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Trophic transfer of trace metals: subcellular compartmentalization in bivalve prey, assimilation by a gastropod predator and *in vitro* digestion simulations

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ABSTRACT: The uptake of trace metals from the diet is a significant route for their entry into marine animals, and the chemical form of trace metals accumulated by food organisms is one potential factor controlling their assimilation from the diet. Therefore, we investigated relationships between the assimilation efficiencies (AE) of the trace metals Cd, Ag and Zn in the neogastropod mollusc *Nassarius festivus* and their subcellular compartmentalized fractionation in selected tissues of 4 species of bivalve prey, seeking to identify the relative trophic availabilities of such different fractions. We also sought parallels between the AE of *N. festivus* and 2 *in vitro* models of digestion, a generalised invertebrate model and a human digestion model. The bivalves were the scallop *Chlamys nobilis*, the clam *Marcia hiantina*, the green mussel *Perna viridis* and the oyster *Saccostrea cucullata*, as these show a range of accumulated trace metal concentrations and detoxificatory binding to subcellular compartments. Measured assimilation efficiencies of *N. festivus* for Cd, Ag and Zn from invertebrate prey tissues are very high in comparison to other marine animals, assimilation occurs from accumulated metal in prey bound in subcellular fractions across the insoluble and soluble spectrum, and assimilation is best modelled *in vitro* by a human digestion model with low pH (1.3) rather than an invertebrate model (pH 5.6). It is concluded that what is trophically available to one predator (feeding on one prey type) is not necessarily trophically available to another (taxonomically separated) predator even if feeding on the same prey, given the variability between invertebrate digestive systems. Variation in the subcellular distribution of accumulated trace metals within different prey will also add variation in trophic availability even for the same metal.

KEY WORDS: Trace metals · Trophic availability · Assimilation efficiency · Fractionation · Neogastropod · Bivalve prey · *Nassarius festivus*

INTRODUCTION

It is now appreciated that the uptake of trace metals from the diet is a significant route for their entry into marine animals (Wang 2002), with the further potential for the metals to be transferred along food chains (Wang 2002, Rainbow et al. 2004, 2006b). It is therefore relevant to identify general principles that govern the trophic bioavailability of trace metals (Wang & Fisher 1999). The chemical form of trace metals accumulated by food organisms is one potential major factor controlling the assimilation of a trace metal from the diet (Reinfelder & Fisher 1991, Wallace & Lopez 1996, 1997, Wallace & Luoma 2003, Wallace et al. 1998, 2003, Ng et al. 2005, Rainbow et al. 2006a).

Reinfelder & Fisher (1991) observed a linear 1:1 relationship between the metal assimilated by marine copepods from diatoms *Thalassiosira pseudonana* and

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various metals partitioned in the cytoplasm of the ingested phytoplankton, suggesting that only metal bound to the soluble fraction in diatoms is available to copepods. This relationship is an oversimplification, however, for filter-feeding invertebrates in general (Wang & Fisher 1996, Decho & Luoma 1996, Xu & Wang, 2001, 2002, 2004). For example, Ng et al. (2005) investigated whether the nature of the binding of Cd, Ag and Zn accumulated by phytoplankton (surface exchangeable metal, soluble incorporated metal, insoluble incorporated metal) could affect their subsequent assimilation efficiencies in 3 filter-feeding benthic invertebrates. None of the 3 fractions isolated represented the sole form of metal that is trophically available to a herbivore, and even trace metals bound to the insoluble fraction in phytoplankton were at least partly trophically available to the herbivores.

With regard to predators feeding on animal prey, the physicochemical form of Cd accumulated by the oligochaete worm *Limnodrilus hoffmeisteri* was found to affect the assimilation of the Cd by the decapod crustacean predator *Palaemonetes pugio* (Wallace & Lopez, 1996, 1997, Wallace et al. 1998). Wallace & Lopez (1997) concluded that while Cd associated with cytosolic proteins was 100% trophically available to *P. pugio* and Cd bound to the Cd-rich granules was unavailable, Cd bound to cell organelles was 70% trophically available. Wallace & Luoma (2003) examined how the subcellular partitioning of Cd and Zn accumulated in the bivalves *Macoma balthica* and *Potamocorbula amurensis* affected their trophic transfer to the decapod crustacean *Palaemon macrodactylus*. The accumulated metals in the soft tissues of the bivalves were fractionated into 5 components (after Wallace et al. 2003)—metal-rich granules (MRG), cellular debris, organelles, metallothioneins (and MT-like proteins or MTLP), which are characteristically relatively heat-resistant, and other (heat-sensitive) proteins (HSP). Wallace & Luoma (2003) could best explain their comparative assimilation results if trace metals bound to cell organelles were added to metal bound to the soluble fraction (HSP and MTLP components) to form what they termed the Trophically Available Metal (TAM) fraction of metals accumulated in the bivalve prey.

More recent studies, however, have shown that there is no simple generalisation defining which fractions of accumulated trace metals are trophically available and which are not in predator–prey relationships. Different subcellular components of accumulated trace metals appear to be trophically available to different degrees to different feeding animals. Cheung & Wang (2005) found a variation in the assimilation efficiency (AE) of the neogastropod mollusc *Thais clavigera* of metals bound in different subcellular fractions in prey, from soluble proteins to insoluble MRG. Rainbow et al. (2006a) also examined the general application of the TAM combined fraction of Wallace & Luoma (2003) in a study of the trophic availability of Ag, Cd and Zn accumulated by 2 populations of the polychaete worm *Nereis diversicolor* to the decapod crustacean *Palaemonetes varians*. In fact, the predator (*P. varians*) assimilated dietary metal from a range of the fractions binding metals in the prey (*N. diversicolor*), with different AEs summated across these fractions. TAM could only explain about 21% of the variation in AE of the metals by the predator, while there was a significant negative correlation (46% variance explained) between the predator's AE and the percentage of accumulated metal present in MRG (Rainbow et al. 2006a).

The TAM fraction defined by Wallace & Luoma (2003) does not, therefore, account for all trophically available metal in the diet of all predators. Nevertheless, the testing of its wider applicability (e.g. Cheung & Wang 2005, Rainbow et al. 2006a, Zhang & Wang 2006) has confirmed that the chemical binding of metals in prey does affect their subsequent assimilation by a predator, with further variation introduced by the digestive capacity of the predator itself. Thus, metals bound in MRG appear to be more susceptible to the assimilatory powers of neogastropod molluscs (Cheung & Wang 2005) than those of palaemonid decapod crustaceans (Wallace & Lopez 1997, Wallace & Luoma 2003, Rainbow et al. 2006a).

In this paper we investigate relationships between the assimilation efficiencies of the trace metals Cd, Ag and Zn in the neogastropod mollusc *Nassarius festivus* and their subcellular compartmentalized fractionation in 4 species of bivalve prey. We seek particularly to identify the relative trophic availabilities of such different fractions to the neogastropod predator.

Neogastropods of the genus *Nassarius* are opportunistic scavengers with remarkable adaptations for detecting food from a distance by chemical stimuli, and for rapid feeding with associated powerful extracellular digestion capacity (Yonge 1925, Morton 1990). *N. festivus* is a common intertidal scavenger on sheltered sandy shores in Hong Kong with rapid detection of food (preferably freshly dead bivalves), swift locomotion and fast feeding to satiation (30% of its tissue weight) (Morton 1990, Britton & Morton 1992, Cheung 1994). Nassariids feed via an eversible proboscis, utilizing secretions from associated salivary glands, including a powerful proteolytic enzyme (Yonge 1925), to commence extracellular digestion of food before final ingestion by sucking into the alimentary tract (Yonge 1925, Cheung 1994). This potentially powerful initial digestion typical of many neogastropods may be the cause of the high trace metal assimilation efficien-

cies observed in these predators (Cheung & Wang 2005), and their recognised ability to assimilate metals from MRG (Cheung & Wang 2005), considered unavailable to several other predators (Wallace & Lopez 1997, Wallace & Luoma 2003). We therefore go further in this study to seek parallels between the AEs of the neogastropod *N. festivus* and 2 *in vitro* models of digestive capacities, a generalised invertebrate model and a human digestion model. The invertebrate digestion simulation uses a pH of 5.6 (as found in most of the alimentary tract of the neogastropod *Buccinum undatum*, Yonge 1925) while the human digestion simulation includes use of a pH of 1.3.

We have chosen 4 species of bivalve as prey for *Nassarius festivus* for 2 reasons. Firstly, bivalves are the preferred food of this neogastropod (Cheung 1994). Secondly, these bivalves are also used locally for human consumption in Hong Kong. The human digestion simulation would therefore also provide information on the potential trophic availability of trace metals in these seafood items for human consumers (see also Bragigand et al. 2004). The following bivalves were chosen: the scallop *Chlamys nobilis*, the clam *Marcia hiantina*, the green mussel *Perna viridis* and the oyster *Saccostrea cucullata*, in the knowledge that these bivalve taxa show a range of trace metal accumulation patterns with differential dependence on soluble (e.g. metallothionein) and insoluble (MRG) detoxification (Langston et al. 1998, Marigomez et al. 2002). For all species we separated out the digestive gland. This is the usual site of trace metal detoxification in bivalves (Langston et al. 1998, Marigomez et al. 2002) and would provide one uniform organ for interspecific comparison. Thereafter we pooled all remaining soft tissues, except in the case of the scallop where we investigated only the large adductor muscle, the specific tissue typically used for human consumption. We used 2 forms of radiolabelling of the bivalves to promote differences in the subcellular fractionation of accumulated radiolabelled metals (see also Rainbow et al. 2006a): from solution (Cd, Ag and Zn) and from a diet of radiolabelled diatoms (Cd, Zn).

MATERIALS AND METHODS

Collection. The neogastropods *Nassarius festivus* were collected by hand from the intertidal sandflat at Hoi Sing Wan in Tolo Harbour, Hong Kong. During low tide, freshly broken open specimens of the clams *Ruditapes philippinarum* were placed in shallow (<1 cm) pools; *N. festivus* were attracted chemically to the clams after they had been stimulated to emerge from being burrowed in the sand (Morton 1990, Morton et al. 1995).

Scallops *Chlamys nobilis* were obtained from Daya Bay, Shenzhen, China. Clams *Marcia hiantina* were purchased from the local seafood market at Saikung, Hong Kong, where they were on sale for human consumption after collection in the (unspecified) local SE China area. Green mussels *Perna viridis* were collected from the rocky shore bordering the sandflat at Hoi Sing Wan, Tolo Harbour, Hong Kong, and oysters *Saccostrea cucullata* from the rocky shore at the Hong Kong University of Science and Technology, near Saikung, Hong Kong.

All collections were made in October and November 2006. After collection, the animals were acclimated in seawater in the laboratory at 18°C for at least 3 d before any experiments.

Radiotracers. Radioisotopes were obtained as follows: ^{109}Cd ($t_{1/2}$ = 462 d, in 0.1 N HCl, from Isotope Products), ^{110m}Ag ($t_{1/2}$ = 249 d, in 0.1 N HNO_3, from Risø National Laboratory) and ^{65}Zn ($t_{1/2}$ = 244 d, in 0.1 N HCl, from Brookhaven Science Associates). All counting (bivalve tissues and separated fractions and digests, live gastropods) was carried out on a Wallac gamma counter. Spillover of radioisotopes was corrected for, and all counts were related to standards for each isotope. The gamma emissions of ^{110m}Ag were determined at 658 keV, ^{109}Cd at 88 keV, and ^{65}Zn at 1115 keV, and counting times adjusted so that the propagated counting errors were typically <5%.

Radiolabelling of bivalves. Each of the 4 bivalve prey species was radiolabelled from solution (Cd, Ag and Zn) and via a radiolabelled dietary source, the diatom *Thalassiosira pseudonana* (Cd, Zn only). To radiolabel the bivalves from solution, 20 individuals of each bivalve were exposed to 2 l 0.2 µm filtered seawater spiked with 11.1 kBq l^{-1} of ^{109}Cd, 3.7 kBq l^{-1} of ^{110m}Ag, and 11.1 kBq l^{-1} of ^{65}Zn (corresponding to ~0.15 µg l^{-1} Cd, 1.1 µg l^{-1} Ag, and 0.1 µg l^{-1} Zn) simultaneously for 20 d at 18°C. The bivalves were fed unlabelled diatoms *T. pseudonana* in non-radiolabelled filtered seawater for 4 h every day. To radiolabel the bivalves from the diet, the diatom *T. weissflogii* was radiolabelled as described in Wang & Rainbow (2000). Briefly, the phytoplankton cells were recovered from culture by filtration (pore size 3 µm), and then resuspended in 0.2 µm filtered seawater enriched with f/2 levels of N, P, Si, vitamins, and f/20 levels of trace metals minus EDTA, Cu, and Zn (Guillard & Ryther 1962). ^{109}Cd (92.5 kBq l^{-1}) and ^{65}Zn (92.5 kBq l^{-1}) were added and the phytoplankton grown for 5 d, allowing the cells to be uniformly radiolabelled. The diatoms were not radiolabelled with Ag because of potential Ag toxicity to the diatoms due to the low specific activity of ^{110m}Ag, which resulted in a high total Ag exposure concentra-

tion. On Day 5, the radiolabelled diatoms were concentrated by filtration. Twenty individuals of each bivalve were then fed radiolabelled *T. pseudonana* for 8 h (at a concentration about 5×10^4 cells ml^{-1}), before depuration in non-radiolabelled filtered seawater for the other 16 h. This feeding regime was repeated for 20 d (except oysters: 10 d). Water was renewed daily during the labelling period. After radiolabelling from either source, the labelled bivalves were finally depurated in filtered seawater for 24 h, rinsed with non-labelled filtered seawater for 1 h, and frozen at –80°C.

Fractionation. The following soft tissues were dissected out (after thawing) from between 3 and 5 bivalves (according to the total number of counts present) which had been radiolabelled from solution or from the diet: digestive glands (all species), adductor muscle (scallop), remaining soft tissues other than the digestive gland (clam, mussel, oyster). The 3 to 5 samples of the same tissue were then pooled, homogenised (30 mM Tris buffer, pH 8.0) and divided into 3 subsamples each. These replicate subsamples were separated into 5 different operational subcellular fractions according to the method of Wallace et al. (1998), with slight modification as described below. The homogenised subsamples were centrifuged at $1500 \times g$ for 15 min at 4°C. The pellets (containing MRG and cellular debris fractions) were digested with 1 M NaOH for 1 h at 100°C before centrifugation for 10 min at $5000 \times g$ at 4°C. This second pellet contained the MRG fraction, while metals originally associated with cellular debris were now present in the associated supernatant. The supernatant from the first centrifugation was further centrifuged at $100\,000 \times g$ for 1 h, producing a pellet containing organelles with associated bound metals. The supernatant from this centrifugation contained all metal bound in soluble form and was subjected to heat treatment (80°C for 10 min) before centrifugation ($30\,000 \times g$, 10 min). This final centrifugation separated the soluble metal fraction into metal bound to heat-resistant proteins in the supernatant (conventionally referred to as the MTLP fraction, although other heat stable proteins may also be present), and the metal bound to other HSP in the pellet. All fractions were then counted for radioactive metal contents.

Where required for further experiments, the MRG fractions were rinsed with Milli-Q water several times to remove all remaining NaOH; organelle fractions were resuspended in 1 ml of original Tris homogenisation buffer; and MTLP fractions were concentrated by ultrafiltration (5 kDa, Ultrafree-4 centrifuge filter unit, Millipore). For incorporation into gelatin packets for feeding to *N. festivus* (see 'Assimilation efficiency'), MRG, organelle or MTLP fractions were mixed with gelatin (Sigma) solution (2.4 g gelatin in 15 ml filtered seawater) at a ratio of 5 to 2. After mixing, 40 µl of fish castor oil (from castor oil capsules) was added and the gelatin cubes allowed to set at –20°C for 3 min.

Ideally, bivalve tissue would have been presented to *Nassarius festivus* fresh, i.e. without freezing. In practice, however, it would have been impossible to carry out the range of experiments undertaken without frozen storage of bivalve tissues. A temperature of –80°C was chosen in the expectation that any theoretical changes in fractionation of the accumulated metals, and any subsequent effect on predator assimilation efficiency, would be kept to a minimum.

Assimilation efficiency. A pulse-chase feeding radiotracer technique was used to follow the assimilation of metals from the diet, according to the method of Wang & Fisher (1999) and Wang & Rainbow (2000). Tissues from 2 or 3 individual radiolabelled bivalves (digestive glands [all species], adductor muscle [scallop], remaining soft tissues other than the digestive gland [clam, mussel, oyster]) were thawed and fed to 8 *Nassarius festivus* in 15 ml filtered seawater in 250 ml beakers for 40 min at 18°C. After the feeding period, each gastropod was rinsed with filtered seawater, wrapped in cellophane to prevent movement and live counted for radioactive metal contents for 60 s. After counting, each gastropod was returned to an individual 100 ml plastic tube with 50 ml filtered seawater with an aluminium foil cap to prevent escape. The radioactivity in each gastropod was monitored at intervals (e.g. after 4, 22, 28, 46 h), the exact hour varying slightly (e.g. 44 to 48 h for the 2 d time point) between groups of gastropods given the differences in starting times of experiments and the finite time necessary to count all gastropods in a counting session. Gastropods were fed with foot tissue of unlabelled clams over this 2 d period; unlabelled water and food were renewed while the radioactivity of gastropods was being counted. Certain tissue experiments were repeated in order to obtain adequate replication when ingested counts were low (see variability in numbers counted in Table 1).

In the case of feeding with gelatin packets containing radiolabelled MRG, organelle or MTLP fractions, 5 to 9 *Nassarius festivus* were allowed to feed for 2 h before the first count, thereby promoting the likelihood of ingestion of sufficient radiolabelled material for future counting.

***In vitro* digestion.** Digestive glands of all 4 of the bivalves, adductor muscles of scallops and remaining soft tissues of clams, mussels and oysters were minced at 4°C. To mimic invertebrate digestion, the tissues were shaken for 2 h at room temperature with

Table 1. *Nassarius festivus*. Assimilation efficiencies (AE, mean percentage ± SD) of Cd, Ag and Zn of the neogastropod feeding on tissues of 4 bivalve species radiolabelled with metal from solution (W) or food (F)

	Cd			Ag			Zn		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
Scallop *Chlamys nobilis*									
Digestive gland (W)	83.5	9.6	8	66.2	12.6	8	79.8	20.9	8
Adductor muscle (W)	62.3	19.5	8	26.8	4.8	2	77.8	21.1	8
Digestive gland (F)	82.1	4.4	8	–	–		85.9	11.0	8
Muscle (F)	68.9	21.8	7	–	–		78.9	9.1	6
Clam *Marcia hiantina*									
Digestive gland (W)	–	–	–	52.9	19.1	14	69.3	19.1	6
Rest soft tissue (W)	–	–	–	–	–		–	–	
Digestive gland (F)	89.3	6.2	6	–	–		90.5	10.8	6
Rest soft tissue (F)	73.7	11.2	6	–	–		92.4	15.1	2
Mussel *Perna viridis*									
Digestive gland (W)	44.8	10.0	5	47.6	20.9	12	57.4	12.7	11
Rest soft tissue (W)	67.9	16.5	7	44.9	26.1	4	62.3	15.1	5
Digestive gland (F)	71.1	19.1	11	–	–		69.6	19.7	13
Rest soft tissue (F)	75.2	19.8	4	–	–		79.4	15.2	7
Oyster *Saccostrea cucullata*									
Digestive gland (W)	81.7	17.9	7	60.0	10.7	5	62.2	23.8	7
Rest soft tissue (W)	80.6	4.9	6	52.9	21.5	3	77.0	7.0	5
Digestive gland (F)	79.5	9.6	7	–	–		69.3	19.6	7
Rest soft tissue (F)	75.4	10.1	5	–	–		71.4	22.4	4

8 ml 1% acetic acid adjusted to pH 5.6 (with ammonia water 30%) per g of bivalve tissue. Organelles and MRG recovered after fractionation were submitted to the same treatment. The purified MRG fraction was suspended in 5 ml Tris buffer (pH 7.6), vortexed and centrifuged at 5000 × *g* for 15 min twice, the final pellets being used for the further extraction. Mimicking of human digestion was carried out according to the procedure described by Versantvoort et al. (2005). Briefly, it consists of a 3-step procedure simulating the digestive processes in the mouth, stomach and small intestine. The food matrix was first exposed to artificial saliva at pH 6.8 for 5 min, then artificial gastric juice at pH 1.3 was added for 2 h, and thirdly a mixture of artificial duodenal juice, bile and HCO_3^- at pH 8.1 to 8.2 was added for a further 2 h. The incubation temperature was 37°C. In each case, after centrifugation at 2800 × *g* for 5 min, the supernatants and pellets were separated and counted for radiolabelled metal content. Extractability was determined as the percentage of metals recovered in the supernatant.

Statistical analysis. Assimilation efficiencies were arcsine-transformed before application of parametric statistics, including comparison by *a priori* analysis of variance (ANOVA) and post hoc *a posteriori* ANOVA using Tukey's HSD test for unequal numbers (Statistica).

RESULTS

Assimilation efficiencies

Fig. 1 shows the percentage retention of Cd by *Nassarius festivus* feeding on tissues of 2 of the 4 bivalves radiolabelled with Cd, either from solution or from the diet, as an example of the typical pattern of retention of ingested Cd. After the defaecation of unassimilated Cd, the retained percentage is the por-

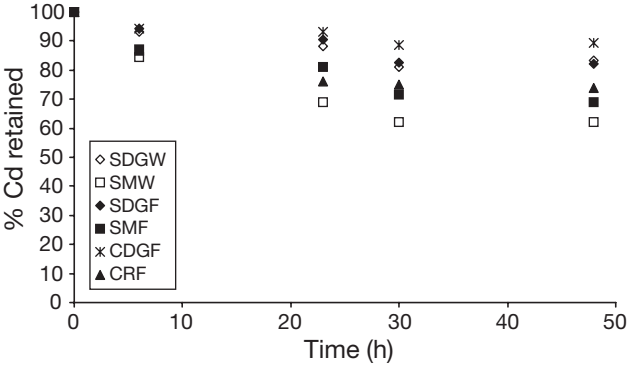

Fig. 1. *Nassarius festivus*. Percentages (mean, n = 6 to 8) of ingested Cd retained by the neogastropod mollusc feeding on tissues (DG: digestive gland, M: adductor muscle, R: remaining soft tissue) of bivalves scallop *Chlamys nobilis* (S) or clam *Marcia hiantina* (C) radiolabelled with Cd either from solution (W) or food (F)

tion that has been assimilated by the neogastropod. This retained percentage is a measure of the AE (Wang & Fisher 1999). Table 1 presents the AEs of Cd, Ag and Zn from all replicates of all experiments involving tissues from all 4 species of bivalves. The depuration curves (e.g. Fig. 1) were the same pattern for all metals for all diets, with steady state always established by Day 2. The time point at which the measured percentage retained was taken to be an estimate of the AE was therefore that of 2 d, the exact hour varying slightly between experiments. As is apparent from Table 1, results are not available for all metals for all tissues, given the variation of accumulated radiolabelled metal loads in (different tissues of) different bivalves and the differential inclination of the neogastropods to feed on particular tissues. For example, in spite of repeating experiments, no gastropod ingested a sufficient amount of remaining soft tissues of clams radiolabelled from solution to give enough counts of any metal to follow assimilation (Table 1).

Trace metal AEs of *Nassarius festivus* are high, particularly for Zn and Cd, with somewhat lower AEs for Ag. The AEs for individual metals appear to vary between tissues ingested. ANOVA on arcsine-transformed percentage AE data confirms that there is a significant difference between AEs across groups for Cd (F_s = 2.982; df = 1,13; p = 0.0013) and Zn (F_s = 2.485; df = 1,14; p = 0.0051), but not for Ag (F_s = 1.532; df = 1,6; p = 0.192). In the case of both Cd and Zn, the AE from the digestive gland of mussels radiolabelled from solution is significantly lower than that of the AE from the digestive gland of the clam radiolabelled from the diet (Table 1). Other AEs for a particular metal do not differ significantly between tissues, given the variability around each mean.

Fractionation

Fig. 2 provides summary data on the mean percentage distributions of accumulated radiolabelled metals in tissues of the 4 bivalves radiolabelled via water (Cd, Ag, Zn) or diet (Cd, Zn) between the 5 subcellular fractions. Metals associated with MRG, cell debris and organelles make up the insoluble metal fraction, while metals bound with MTLP and other HSP constitute the soluble metal fraction. Interspecific comparisons of the fractionation data (Fig. 2) confirm major differences in the subcellular distribution of accumulated metal into the different fractions in the same tissue of different bivalve species, as well as between species. For example, MRG was the predominant site for Cd and Ag binding in the oysters, whereas MTLP was the major site for Cd binding in the mussels. In contrast, only a small fraction of Cd was measured in the MRG fraction in the scallops and clams. Cellular debris played the most important role for Zn sequestration in scallops, mussels and oysters.

In vitro digestion

Fig. 3 shows the comparative extraction of each radiolabelled metal from the different bivalve tissues investigated using *in vitro* models of invertebrate and human digestion. The results for Ag extractability are striking, with much more extraction of Ag into solution by the human digestion model in every comparison. Differences between invertebrate and human digestion models are not so clear cut for Cd and Zn. The clearest difference between invertebrate and human digestion models for both Cd and Zn was evident for the extractability of metal from scallop adductor muscle.

Correlations

Correlations between assimilation efficiencies

The availability of AE data for more than one metal from the same food source for individual *Nassarius festivus* allowed us to seek correlations between AEs of this predator for the different metals. There were significant correlations between AEs of Cd and Ag ($p < 0.01$) and AEs of Zn and Cd ($p < 0.001$), but not between AEs of Zn and Ag (Table 2).

Correlations between assimilation efficiencies and subcellular fractionation in prey

The first correlations investigated were those between the percentage distribution of accumulated metals in the soluble fraction and the subsequent AE of *Nassarius festivus*. Using data for all tissues, there was no significant correlation for Cd or Ag, but there was a significant positive correlation in the case of Zn and for all metals together (AEs for the 3 separate metals treated as a single data set) (Table 3). In order to eliminate confounding factors introduced by the inclusion of different tissues in the whole data set, data for the digestive glands alone were also used for comparisons. In the digestive gland data set alone, there was again no correlation between the percentage distribution of accumulated metals in the soluble fraction and the subsequent AE of *N. festivus* for Cd and Ag, but there was for Zn (Table 3). There was also a significant cor-

relation for the percentage distribution of accumulated metal in the soluble fraction in the digestive glands and subsequent AE for the data set for all metals together (Table 3).

Correlations were also sought between the percentage distribution of accumulated metals in the TAM fraction (soluble plus organelle-associated metal) of Wallace & Luoma (2003) and the subsequent AE of *Nassarius fes-*

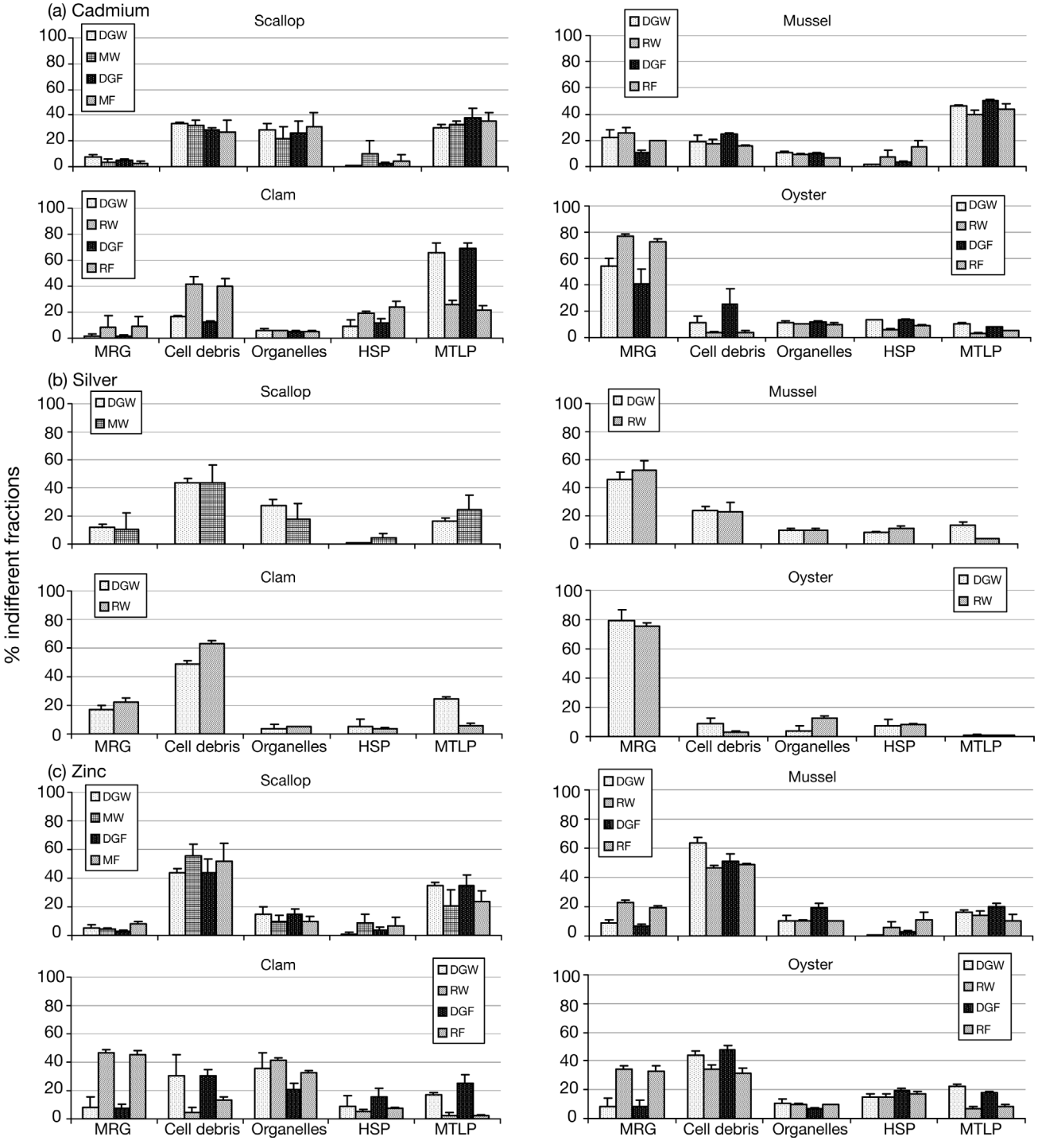

Fig. 2. *Nassarius festivus*. Percentage distributions (mean ±1 SD) of accumulated radiolabelled (a) Cd, (b) Ag and (c) Zn between 5 subcellular factions: metal-rich granules (MRG), cell debris, organelles, heat-sensitive proteins (HSP), metallothionein-like proteins (MTLP) in tissues (DG: digestive gland, M: adductor muscle, R: remaining soft tissues) of 4 bivalves (scallop *Chlamys nobilis*, clam *Marcia hiantina*, mussel *Perna viridis*, oyster *Saccostrea cucullata*) after uptake from water (W) or food (F)

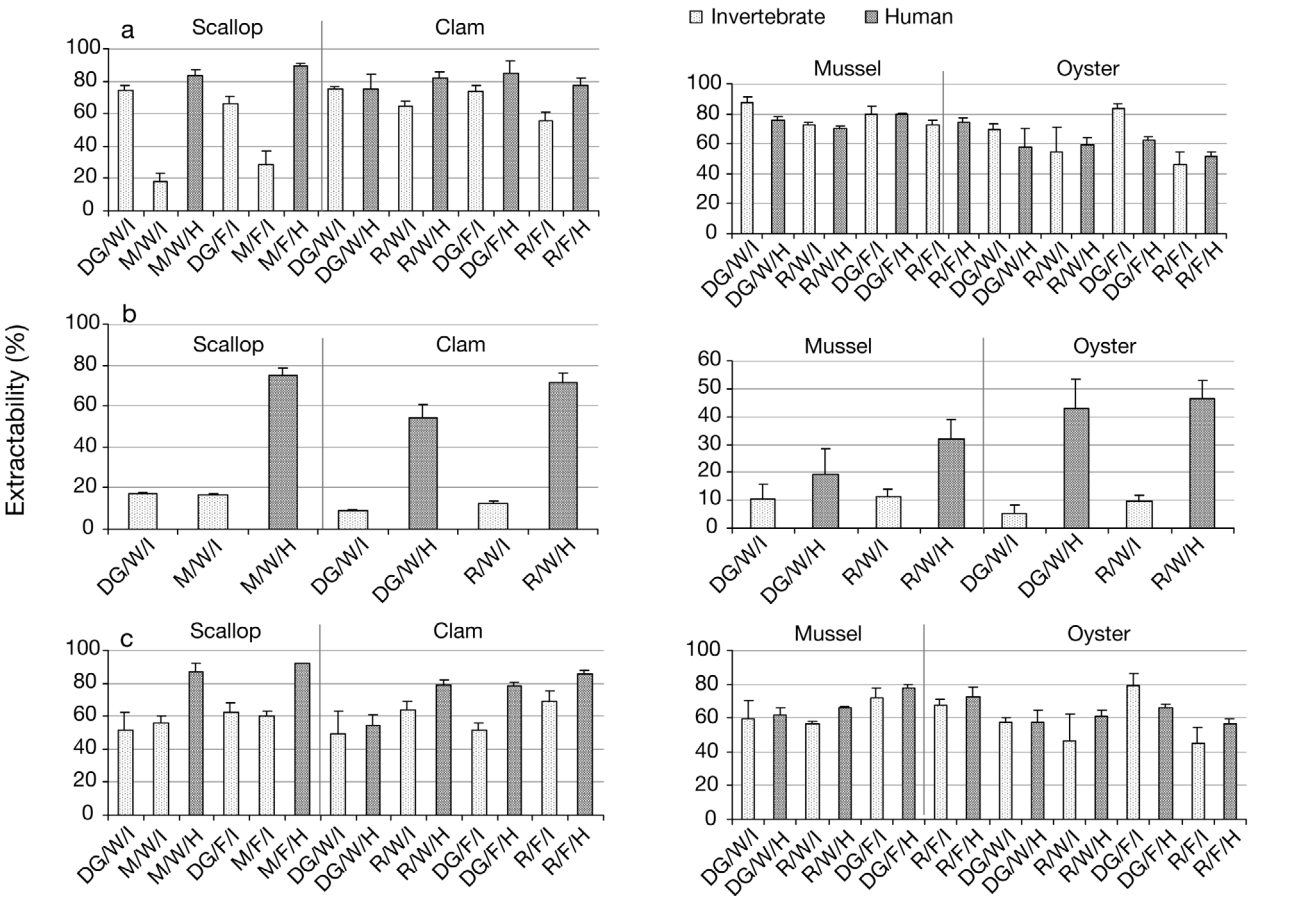

Fig. 3. *Nassarius festivus*. Comparative *in vitro* extractability by an invertebrate (I) or human (H) model of digestion of accumulated radiolabelled (a) Cd, (b) Ag and (c) Zn from tissues (DG: digestive gland, M: adductor muscle, R: remaining soft tissues) of 4 bivalves (scallop *Chlamys nobilis*, clam *Marcia hiantina*, mussel *Perna viridis*, oyster *Saccostrea cucullata*) after uptake from water (W) or food (F)

tivus. Using data for all tissues, there was no significant correlation for Cd or Ag, but there was a significant positive correlation in the case of Zn and for all metals together (Table 3). When only data for the digestive glands were used, there was again no correlation for Cd or Ag, but there was for Zn and all metals together (Table 3).

In the case of the metal-rich granules specifically, there were no significant correlations between the percentage distribution of accumulated metals in the MRG fraction and the subsequent AE of *Nassarius festivus* for any of the individual metals when the data set of all tissues was examined, but there was for all metals together (Table 3). In the digestive gland data set, there were again no significant correlations for Cd nor Ag, although there were significant negative correlations for Zn, all 3 metals together and the combined data sets for Ag plus Zn, and Ag plus Cd (Table 3).

These results indicate that metals associated with specific combinations of fractions (soluble, TAM) do not explain all metal assimilation by *Nassarius festivus*, indicating assimilation from potentially all fractions, but less so from the MRG fraction (Table 3). Assimilation of Zn in particular is biased to assimilation from soluble and TAM fractions as opposed to assimilation from MRG (Table 3).

Table 2. *Nassarius festivus*. Correlations between the assimilation efficiencies (AE) of Cd, Ag and Zn. Data were fitted with the linear regression model $y = a + bx$. R^2 is the correlation coefficient, and significance of regression coefficient was tested by AVOVA: NS, not significant at 95% level; ** significant at 99%; ***significant at 99.9%

y	*x*	Intercept (*a*)	Regression coefficient (*b*)	n	ANOVA	R^2
Ag AE (%)	Cd AE (%)	9.11	0.549	27	**	0.309
Cd AE (%)	Zn AE (%)	44.29	0.409	85	***	0.213
Ag AE (%)	Zn AE (%)	41.74	0.139	36	NS	0.017

Table 3. *Nassarius festivus*. Correlations between the percentage of accumulated metal in bivalve tissue in a designated subcellular fraction and assimilation efficiency. Data were fitted with linear regression models. R^2 is the correlation coefficient, and significance of regression coefficient was tested by ANOVA: NS, not significant at 95% level; *significant at 95% level; **significant at 99%; ***significant at 99.9%; SE: standard error. TAM: trophically available metal; MRG: metal-rich granules

Percentage in subcellular fraction	Regression coefficient	SE	ANOVA	R^2
Soluble fraction				
All tissues				
Cd	−0.075	0.100	NS	0.006
Ag	−0.185	0.366	NS	0.006
Zn	0.537	0.220	*	0.056
All metals	0.248	0.087	**	0.032
Digestive glands				
Cd	−0.033	0.132	NS	0.001
Ag	−0.157	0.407	NS	0.004
Zn	0.880	0.263	**	0.149
All metals	0.401	0.108	***	0.082
TAM fraction				
All tissues				
Cd	−0.071	0.098	NS	0.006
Ag	0.235	0.295	NS	0.014
Zn	0.465	0.171	**	0.068
All metals	0.301	0.077	***	0.059
Digestive glands				
Cd	0.051	0.149	NS	0.002
Ag	0.471	0.316	NS	0.057
Zn	0.744	0.208	***	0.167
All metals	0.543	0.095	***	0.173
MRG fraction				
All tissues				
Cd	0.065	0.075	NS	0.009
Ag	−0.100	0.117	NS	0.016
Zn	0.030	0.189	NS	0.000
All metals	−0.130	0.059	*	0.020
Digestive glands				
Cd	−0.051	0.131	NS	0.003
Ag	−0.176	0.130	NS	0.048
Zn	−3.967	1.209	**	0.144
All metals	−0.292	0.086	***	0.070
Ag + Zn	−0.436	0.102	***	0.150
Ag + Cd	−0.278	0.100	**	0.080
Zn + Cd	0.000	0.127	NS	0.000

These data can also be presented as ratios of percentage distribution of a metal in a different combination of fractions in the diet to the percentage AE of the predator (Fig. 4). A ratio of 1 indicates a perfect 1:1 ratio that could explain all assimilation; a ratio <1 indicates that metal in addition to that in the combined fractions is being assimilated, and a ratio >1 indicates that less metal than contained in the combined fraction is being assimilated by *Nassarius festivus* (Fig. 4). Thus, in the case of Zn, much more metal than is present in the TAM fraction is assimilated from every food source, but usually less metal than in a combination of TAM and cell debris fractions (Fig. 4). The data for Ag reflect the lower AEs measured for this metal, but again, more metal is usually assimilated than is present in the TAM fraction (Fig. 4). Moreover, adding Ag present in the cellular debris is still insufficient to explain Ag AE in both organs of oysters. For Cd, metal associated with TAM is usually less than the amount assimilated by the neogastropod (Fig. 4) and again, adding Cd present in the cellular debris is still insufficient to explain Cd AE in both the digestive gland and remaining soft tissues of oysters.

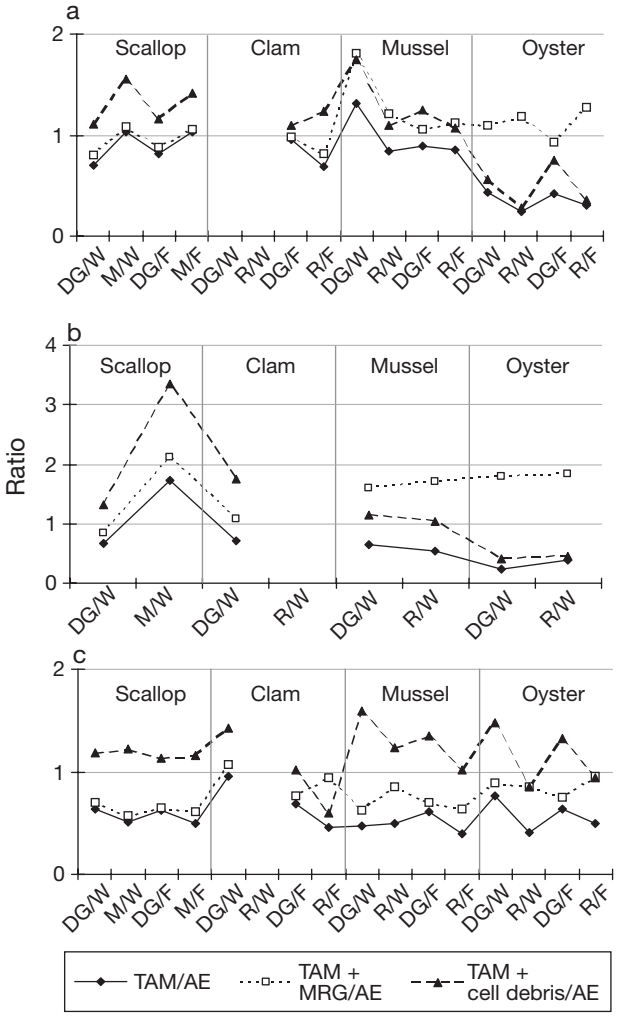

Fig. 4. *Nassarius festivus*. Ratios of the percentage distributions of accumulated radiolabelled, (a) Cd, (b) Ag and (c) Zn in the combined subcellular fractions of trophically available metal (TAM), TAM + metal-rich granules (MRG), and TAM + cell debris in tissues (DG: digestive gland, M: adductor muscle, R: remaining soft tissues) of 4 bivalves (scallop *Chlamys nobilis*, clam *Marcia hiantina*, mussel *Perna viridis*, O: oyster *Saccostrea cucullata*) after uptake from water (W) or food (F), to percentage assimilation efficiency (AE)

Correlations between digestion models and subcellular fractionation in tissues

We also sought correlations between the results of the *in vitro* digestive models and the subcellular fractionation of accumulated metals in the tissues of the prey. Fig. 5, for example, compares extractability by the invertebrate digestion model against the distribution of metal into the TAM fraction of prey tissues, compared against a 1:1 ratio (see diagonal line). There were no significant relationships for Cd and Zn, but there was for Ag (Fig. 5). The best-fit line for Ag was below the 1:1 ratio line (Fig. 5), indicating that the invertebrate digestion model was not strong enough to extract all metal in the TAM fraction into solution.

Fig. 6 compares the ratios of metal AE to both *in vitro* digestion models, and it is clear that the human digestion model is the better model for the assimilation of metals by *Nassarius festivus*. The invertebrate digestion model consistently underestimates Zn AE, but spectacularly so for Ag AE (by factors up to 10); there is better agreement of both digestion models with *N. festivus* AE in the case of Cd (Fig. 6). The human digestion model appears to provide a better fit with *N. festivus* AE (ratio closer to 1) for all 3 metals (Fig. 6). Analysis of the correlation between extractability by the human digestion model analysis and *N. festivus* AE (Fig. 7) shows that the correlation is significant in the case of Zn.

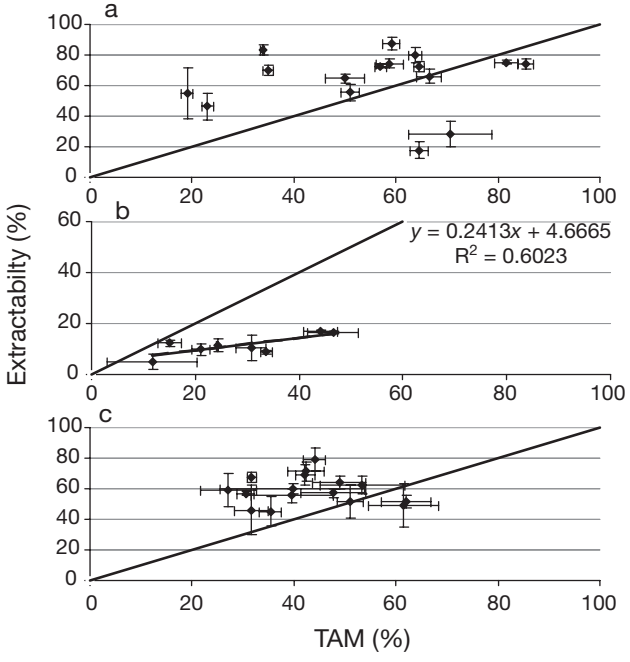

Fig. 5. *Nassarius festivus*. Correlations of *in vitro* extractability by an invertebrate model of digestion of accumulated radiolabelled (a) Cd, (b) Ag and (c) Zn from tissues of 4 bivalves after uptake from water or food, with the percentage distribution of accumulated radiolabelled metal into the trophically available metal (TAM) fraction. 1:1 ratio line (diagonal) shown for each metal. Only the Ag correlation was significant; details of the best fit linear regression line are shown

Assimilation efficiency and *in vitro* invertebrate digestion of isolated fractions

AE and *in vitro* digestion of isolated fractions by the invertebrate model were also tested directly, in the first instance by packaging isolated radiolabelled fractions in gelatin for ingestion by *Nassarius festivus*. However, the availability of tissues with sufficient radioactive counts after isolation of fractions, packaging in gelatin and ingestion by the predator severely restricted the

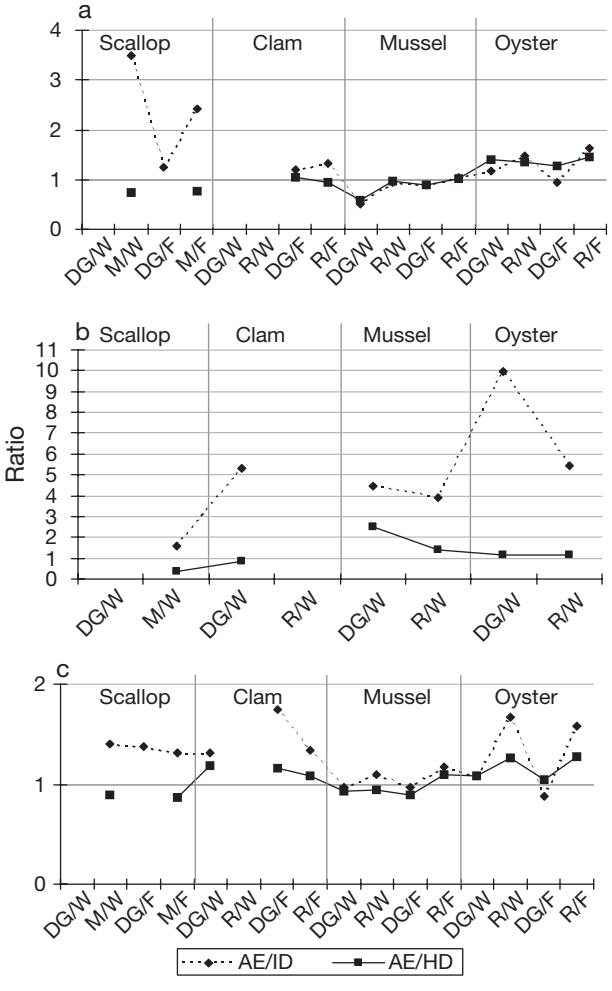

Fig. 6. *Nassarius festivus*. Ratios of the percentage assimilation efficiency (AE) to percentage *in vitro* extractabilities by invertebrate (ID) and human (HD) models of digestion of accumulated radiolabelled (a) Cd, (b) Ag and (c) Zn from tissues (DG: digestive gland, M: adductor muscle, R: remaining soft tissues) of 4 bivalves (scallop *Chlamys nobilis*, clam *Marcia hiantina*, mussel *Perna viridis*, oyster *Saccostrea cucullata*) after uptake from water (W) or food (F)

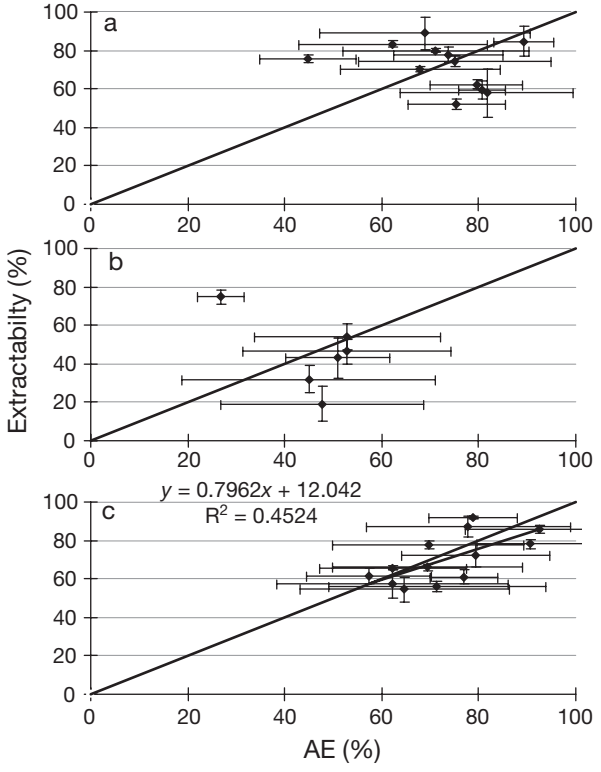

Fig. 7. *Nassarius festivus*. Correlations of *in vitro* extractability by the human model of digestion of accumulated radiolabelled (a) Cd, (b) Ag and (c) Zn from tissues of 4 bivalves after uptake from water or food, with the percentage assimilation efficiency (AE) of these metals from the tissues. 1:1 ratio line (diagonal) shown for each metal; details of the significant best fit linear regression presented for Zn

number of estimates of AE that could be made. Table 4 confirms that Cd and Ag can be assimilated from organelles and MRG, in addition to MTLP (confirmed also for Zn). For all metals, AEs of MTLP-associated metal were extremely high, approaching 100%, certainly for Cd and Zn. Assimilation of Cd and Ag from organelles and MRG was also strong, with some indication in scallops of lower AE of Ag from MRG than organelles and MTLP (Table 4).

The results for radiolabelled metal extractability by the invertebrate digestion model from organelles and MRG separated from the different bivalve tissues are shown in Table 5. Cd and zinc extractability from organelles which are part of the TAM fraction varied from 40 to 67% depending on the species and the source of labelling (water or food). For Ag, only one sample was available for *in vitro* digestion and showed a very high extractability (99.3%). Strikingly, all 3 metals were extractable from MRGs, which are not considered part of the TAM fraction. For Zn, extractability even reached 100% when mussel and oyster MRGs isolated from the digestive gland had taken up this metal from food.

DISCUSSION

It is clear that the neogastropod *Nassarius festivus* shows very strong assimilatory powers for trace metals accumulated by prey organisms. Measured assimilation efficiencies of *N. festivus* for Cd, Ag and Zn from invertebrate prey tissues are very high, assimilation occurs from accumulated metal in prey bound in subcellular fractions across the insoluble and soluble spectrum, and assimilation from different bivalve tissues is more comparable to results from an *in vitro* human digestion model involving low pH (1.3) rather than an invertebrate model (pH 5.6).

These results are explicable in terms of the predator's biology. *Nassarius festivus* is a scavenger with a preference for bivalve tissue (Britton & Morton 1992), feeding rapidly to satiation (Morton 1990, Britton & Morton 1992, Cheung 1994). To this end, nassariids feed via an eversible proboscis, using secretions from associated salivary glands to start digestion of food be-

Table 4. *Nassarius festivus*. Assimilation efficiencies (AE, mean percentage ± SD) of Cd, Ag and Zn of the neogastropod feeding on gelatin packeted isolated subcellular fractions of accumulated metal in the digestive glands of 2 bivalve species radiolabelled with metal from solution (W) or food (F). MTLP: metallothionein-like proteins; MRG: metal-rich granules

	Cd			Ag			Zn		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
Scallop *Chlamys nobilis* digestive gland									
MTLP (W)	96.6	9.2	7	79.5	6.6	8	89.3	11.0	2
MTLP (F)	80.5	12.5	8	–	–	–	81.5	8.6	2
Organelles (W)	77.7	4.4	4	82.1	5.7	4	–	–	–
Organelles (F)	84.4	5.8	4	–	–	–	–	–	–
MRG (W)	–	–	–	57.8	14.1	5	–	–	–
Oyster *Saccostrea cucullata* digestive gland									
MRG (W)	75.2	13.8	2	73.5	5.6	3	–	–	–

Table 5. Extractability (%) by the invertebrate digestion model of radiolabelled metals from subcellular fractions of tissues of bivalves exposed to radiolabelled metals in water (W) or food (F)

	Cd	Ag	Zn
Scallop *Chlamys nobilis* digestive gland			
Organelles (F)	42.8	–	52.3
Scallop *Chlamys nobilis* muscle			
MRG (W)	49.9	45.9	42.5
MRG (F)	85.3		51.4
Clam *Marcia hiantina* digestive gland			
MRG (W)	35.3	23.2	50.0
MRG (F)	49.7	–	24.4
Mussel *Perna viridis* digestive gland			
Organelles (F)	47.8	–	40.2
MRG (W)	46.9	25.6	44.3
MRG (F)			100
Oyster *Saccostrea cucullata* digestive gland			
Organelles (W)	66.9	99.3	56.4
MRG (F)	68.2	–	100

fore ingestion by sucking (Cheung 1994). This strong external initial digestion, typical of many neogastropods, probably underlies the better fit of the stronger human digestion model to what we found in *N. festivus*. Similarly, these strong initial digestive powers are probably the cause of the high trace metal assimilation efficiencies observed in neogastropods (Cheung & Wang 2005), and their ability (confirmed here) to assimilate metals from MRG (Cheung & Wang 2005).

The metal AEs of *Nassarius festivus* from bivalve tissues measured here (45 to 89% for Cd, 27 to 66% for Ag, 57 to 92% for Zn, Table 1) approach those of another neogastropod *Thais clavigera* (76 to 94% Cd, 60 to 87% Ag, 53 to 92% Zn) feeding on a range of invertebrate digestive glands (Cheung & Wang 2005). In contrast, AEs of the palaemonid decapod crustacean *Palaemonetes varians* feeding on the polychaete worm *Nereis diversicolor* were lower (34 to 63% Cd, 7 to 50% Ag, 41 to 84% Zn) (Rainbow et al. 2006a); although decapod crustacean gut fluid pH usually ranges from 5 to 7 (van Weel 1970, Dall & Moriarty 1983), similar to the pH range (5.4 to 7.0) in most of the gut of the neogastropod *Buccinum undatum* (Yonge 1925). Indeed, limited data for the crab *Carcinus maenas* (a brachyuran, as opposed to a palaemonid decapod crustacean) do indicate higher metal AEs: 41 to 86% for Cd in crabs feeding on labelled mussels *Mytilus edulis* (Bjerregaard et al. 2005). Fish have much lower metal AEs. For example, the grunt *Terapon jarbua* had AEs of 3 to 9% for Cd and 2 to 52% for Zn when feeding on a variety of invertebrate prey and fish viscera (Zhang & Wang 2006).

Neogastropod molluscs have such strong digestive powers that their AEs are high and much of the accumulated trace metals in prey appears to be trophically available to them. It is likely, therefore, that the dietary uptake route for trace metals is particularly important for these invertebrates (Cheung & Wang 2005, Luoma & Rainbow 2005). Despite the strong digestive and assimilatory capacity of neogastropod molluscs, subcellular fractionation does still retain some effect on predator AE, although this appears to be less significant than, for example, for palaemonid decapod predators (Wallace & Luoma 2003, Rainbow et al. 2006a). Our results have shown that *Nassarius festivus* probably assimilates trace metals from all of the operational fractions identified by Wallace et al. (1998), with lower but significant assimilation even from MRG (confirmed for Cd and Ag, Table 4). There are inevitably differences between metals and tissues (Table 1), as different metals are bound in different soluble and, particularly, insoluble forms to different extents in different tissues with strong intertaxon differences even within bivalve molluscs (Langston et al. 1998, Marigomez et al. 2002).

The combined metal data set showed significant correlations between the percentage held in the soluble fraction in the digestive glands of the bivalves and subsequent AE by *Nassarius festivus*, but this explained only 8% of the observed variance (Table 3). There were similar weak but significant positive correlations for combined metals in the TAM fraction and predator AE for all tissues (6% variance explained) and for digestive glands only (17% variance explained) (Table 3). Data for Zn alone show similar correlations — 7% variance explained for all tissues and 17% for digestive gland data (Table 3) — probably because of the low percentage distributions of accumulated Zn in MRG fractions (Fig. 2). Thus, again, it can be concluded that more metal than bound to the soluble (protein) fraction and the organelles in the bivalve tissues is assimilated by *N. festivus*. The significant negative correlation between percentage of metal in MRG and predator AE for Zn alone of the individual metals (Table 3) does suggest that the AE for Zn in MRG is well below that of Zn in the other subcellular fractions, although it has not been proved here that there is no assimilation from Zn in MRG. Indeed, integration of all our results does suggest that, like Cd and Ag, Zn in MRG is also trophically available to this predator.

The TAM fraction best explained the relatively limited data of Wallace & Luoma (2003) for the decapod *Palaemon macrodactylus* feeding on bivalve prey. A more extensive data set showed that its palaemonid decapod relative *Palaemonetes varians* assimilated metal from more fractions than the TAM from polychaete worm prey (Rainbow et al. 2006a). As confirmed here, neogastropods assimilate trace metals to

an even greater extent than palaemonid decapods, including metals associated with MRG fraction (Cheung & Wang 2005). Thus, the TAM fraction identified by Wallace & Luoma (2003) for *P. macrodactylus* feeding on bivalves is applicable as such for that predator (possibly even restricted to those prey types), but cannot be applied generally to a range of (invertebrate) predators (Cheung & Wang 2005, Rainbow et al. 2006a). What is trophically available to one predator (feeding on one prey type) is not necessarily trophically available to another (taxonomically separated) predator even if feeding on the same prey, given the variability between invertebrate digestive systems. Variation in the subcellular distribution of accumulated trace metals within different prey also adds variation in trophic availability, even for the same metal.

Metals bound in MRG were considered not to be trophically available to palaemonid decapods feeding on invertebrate prey by Wallace & Lopez (1997) and Wallace & Luoma (2003). Rainbow et al. (2004) also showed that copper detoxified in Cu-rich granules in the polychaete *Nereis diversicolor* from Restronguet Creek, UK, a site strongly contaminated with metals, passes through the gut of a predatory polychaete worm *Nereis virens* without any apparent change in elemental composition. Similarly, Nott & Nicolaidou (1990) showed that metals in trace metal-rich phosphate granules of prey animals remain in the same insoluble form during passage through the gut of molluscan carnivores, but that granules containing both Mg and Ca as CO_3, which do not contain metals other than Mg and Ca, were leached and demineralised by the process of digestion. Cheung & Wang (2005), on the other hand, found that the trace metals Ag, Cd and Zn bound in MRG from gastropod and oyster prey were trophically available to the neogastropod predator *Thais clavigera* in line with our results here for the neogastropod *Nassarius festivus*.

Extractability determined by the *in vitro* model for human digestion has shown differences between prey species, tissues and metals. These extractability results suggest that the bioavailabilities of trace metals in bivalves to human consumers are high, extractability generally being higher than half of the total content and reaching even more than 80% for Cd and Zn. It can be concluded, therefore, that food safety regulations based on total toxic metal concentrations in food are not overly conservative.

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