelles) in conjunction with loss from the cellular debris. Whole body loss of Zn from *P. amurensis*, however, was somewhat slowed by the redistribution into BDM (i.e. MT). A net result of this redistribution into MT may be a faster recovery from adverse Zn exposures than would be indicated from whole body loss alone. Some organisms have been shown to regulate Zn body burdens (Rainbow et al. 1990). The rapid accumulation and loss of Zn by *P. amurensis* suggests such regulation. Furthermore, because the organelle fraction was the site of Zn accumulation and loss, components within this fraction may play a role in the regulation of this metal. Retention of Cd in *P. amurensis*, however, was not marked by any appreciable redistribution, and this could suggest vulnerability to adverse Cd exposures.

The rapid loss of Cd from *Macoma balthica* results from removal of metal from MSF (i.e. organelles and 'enzymes') as well as depuration from BDM (i.e. MRG). Although loss from MSF was the greatest, it was somewhat surprising to observe the loss of Cd from MRG, as it was thought that these concretions would simply be a 'sink' for accumulated metal. The retention of Zn by *M. balthica*, however, was marked by removal from the MSF (i.e. 'enzymes') with redistribution into the BDM (i.e. MRG). Slow depuration of Zn from *M. balthica* would therefore not necessarily represent an inability to recover from Zn stress.

Size dependence in accumulation and subcellular partitioning

Relationships between tissue metal concentrations and animal size have long been observed in invertebrates, and their complexity is well known (Strong & Luoma 1981). Three different circumstances can characterize such relationships, and mechanisms that help explain each of them can be found in the subcellular partitioning of Cd and Zn in *Macoma balthica* and *Potamocorbula amurensis*.

An inverse relationship between metal accumulation and clam size

The enhanced accumulation of metal by small (young) animals over that of large (old) counterparts is often observed in aquatic invertebrates (Fowler et al. 1978, Lee et al. 1998) and has been attributed to size-specific metabolic rates (Ringwood 1989, Newman & Heagler 1991). This rapid accumulation of metal by smaller individuals often results in size-dependent toxicity (Ringwood 1989, Kiffney & Clements 1996). Studies have shown that smaller individuals of some marine invertebrates possess higher concentrations of MT as compared with older counterparts (Luk'yanova & Evtushemkoi 1982, Mouneyrac et al. 2000). Our observation of a reduced binding of both Cd and Zn to the MT fraction with increased size for *Macoma balthica* is in keeping with these results. The production of MT can be rapidly induced (Roesijadi 1992), whereas longer time frames may be required for the development of MRG detoxification pathways (Brown 1982). It therefore seems reasonable that younger animals would rely on MT for metal detoxification. There may, however, be toxicological consequences of not having an efficient MRG detoxification system. Specifically, if younger individuals only have access to MT for the sequestration of accumulated metal, size-dependent metal toxicity may result from a saturation of this singular detoxification pathway.

A positive relationship between metal accumulation and clam size

Size dependence in metal concentrations has often been observed in invertebrates, particularly in bivalves inhabiting contaminated sites, and the storage of metal into metal concretions (i.e. MRG) has been cited as a possible mechanism to explain these observations (Johnels et al. 1967, Strong & Luoma 1981). The accumulation of metal into MRG has been suggested as a mechanism for the long-term storage of

essential metals, and the retention of metal in granular deposits results in extremely high metal body burdens (Brown 1982, Klerks & Bartholomew 1991). The storage and retention of metal in MRG could therefore explain size dependence in tissue concentrations if chronically exposed populations progressively accumulate metals in these concretions over time.

This progressive accumulation of metal into MRG was observed in this current study. Specifically, Zn was shunted into the MRG fraction of *Macoma balthica* during both uptake and loss, and release was very slow. The continued accumulation and retention of Zn in MRG of *M. balthica* could lead to the size dependence in Zn concentrations reported in other studies (Strong & Luoma 1981). Additionally, even though the relationship for the storage of Cd in MRG for *M. balthica* is not statistically significant, clams smaller than 0.3 g wet wt did not have Cd associated with this fraction. Storage of Cd within MRG of large *M. balthica* may therefore also explain the size dependence in Cd concentrations often observed for this bivalve. When considering the subcellular distributions of stable metal, calculations indicate that a majority of the Cd and Zn in large *M. balthica* is associated with MRG and the cellular debris. Although other subcellular fractions do exhibit enhanced binding in large clams, the preferential binding to these 2 fractions is what drives size-dependent tissue concentrations. Beyond size dependence in tissue concentrations, the preferential binding of Cd and Zn to MRG of large *M. balthica* may also confer enhanced detoxification leading to greater tolerance. This however, has yet to be studied in chronically exposed populations, where such effects would be most obvious.

No correlation between metal accumulation and clam size

Lack of size dependence in metal concentrations is also a common observation (Boyden 1974, Strong & Luoma 1981). If the suggestion of progressive accumulation into MRG resulting in size-dependent metal burdens holds, one cause for the absence of size-dependent metal concentrations could be related to the lack of an efficient MRG detoxification system. This is precisely what is seen for *Potamocorbula amurensis*, where size dependence in tissue concentrations is rare (Brown & Luoma 1995). Even though *P. amurensis* exhibits size dependence in the storage of Zn into MRG, Zn is not shunted into this fraction during loss. The importance of this fraction, therefore, does not increase over time. Additionally, a large fraction of accumulated Zn was associated with rapidly exchanging pools of the MSF compartment, resulting in a rapid loss of Zn.

Although calculations show that some fractions of large *Potamocorbula amurensis* do exhibit enhanced binding over that of smaller individuals (i.e. 6× Cd in MT and 9× Zn in MRG), others display diminished binding (i.e. 0.2 to 0.5× Cd and Zn in organelles). The interplay of this fraction-specific binding within *P. amurensis* results in no 'net gain' in total tissue concentrations with increased size. Therefore, unlike *Macoma balthica*, Cd and Zn concentrations of *P. amurensis* do not increase with clam size. Interestingly, whereas large *M. balthica* relied on MRG for the storage and perhaps detoxification of both metals, large *P. amurensis* detoxified Cd through binding with MT and sequestered Zn via storage in MRG.

CONCLUSIONS

We have shown that metal, species and size dependence in the accumulation and loss of Cd and Zn in *Macoma balthica* and *Potamocorbula amurensis* can be interpreted by examining not only subcellular partitioning into individual fractions, but also subcellular compartmentalization as MSF and BDM. This approach has highlighted a number of important patterns in metal accumulation and loss that should provide avenues for continued research. For instance, the usefulness of bioindicator species (based on responsiveness to contamination) may be linked to rapid uptake of metal with subsequent storage into detoxification products. Regulation of metals (i.e. Zn) or poor responsiveness to environmental change, as reported by others (Rainbow et al. 1990), may be partly related to accumulation into and release from various organelles. MSF metals tend to be lost more rapidly than BDM metals, although details are metal- and species-specific, and these differences may have important toxicological implications. Additionally, the lability of metal in MSF may have implications for size dependence in bioaccumulation as well as for recovery from adverse exposures. Storage into MRG may be especially important in driving size dependence in metal body burdens. Finally, size-dependent uptake in conjunction with size-dependent partitioning to BDM (i.e. MT and MRG) may both play a role in the increased sensitivity of smaller bivalves, although this needs to be investigated further.

The use of operationally defined subcellular compartments as set forth in this study could aid interpretations of differences in tolerance and toxicity observed among aquatic as well as terrestrial soft-bodied invertebrates. For instance, all else being equal, greater storage of metal as BDM (i.e. MT and MRG) in one species or population compared to another may relate to metal tolerance, while greater storage in MSF

(i.e. organelles and 'enzymes') may relate to metal vulnerability. This subcellular compartmentalization approach is similar to the 'spillover' concept aimed at linking the saturation of MT and subsequent binding of metal to high molecular proteins to the onset of toxic responses (Brown & Parsons 1978, Sanders et al. 1983); however, it considers additional mechanisms of detoxification (i.e. MRG) as well as additional targets of metal toxicity (i.e. organelles).

Although many of the above patterns are speculative and further research is required, the application of this subcellular compartmentalization approach, focusing on metal associated with MSF (organelles and 'enzymes') and BDM (MT and MRG) could be used: (1) to develop hypotheses to guide future mechanistic studies; and (2) to interpret previously observed differences in toxicity. Finally, with the inclusion of a third compartment relating subcellular distributions to metal trophic transfer (TAM: organelles, 'enzymes' and MT; Wallace & Luoma 2003), this subcellular compartmentalization approach could provide valuable information on factors controlling the accumulation, toxicity and trophic transfer of metal in aquatic, and perhaps even terrestrial, ecosystems.

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