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Physico-chemical form of trace metals accumulated by phytoplankton and their assimilation by filter-feeding invertebrates

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ABSTRACT: This study investigated whether the nature of the binding of the trace metals cadmium, silver and zinc accumulated by phytoplankton can affect their subsequent assimilation efficiencies (AE) in 3 filter-feeding benthic invertebrates, the green mussel *Perna viridis*, the clam *Ruditapes philippinarum* and the barnacle *Balanus amphitrite*. Seven phytoplankton species were chosen from a wide systematic range to ensure large differences in the partitioning of their accumulated trace metals into 3 fractions: (1) exchangeable metal adsorbed on the outside of the cells, as defined by extraction with the chelating agent 8-hydroxyquinoline-5-sulphonate; (2) incorporated metal that is in a soluble form; and (3) insoluble incorporated metal. There were few significant correlations between AE and the percentage of phytoplankton metal incorporated into any 1 fraction or combination of fractions. There is no support, therefore, for a generalised conclusion that any of the 3 fractions isolated represents the sole form of phytoplankton metal that is bioavailable for trophic transfer to a herbivore. Even trace metals bound to the insoluble fraction in phytoplankton may be bioavailable to herbivores. Furthermore, there were no consistent effects of phytoplankton concentration on metal AEs in 1 of the herbivores—*P. viridis*. There was no evidence that the AE of any of the 3 trace metals was changed when the herbivores were feeding on the phytoplankton species (*Thalassiosira weissflogii*) on which they were fed during acclimation.

KEY WORDS: Trace metal assimilation · Phytoplankton · Filter-feeding invertebrates

INTRODUCTION

It is appreciated that many marine invertebrates gain a significant (often majority) part of their intake of trace metals from their food supply rather than from solution, and filter-feeding benthic invertebrates ingesting phytoplankton are no exception (Wang 2002). Clearly the total amount of metals accumulated by phytoplankton will affect the quantity of trace metals transferred trophically to the invertebrates, but the nature of the binding of metals in the phytoplankton also has the potential to have a significant effect on trophic transfer (Reinfelder & Fisher 1991, Amiard-Triquet et al. 1993, Wang & Fisher 1999, Chong & Wang 2000, Wang 2002).

Reinfelder & Fisher (1991) first demonstrated a linear 1:1 relationship between the metal assimilated by marine copepods from a diet of diatoms and the metal partitioned in the cytoplasm of the ingested diatoms. There were also later extensive studies into this relationship in bivalves and copepods (Hutchins et al. 1995, Wang & Fisher 1996, Xu & Wang 2002, 2004), but still no generalisations can be made on the relationship between metal partitioning in phytoplankton and metal assimilation in the herbivores feeding on them. In particular, very few comprehensive studies have attempted to study this relationship in several marine herbivores feeding on different phytoplankton species simultaneously.

To study the metal distribution in the phytoplankton, the phytoplankton is conventionally separated into

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several fractions by differential centrifugation (Fisher et al. 1983, Reinfelder & Fisher 1991)—the heaviest fraction (cell walls and membranes), the moderate fraction (organelles) and the lightest fraction or the supernatant (cytoplasm). In this study, we investigated the subcellular metal distribution in 7 different phytoplankton species (2 diatoms, 1 dinoflagellate and 4 flagellates). We separated the phytoplankton into 3 fractions: (1) exchangeable metal adsorbed on the outside of the cells, as defined by extraction with the chelating agent 8-hydroxyquinoline-5-sulphonate (after Price & Morel 1990); (2) incorporated metal that is in a soluble form; and (3) insoluble incorporated metal. This fractionation scheme is somewhat different from that frequently used in the past: the division into cytoplasmic versus cell-wall fractions. In the present study we have investigated the bioavailability of the easily exchangeable fraction that is rarely examined. Metals adsorbed on the cell walls of phytoplankton are very reactive and may be bioavailable to filter-feeding herbivores. In addition, we have combined the cell-wall fractions and organelles, to form the insoluble fraction, while the soluble fraction consists of cytosol.

We seek generalities in the relationships between metal partitioning in phytoplankton and the trophic transfer of trace metals from phytoplankton to filter-feeding herbivores, as indicated by the assimilation efficiencies (AEs) of the trace metals. We employed 3 filter-feeding benthic invertebrates, namely the green mussel *Perna viridis*, the clam *Ruditapes philippinarum* and the barnacle *Balanus amphitrite*, for each of which we have considerable experience in measuring AEs (Chong & Wang 2001, Rainbow et al. 2003, 2004, Shi et al. 2003, Ng & Wang 2004, Shi & Wang 2004). We chose 7 phytoplankton species from a wide range of systematic groupings, in order to increase the chances of large differences in the fractionation of accumulated trace metals in the phytoplankton and, thus, to increase the variations of AEs. We have used 3 trace metals—cadmium, zinc and silver, which have gamma-emitting radioisotopes suitable for simultaneous counting in experiments. We also investigated the effects of phytoplankton concentration on metal AEs in 1 of the herbivores—*P. viridis*. Finally, we took the opportunity to see whether AEs were raised when the herbivores were feeding on the phytoplankton species (*Thalassiosira weissflogii*), on which they were fed during acclimation.

MATERIALS AND METHODS

Phytoplankton cultures. Seven phytoplankton species (Table 1), originating from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, were batch-cultured in *f*/2 medium (Guillard & Ryther 1962) at 18°C (*Thalassiosira weissflogii*, *Phaeodactylum tricornutum*, *Tetraselmis* sp., *Dunaliella tertiolecta*) and 24°C (*Prorocentrum minimum*, *Isochrysis galbana*, *Rhodomonas salina*) and a light illumination of 100 µmol m^{-2} s^{-1} with a 14 h light:10 h dark cycle. The phytoplankton with different growth rates were cultured at 2 temperatures, in order to reach the late exponential phase simultaneously.

Table 1. Cell dimensions of the phytoplankton species used in the metal-assimilation experiments, with accession numbers from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (CCMP). Cell density measured at the end of the radiolabelling is also given (mean ± SD, n = 3 experiments)

	Cell dimensions (µm)	Cell density (10^6 cells ml^{-1})
Bacillariophyceae (diatoms)		
Thalassiosira weissflogii (CCMP 1587)	8–10 × 14–18	0.10 ± 0.01
Phaeodactylum tricornutum (CCMP 630)	4–5 × 24–29	3.06 ± 0.36
Dinophyceae (dinoflagellates)		
Prorocentrum minimum (CCMP 696)	10–12 × 13–16	0.06 ± 0.01
Prasinophyceae		
Tetraselmis sp. (CCMP 896)	6–10 × 10–12	0.36 ± 0.17
Chlorophyceae		
Dunaliella tertiolecta (CCMP 1320)	1–3 × 6–9	0.48 ± 0.17
Prymnesiophyceae		
Isochrysis galbana (CCMP 1323)	2–4 × 4–6	1.93 ± 0.08
Cryptophyceae		
Rhodomonas salina (CCMP 1319)	6–8 × 5–13	0.68 ± 0.07

Filter-feeding invertebrates. Three filter-feeding benthic invertebrates were used in this study—2 bivalves, *Perna viridis* and *Ruditapes philippinarum*, and the barnacle *Balanus amphitrite*. *B. amphitrite* was collected from Tung Chung on Lantau Island, Hong Kong, whereas *P. viridis* and *R. philippinarum* were collected from Tolo Harbour, Hong Kong. Mussels were cleaned of epibionts, and individual barnacles were isolated on small pieces of mussel shell. In the laboratory, the invertebrates were maintained at 23°C in aerated seawater (33 psu salinity) and fed the diatom *Thalassiosira weissflogii* for >1 wk before experiments began.

Radiotracers. Radioisotopes ^{110m}Ag ($t_{1/2}$ = 249.8 d), ^{109}Cd ($t_{1/2}$ = 462 d) and ^{65}Zn ($t_{1/2}$ = 244 d) were obtained from New England Nuclear, Boston, USA, or Riso National Laboratory, Denmark. Counting of phytoplankton fractions and individual live animals was car-

ried out on a Wallac gamma counter. Spillover of radioisotopes was corrected, 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 were adjusted so that the propagated counting errors were typically <5%.

Phytoplankton metal labelling. For 3 of 4 experiments, each phytoplankton species was radiolabelled with the 3 metal radioisotopes simultaneously in 4 replicated bottles (1 bottle for radioactive pulse feeding to the invertebrates and 3 bottles for determination of metal subcellular fractionation). Cultures were replicated on 4 different dates, to be ready, in turn, to be fed to the filter-feeding invertebrates in 4 separate experiments. The phytoplankton was radiolabelled as described in Wang & Rainbow (2000). Briefly, the phytoplankton cells were collected by centrifugation at 3836 × *g* for 15 min, except *Thalassiosira weissflogii*, which was recovered by filtration (pore size: 3 µm). The cells were then resuspended in 0.2 µm filtered seawater enriched with *f*/2 levels of N, P, Si and vitamins and with *f*/20 levels of trace metals minus EDTA, Cu and Zn (Guillard & Ryther 1962). Each radioisotope was added at 0.2 to 0.75 µCi (Ag), 0.1 to 0.75 µCi (Cd), or 0.2 to 0.75 µCi (Zn) (radioactivity varied for mussel, clam and barnacle experiments) on Day 0 for ^{109}Cd and ^{65}Zn and on Day 4 for ^{110m}Ag. The phytoplankton were grown for 5 d, allowing the cells to be uniformly radiolabelled.

On Day 5, the phytoplankton were collected by a similar method to that described above. The cell density was counted microscopically. Aliquots of each culture of each phytoplankton species were then added to the feeding containers (see 2 subsections below) to give suspended phytoplankton concentrations of either 0.5 or 3 mg l^{-1} for each species. Phytoplankton were also collected for fractionation analysis (3 replicates per flask). Preliminary tests showed that Day 5 corresponds to the exponential phase in all species except in the case of *Prorocentrum minimum*, the growth of which proved slightly slower.

Phytoplankton metal fractionation analysis. Phytoplankton cells to be used to assess metal partitioning (3 replicates in each of 3 experiments, except for *Perna viridis* at 3 mg l^{-1}) were resuspended for 10 min in 20 ml of a chelating agent (8-hydroxyquinoline-5-sulphonate, 1 mM) to extract any exchangeable metals loosely bound onto the cell wall, according to the method of Price & Morel (1990). The phytoplankton was centrifuged again (2180 × *g* for 20 min at 20°C), and the supernatant (S1 containing exchangeable radiolabelled metals) and the pellet (P1 containing incorporated radiolabelled metals) were separated. The metal fractionation of these pellets into the insoluble fraction (P2) and cytosol (S2) was completed according to the procedure described by Ettajani et al. (2001). Gamma counting was carried out on P1, P2, S1 and S2.

Assimilation experiments. The AEs of the 3 metals in the invertebrates were determined with a pulse–chase feeding technique, as described in Wang & Fisher (1999) and Wang & Rainbow (2000). Then, 8 bivalves or 10 barnacles were placed in 500 ml (bivalves) or 100 ml (barnacles) of 0.2 µm filtered seawater and fed (30 min for bivalves, 2 h for the smaller barnacles) on each of the radiolabelled phytoplankton at a cell density of 0.5 mg l^{-1} (all 3 invertebrates) or 3 mg l^{-1} (*Perna viridis* only). The first phytoplankton concentration represents typical phytoplankton concentrations in Hong Kong waters; the second concentration is the maximum phytoplankton concentration at which *P. viridis* does not produce pseudofaeces. Individual invertebrates were rinsed with non-radiolabelled water, and their radioactivity was counted. The 5 individuals (up to 8 for some barnacle samples) with the highest counts in each feeding treatment were chosen for AE estimation. The bivalves were placed in individual containers in a 10 l enclosed recirculating aquarium and fed with unlabelled diatoms (*Thalassiosira weissflogii*) to promote depuration of ingested radiolabelled food. The radioactivity in each bivalve was counted at frequent time intervals over 48 h. At each count faecal pellets were removed to minimise desorption of radiotracers from faecal material, and the aquarium was covered to prevent light disturbance. The barnacles were placed individually in beakers containing 100 ml filtered seawater and fed with unlabelled *T. weissflogii*. The radioactivity remaining in each barnacle was measured at frequent time intervals over a period of 48 h; faeces were removed, and water and food were renewed at each count. All experiments were carried out at 23°C using filtered (0.2 µm) seawater at 33 psu salinity.

RESULTS

Metal accumulation in phytoplankton

Metal accumulation in the phytoplankton was estimated from all 3 replicates of each of 3 cultures for each species. The concentration factor (l g^{-1}) is the ratio of the total radioactivity in all 3 fractions (exchangeable, soluble and insoluble) of the phytoplankton over the radioactivity in the culture medium. The phytoplankton species had significantly different metal accumulation concentration factors ($p < 0.001$). *Thalassiosira weissflogii* had the highest

cadmium, zinc and silver concentration factors, and the silver concentration factor of *Prorocentrum minimum* was not significantly different from that of *T. weissflogii* (Fig. 1). All the other phytoplankton species had comparable metal concentration factors. The ranges of concentration factors were 6 to 63 l g^{-1} for cadmium, 6 to 64 l g^{-1} for zinc and 3 to 39 l g^{-1} for silver.

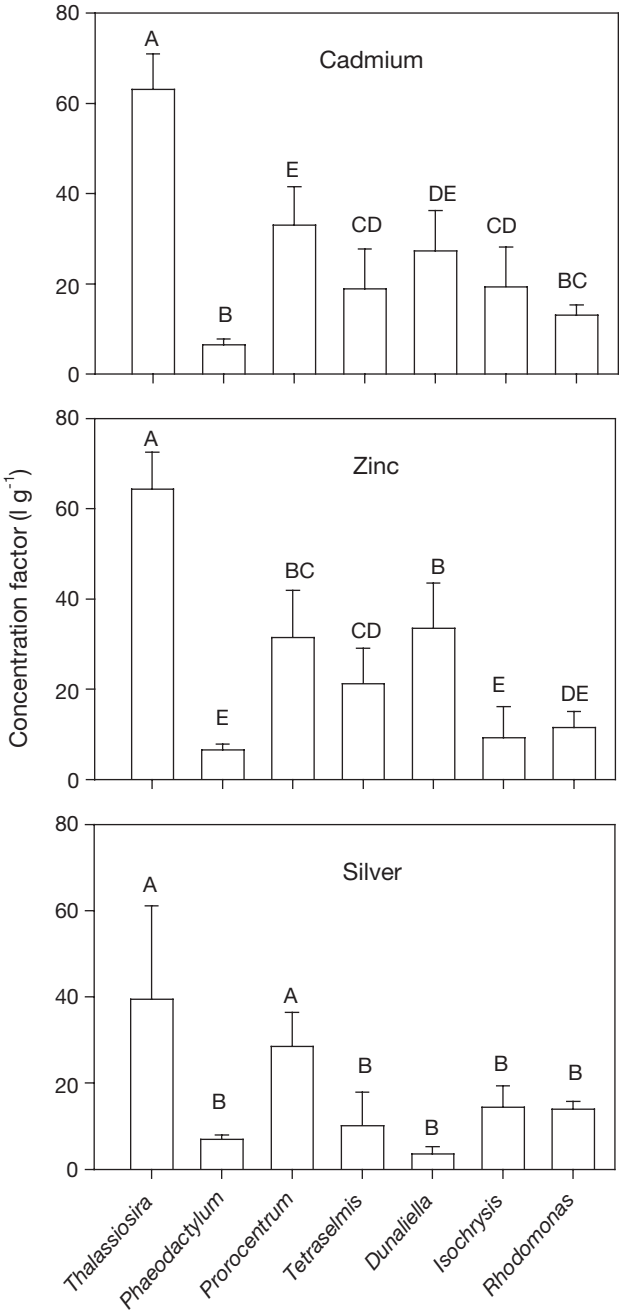

Fig. 1. Concentration factors (l g^{-1}) of cadmium, zinc and silver in 7 species of phytoplankton. Means and standard deviations (n = 9). For each metal, means that share a letter were not significantly different

Metal fractionation in phytoplankton

The distributions of labelled Cd, Zn and Ag in exchangeable, soluble and insoluble fractions are depicted in Fig. 2. The percentage of exchangeable cadmium was low for all the species except *Prorocentrum minimum*, in which it reached 21%. For Zn, the most important exchangeable fraction was also detected in *P. minimum* (31%), but it was also relatively high in *Thalassiosira weissflogii* (23%) and *Rhodomonas salina* (16%). On the other hand, no zinc was detectable in the exchangeable fraction of *Phaeodactylum tricornutum.* In most of the species, however, an important part of the silver taken up was present in the exchangeable fraction, particularly in *Isochrysis galbana* (59%), although in contrast little silver (<10%) was exchangeable in *P. tricornutum* and *R. salina*.

The distribution of metals readily incorporated in the phytoplankton cells between soluble and insoluble fractions was also species-dependent (Fig. 2, Table 2). Incorporated cadmium was mainly present in insoluble form in the diatoms and the dinoflagellate, whereas all other species stored this element mainly in soluble form; this was less distinctly so in *Rhodomonas salina*. The distribution pattern between soluble and insoluble fractions was relatively similar for zinc, except for *R. salina*, which stored most zinc in insoluble form. The distribution pattern of silver again showed a predominance of storage in insoluble form in the diatoms and the dinoflagellate. In the other species, incorporated silver was distributed relatively equally between soluble and insoluble forms, with the exception of *Dunaliella tertiolecta*, which stored silver mainly in the soluble fraction.

Assimilation efficiencies

Four experiments measured the AEs of Cd, Zn and Ag in 3 filter-feeding invertebrates — the green mussel *Perna viridis*, the clam *Ruditapes philippinarum* and the barnacle *Balanus amphitrite*, feeding on up to 7 phytoplankton species. All 3 filter feeders were presented with each phytoplankton species at a concentration of 0.5 mg l^{-1}. The green mussel was also presented with phytoplankton at 3 mg l^{-1}. In the event there was insufficient radioactivity present to measure Ag assimilation in *P. viridis* feeding on any of the phytoplankton at the lower concentration. The barnacles did not feed on either *Isochrysis galbana* or *Rhodomonas salina*. Figs. 3 to 5 show the mean retention of Cd, Zn and Ag (where appropriate) in *P. viridis* at the 2 phytoplankton concentrations and in *R. philippinarum* and *B. amphitrite* at 0.5 mg l^{-1}, broken down by individual phytoplankton species as

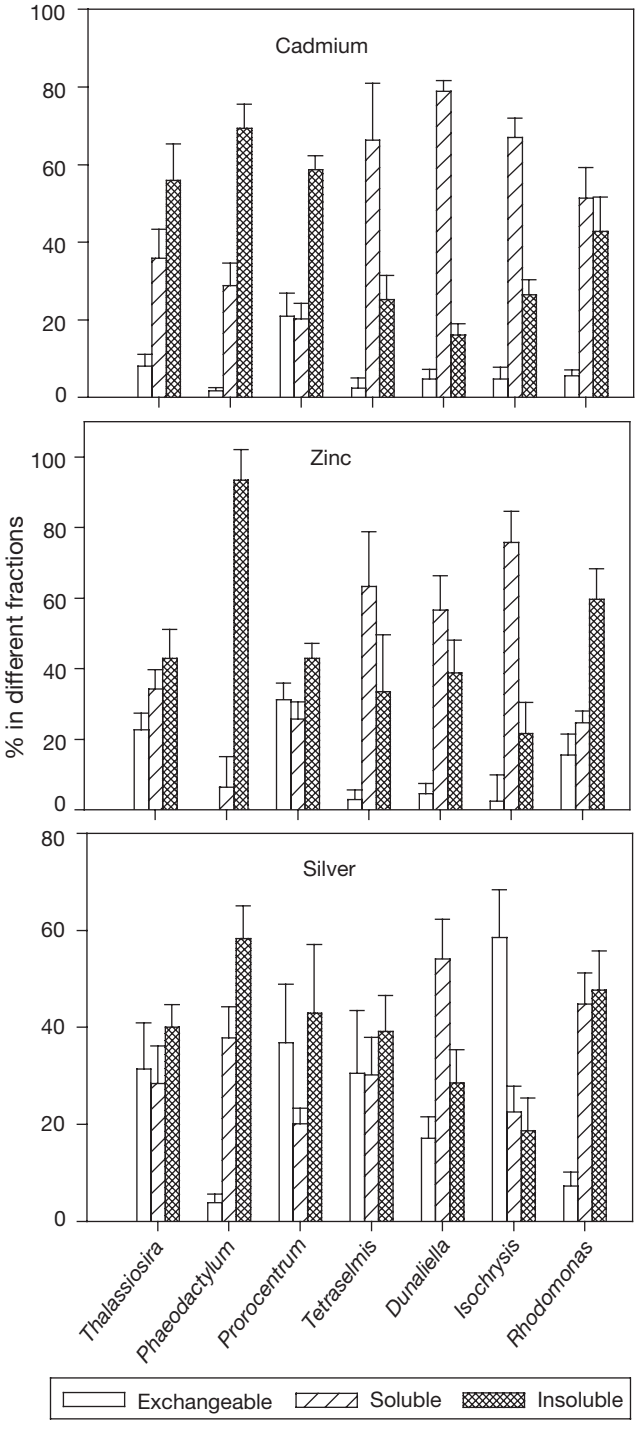

Fig. 2. Percentage distributions of labelled cadmium, zinc and silver in 3 fractions (exchangeable, soluble, insoluble) in 7 phytoplankton species. Mean percentages and standard deviations (n = 9)

food sources. The AEs of metals from phytoplankton in different herbivores are shown in Fig. 6. AE was determined as the percentage of initial radioactivity retained in the barnacles after 24 h (Wang & Rainbow 2000, Rainbow et al. 2003, 2004), but after 48 h in the mussels and clams, the longer period for the bivalves being in line with earlier work in the laboratory (Chong & Wang 2001, Shi et al. 2003, Ng & Wang 2004).

Statistical details from Figs. 3 to 6 are shown in Table 3. There were significant differences in AEs of Cd and Zn (but not Ag) between the invertebrates feeding on the same phytoplankton species at the same concentration (Table 3a, Fig. 6), for each invertebrate feeding on the different phytoplankton species at the same concentration (Table 3b, Fig. 6), and between the Cd and Zn AEs of the same species (*Perna viridis*) feeding on the same phytoplankton species at different concentrations (Table 3c, Fig. 6). Generally the green mussel *P. viridis* had the lowest AEs for both Cd and Zn of the 3 invertebrates feeding on phytoplankton at 0.5 mg l^{-1}, and the Cd, Zn and Ag AEs of *Ruditapes philippinarum* were never significantly exceeded by the AE of another invertebrate (Table 3a, Fig. 6).

Perna viridis showed no significant difference in Cd AE between phytoplankton species at high concentration, nor in Zn AE between phytoplankton at low concentration (Table 3b, Fig. 6). There were significant a priori differences in Cd AE between phytoplankton at low concentration, in Zn AE at high concentration and in Ag AE at high concentration, but in the former 2 cases the effect was not strong enough to distinguish diets a posteriori (Table 3b, Fig. 6). *Ruditapes philippinarum* showed no significant differences in Zn or Ag AEs between phytoplankton diets, but Cd AE did vary significantly (Table 3b, Fig. 6). *Balanus amphitrite* showed significant differences in AE between phytoplankton diets for Cd and Zn, but not for Ag (Table 3b, Fig. 6).

In 12 of 14 comparisons (Table 3c), there was no significant difference between the Cd and Zn AEs of *Perna viridis* feeding at low (0.5 mg l^{-1}) and high (3 mg l^{-1}) phytoplankton concentrations. The 2 exceptions did not follow the same pattern. The Cd AE of *P. viridis* feeding on *Rhodomonas salina* was higher in the low-diet mussels, but the Zn AE of *P. viridis* feeding on *Prorocentrum minimum* was higher in the high-diet mussels (Table 3c).

The 3 herbivores had been feeding on the diatom *Thalassiosira weissflogii* prior to the experiments. As Table 3b and Fig. 6 show, there was no evidence that the AE of any of the 3 metals was raised significantly above all others when the experimental food source was also *T. weissflogii*. It is true, however, that the AE of each metal when an invertebrate was feeding on *T. weissflogii* was only exceeded once—in the case of the Ag AE of *Perna viridis* feeding on *Phaeodactylum tricornutum* at high concentration.

Table 2. Comparisons (ANOVA and Tukey multiple-comparison tests) between distribution of Cd, Zn and Ag in 7 species of phytoplankton, using arcsine-transformed percentage data. Columns under the *a posteriori* heading sharing the same letter within a row are not different from each other ($p > 0.05$). ***$p < 0.001$

	ANOVA		*a priori*	*a posteriori*					
	F	df	p	*Thalassiosira*	*Phaeodactylum*	*Prorocentrum*	*Tetraselmis*	*Dunaliella*	*Isochrysis*	*Rhodomonas*
Cadmium										
Exchangeable	30.166	6,56	0 000***	B	D	A	D	B, C, D	C, D	B, C
Soluble	75.354	6,56	0.000***	D	D, E	E	B	A	B	C
Insoluble	51.202	6,56	0.000***	B, C	D	C, D	A	A	A	B
Zinc										
Exchangeable	60.794	6,56	0.000***	D, E	C	D	A, B	A	B, C	E
Soluble	58.871	6,56	0.000***	D	A	D	B, C	B	C	D
Insoluble	51.864	6,56	0.000***	B, C	A	B, C	C, D	C	D	B
Silver										
Exchangeable	50.674	6,55	0.000***	D	C	D	D	A	B	C
Soluble	30.167	6,55	0.000***	C, D, E	A	E	B, C	C, D	D	A, B
Insoluble	21.197	6,55	0.000***	B, C	A	B	B, C	C, D	D	A, B

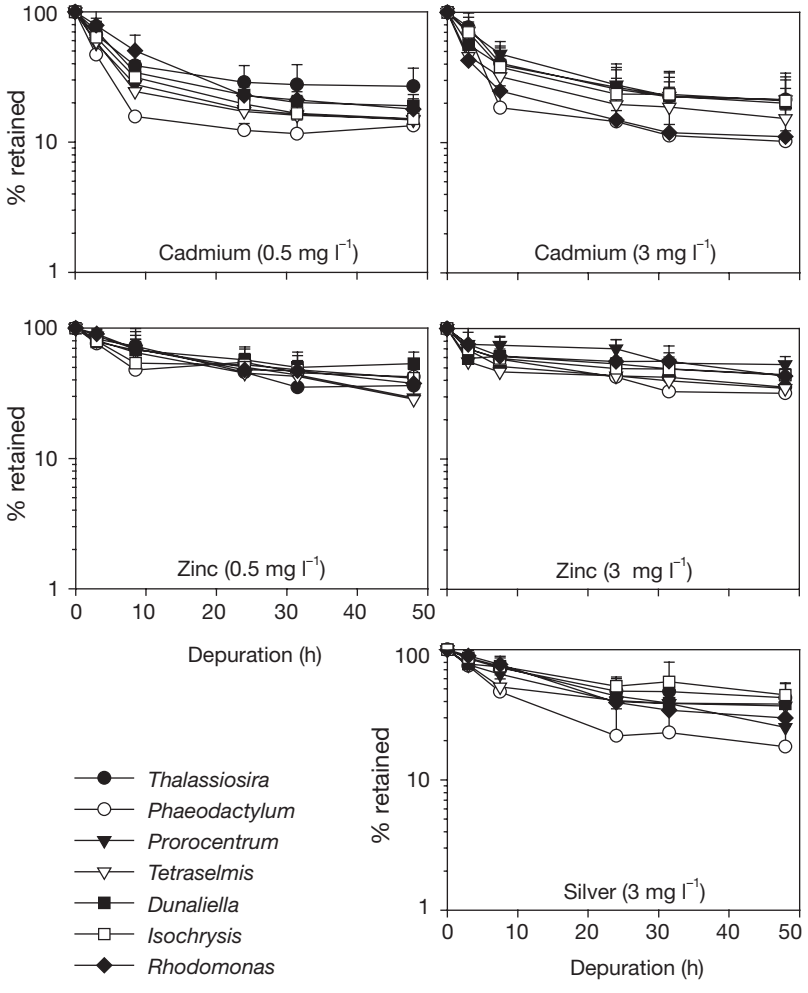

Fig. 3. *Perna viridis*. Retention of cadmium, zinc and silver (3 mg l^{-1} only) by green mussels following pulse-feeding on each of 7 phytoplankton species at 0.5 and 3 mg l^{-1}. Means and standard deviations (n = 5)

Phytoplankton metal fractionation and assimilation efficiencies

Correlations were sought between characteristics of the fractionation of accumulated Cd, Zn and Ag in the 7 phytoplankton species and the subsequent AE of the metal by each of the filter feeders. Out of a potential 48 correlations in the whole data set, only 8 were significant, and details of these are presented in Table 4.

The AE of Ag in *Perna viridis* feeding at 3 mg l^{-1} (but not at 0.5 mg l^{-1}) correlated positively with the percentage of exchangeable Ag in the phytoplankton (Table 4); this correlation caused a similar correlation in the combination of soluble and exchangeable fractions. The negative correlation of the AE with the percentage of Ag content in insoluble form is a mathematical consequence of this latter positive correlation. In the case of the AE of Cd in *Ruditapes philippinarum*, there was a positive correlation with the percentage of phytoplankton Cd in soluble form (Table 4), carried forward to a similar correlation with the combined soluble and exchangeable fractions. The barnacle *Balanus amphitrite* showed a negative correlation between Cd AE and the percentage of phytoplankton Cd in exchangeable form (Table 4). On the other hand, the Zn AE

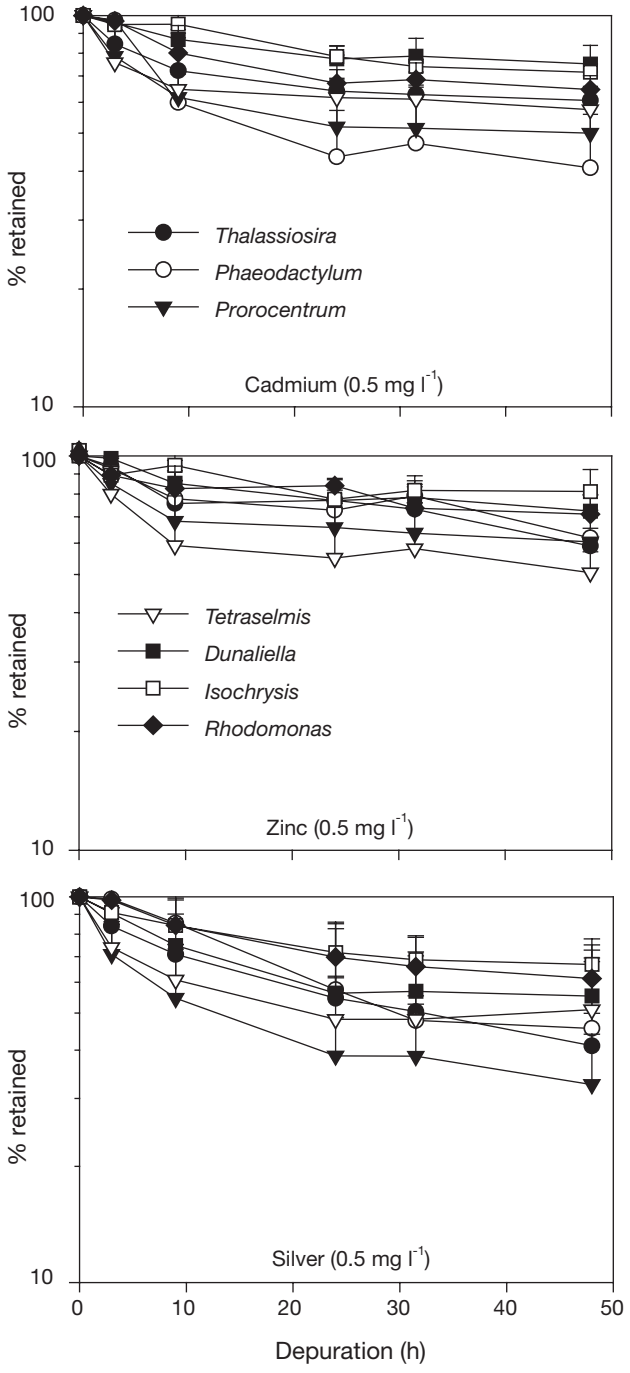

Fig. 4. *Ruditapes philippinarum*. Retention of cadmium, zinc and silver by clams following pulse-feeding on each of 7 phytoplankton species at 0.5 mg l^{-1}. Means and standard deviations (n = 5)

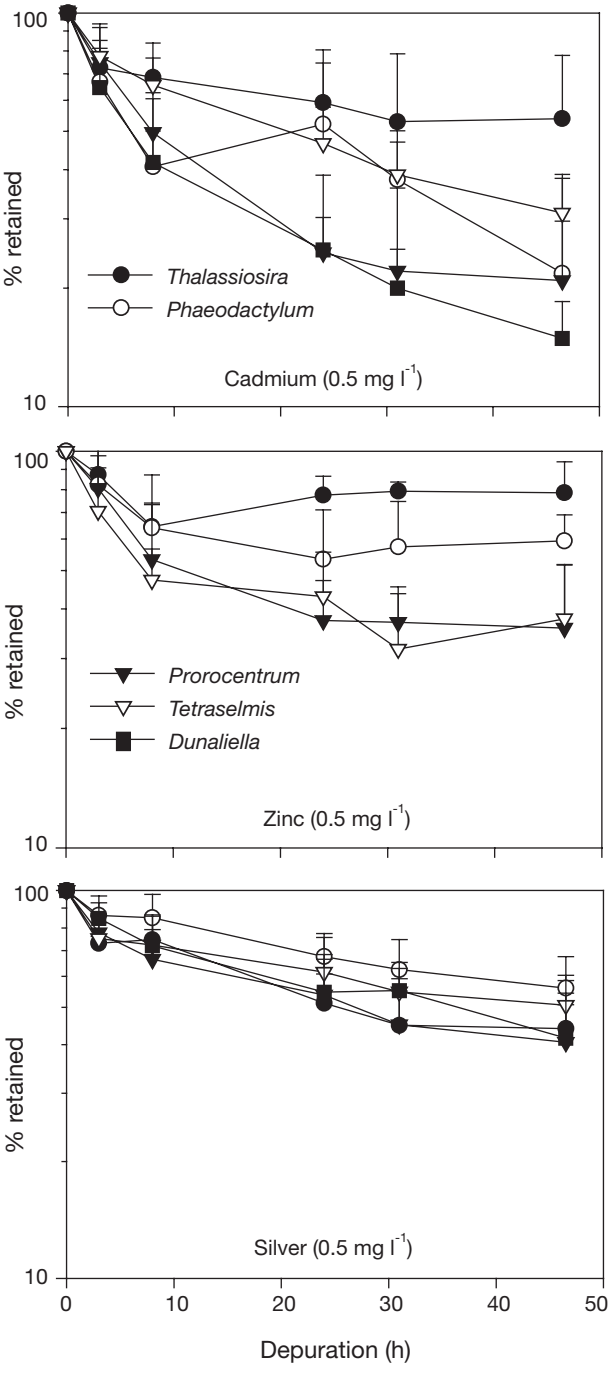

Fig. 5. *Balanus amphitrite*. Retention of cadmium, zinc and silver by barnacles following pulse-feeding on each of 5 phytoplankton species at 0.5 mg l^{-1}. Means and standard deviations (n = 8)

of *B. amphitrite* showed a positive correlation with the percentage of phytoplankton Zn in exchangeable form, but a negative correlation with the percentage of soluble phytoplankton Zn (Table 4). The latter negative correlation was maintained when soluble and exchangeable fractions were combined.

Table 5 presents ratios of the quantities of radiolabelled metals calculated by proportion (Fig. 2) to be in the exchangeable plus soluble fractions in the ingested diet at Time 0 by the clam *Ruditapes philippinarum*, to those assimilated at 48 h. If this ratio is <1, then at least some of the radiolabelled metal assimilated by the

Table 3. Comparisons (ANOVA or *t*-test) between assimilation efficiencies (AEs) of Cd, Zn and Ag in 3 invertebrates feeding on up to 7 species of phytoplankton, using arcsine-transformed percentage data. Columns under the *a posteriori* heading sharing the same letter within a row are not significantly different from each other ($p > 0.05$). NS: $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

(a) AEs of different invertebrates feeding on the same phytoplankton species at 0.5 mg l^{-1}

	ANOVA (*t*-test)		*a priori*	*a posteriori*		
	F (*t*)	df	p	*Ruditapes*	*Balanus*	*Perna*
Cadmium						
Thalassiosira	5.998	2, 9	0.022*	A, B	A	B
Phaeodactylum	2.625	2, 7	0.141	A	A	A
Prorocentrum	9.170	2, 13	0.003**	A	B	B
Tetraselmis	23.83	2, 10	0.000***	A	A	B
Dunaliella	99.03	2, 15	0.000***	A	B	B
Isochrysis	318.4	1, 6	0.000***	A	–	B
Rhodomonas	166.6	1, 7	0.000***	A	–	B
Zinc						
Thalassiosira	11.69	2, 10	0.002**	A, B	A	B
Phaeodactylum	2.059	1, 3	0.247	A	–	A
Prorocentrum	7.785	2, 10	0.009**	A	A, B	B
Tetraselmis	2.659	2, 9	0.124	A	A	A
Dunaliella	11.68	2, 15	0.01**	A	B	B
Isochrysis	32.32	1, 6	0.001**	A	–	B
Rhodomonas	11.65	1, 3	0.042*	A	–	A
Silver						
Thalassiosira	(1.260)	5	NS	A	A	–
Phaeodactylum	(1.689)	3	NS	A	A	–
Prorocentrum	(1.003)	9	NS	A	A	–
Tetraselmis	(1.065)	6	NS	A	A	–
Dunaliella	(0.118)	11	NS	A	A	–

(b) AEs of 1 invertebrate feeding on different phytoplankton species at 0.5 mg l^{-1} (low) or 3 mg l^{-1} (high)

	ANOVA		*a priori*	*a posteriori*						
	F	df	p	*Thalassiosira*	*Phaeodactylum*	*Prorocentrum*	*Tetraselmis*	*Dunaliella*	*Isochrysis*	*Rhodomonas*
Cadmium										
Perna (low)	3.180	6, 23	0.020*	A	A	A	A	A	A	A
Perna (high)	1.589	6, 28	0.187	A	A	A	A	A	A	A
Ruditapes (low)	5.325	6, 15	0.002**	A, B, C	C	B, C	A, B, C	A	A, B	A, B, C
Balanus (low)	6.166	4, 25	0.001**	A	A, B	B	A, B	B	–	–
Zinc										
Perna (low)	2.089	6, 23	0.094	A	A	A	A	A	A	A
Perna (high)	2.624	6, 24	0.042*	A	A	A	A	A	A	A
Ruditapes (low)	3.139	6, 15	0.034*	A	A	A	A	A	A	A
Balanus (low)	12.12	3, 18	0.000***	A	-	B	B	B	–	–
Silver										
Perna (high)	4.020	6, 25	0.006**	B	A	A, B	A, B	A, B	A, B	A, B
Ruditapes (low)	2.157	6, 16	0.103	A	A	A	A	A	A	A
Balanus (low)	1.612	4, 26	0.201	A	A	A	A	A	–	–

(c) AEs of *Perna viridis* feeding on one phytoplankton species at 0.5 mg l^{-1} (low) and 3 mg l^{-1} (high)

Taxon	Cadmium				Zinc			
	t	df	p	Conclusion	*t*	df	p	Conclusion
Thalassiosira	0.891	8	NS	low = high	0.358	8	NS	low = high
Phaeodactylum	0.541	5	NS	low = high	0.951	5	NS	low = high
Prorocentrum	1.094	8	NS	low = high	4.234	7	**	low < high
Tetraselmis	0.110	8	NS	low = high	0.951	8	NS	low = high
Dunaliella	0.191	8	NS	low = high	0.653	8	NS	low = high
Isochrysis	1.702	7	NS	low = high	0.602	7	NS	low = high
Rhodomonas	3.944	7	**	low > high	2.590	4	NS	low = high

clam must have been derived from metal bound to the insoluble fraction of the phytoplankton. It is evident from Table 5 that zinc must have been assimilated by *R. philippinarum* from the insoluble fractions of *Phaeodactylum tricornutum*, *Rhodomonas salina* and *Dunaliella tertiolecta*. Cadmium has also been assimilated by the clam from the insoluble fractions in the first 2 of these 3 phytoplankton species and in *Thalassiosira*

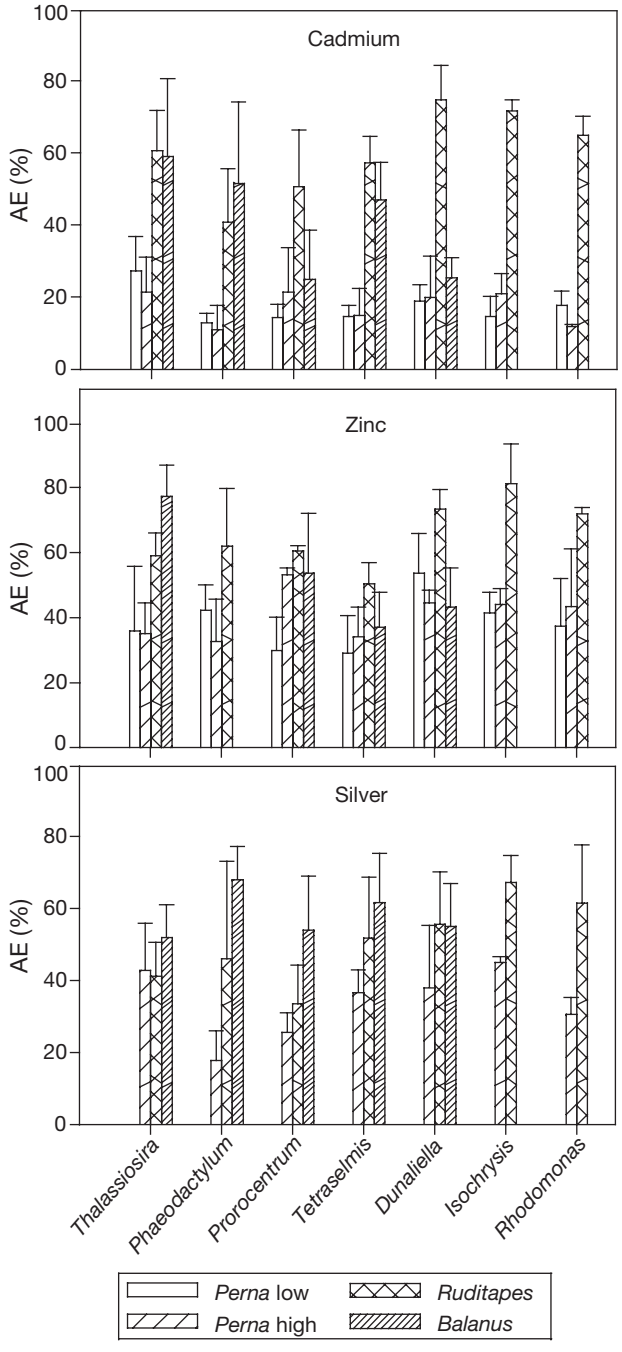

Fig. 6. Assimilation efficiencies (AE, %) of cadmium, zinc and silver from 7 phytoplankton species in the mussels *Perna viridis*, clams *Ruditapes philippinarum* and barnacles *Balanus amphitrite*. AE experiments for clams and barnacles were conducted at phytoplankton concentrations of 0.5 mg l^{-1}, whereas those for mussels were conducted at both low (0.5 mg l^{-1}) and high (3 mg l^{-1}) phytoplankton concentrations. Means and standard deviations (n = 5 to 8)

weissflogii (Table 5). In the case of the mussel *Perna viridis*, this ratio was clearly <1 (95% CL do not overlap 1.0) only for Zn in specimens fed *Prorocentrum minimum* (0.19 ± 0.05). In the barnacle *Balanus amphitrite*, the ratio was clearly <1 only in specimens fed *P. minimum* and then for all 3 metals (Cd: 0.65 ± 0.30; Zn: 0.17 ± 0.10; Ag: 0.66 ± 0.08). It can thus be concluded that trace metals bound to the insoluble fraction in phytoplankton can be bioavailable to herbivores.

Table 4. Correlation between the percentage of phytoplankton metal that is exchangeable, soluble, or insoluble versus assimilation efficiencies of the herbivores. Data were fitted with regression models. R^2 is the correlation coefficient, and significance of regression coefficient was tested by ANOVA ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Most relationships (40 from 48) were not significant (NS), and the remaining 36 are not shown (SE: standard error)

Percentage form of storage	Regression coefficient	SE	ANOVA	R^2
Perna viridis (3 mg l^{-1})				
Silver				
Exchangeable	0.405	0.167	*	0.164
Soluble	0.000	0.183	NS	0.000
Insoluble	−0.585	0.148	***	0.342
Ruditapes philippinarum (0.5 mg l^{-1})				
Cadmium				
Exchangeable	−0.244	0.198	NS	0.595
Soluble	0.671	0.151	***	0.450
Insoluble	−0.701	0.146	***	0.491
Balanus amphitrite (0.5 mg l^{-1})				
Cadmium				
Exchangeable	−0.372	0.175	*	0.139
Soluble	−0.127	0.187	NS	0.016
Insoluble	0.273	0.182	NS	0.075
Zinc				
Exchangeable	0.570	0.184	**	0.324
Soluble	−0.602	0.178	**	0.363
Insoluble	0.635	0.173	**	0.404

Table 5. Ratios of the quantities of radiolabelled metals calculated to be in exchangeable plus soluble forms ingested by the bivalve *Ruditapes philippinarum* to the quantities assimilated at 48 h. Mean and confidence intervals at the 95% level

Food source	Cadmium	Zinc	Silver
Thalassiosira	0.74 ± 0.17	0.97 ± 0.12	1.24 ± 0.55
Phaeodactylum	0.62 ± 0.32	0.09 ± 0.04	0.84 ± 0.40
Prorocentrum	0.82 ± 0.36	0.85 ± 0.17	1.78 ± 0.96
Tetraselmis	1.47 ± 0.68	1.75 ± 1.20	1.16 ± 0.27
Dunaliella	1.13 ± 0.11	0.85 ± 0.07	1.36 ± 0.28
Isochrysis	0.97 ± 0.09	0.92 ± 0.11	1.19 ± 0.12
Rhodomonas	0.89 ± 0.07	0.49 ± 0.08	0.91 ± 0.25

DISCUSSION

The choice of the wide systematic range of phytoplankton for this study has been reflected successfully in the observed variation in the trace-metal accumulation powers of the different phytoplankton species and

in the differential fractionation of the accumulated metals in the phytoplankton cells. The concentration factors of metals in phytoplankton species have been well summarised by Fisher & Reinfelder (1995). Our measured concentration factors in different phytoplankton species were comparable to those of these previous studies. The features of the cell wall of the phytoplankton may affect metal accumulation. However, the high accumulation ability of *Thalassiosira weissflogii* may not be solely related to the external features of the diatom or size of the cells, because the other diatom, *Phaeodactylum tricornutum*, of similar size to *T. weissflogii*, only accumulated low amounts of metals.

The distribution of accumulated metals on and in the phytoplankton cells, as reflected in the percentage contributions to total content of exchangeable, soluble and insoluble incorporated metals, was extremely variable. In our study, most Cd and Zn were incorporated into all the phytoplankton cells as opposed to remaining in exchangeable form externally. Incorporated Cd and Zn were predominantly in insoluble form in the 2 diatoms and in the dinoflagellate, and were mostly soluble in the remaining species. In fact, the insoluble component of incorporated metal includes metal tightly bound to cell walls and cell debris, as well as to specific metal-binding insoluble deposits, such as phosphate granules or lysosomal residual bodies. In addition, the siliceous frustule of the diatom cell walls may incorporate Cd and Zn strongly, contributing to the high percentage in the insoluble fraction. However, the reason for the similar distribution in the dinoflagellate is still unclear. Metal in the exchangeable component was much more evident for silver. This is probably due to the high particle reactivity of silver, which, once it was incorporated into the cells, distributed relatively evenly between soluble and insoluble forms in all phytoplankton.

The AEs of Cd and Zn showed significant variation between the 3 invertebrates feeding on the same phytoplankton species at the same concentration, while Ag AE did not differ significantly between *Ruditapes philippinarum* and *Balanus amphitrite* feeding on the same phytoplankton (Table 3). The AEs of *R. philippinarum* were usually the highest and those of *Perna viridis* the lowest, in agreement with earlier work (Chong & Wang 2001, Shi et al. 2003, Ng & Wang 2004). Barnacles, including *B. amphitrite*, are considered to have high AEs in comparison to many invertebrates (Wang et al. 1999a,b, Wang & Rainbow 2000, Rainbow & Wang 2001), and so the AEs of *R. philippinarum* are particularly high.

The differential assimilation of trace metals by invertebrates from different phytoplankton diets is well established, notably in bivalve molluscs (Wang & Fisher 1999) including *Perna viridis* and *Ruditapes philippinarum* (Chong & Wang 2000) and barnacles (Wang & Rainbow 2000, Rainbow & Wang 2001). *P. viridis*, in fact, showed no differential assimilation of Cd and Zn between phytoplankton diets at 0.5 or 3 mg l^{-1} (Table 3). *R. philippinarum* showed differential assimilation of Cd between phytoplankton at 0.5 mg l^{-1}, but not of Zn or Ag (Table 3). *Balanus amphitrite* only fed on 5 of the 7 phytoplankton species, but still showed differences between phytoplankton for AEs of Cd and Zn, but not Ag (Table 3).

In oysters fed Cu-enriched diatoms (*Haslea ostrearia*), it has been shown that the percentage of metal retained by bivalves increased from 13 to 79% and to 93% after 1, 2 and 3 wk of experimental feeding, respectively (Ettajani et al. 1992). Temporal changes in Cu retention in oysters may be attributed to differential absorption of biochemical components by these bivalves in response to changes in their food regime, as shown in other species, *Mytilus chilensis* and *Mulinia edulis*. When their typical diets were changed, over a period of 7 d, the energy absorbed declined by 32% in *M. chilensis* and 64% in *M. edulis* (Navarro et al. 2003). However, absorption efficiency involved acclimation (which was not completed at Day 7), indicating the capacity of these species to modulate their enzymatic-digestive activity depending on food composition (Labarta et al. 2002). In the present work the filter feeders had been fed diatoms (*Thalassiosira weissflogii*) during acclimation in the laboratory. There was no evidence that the AEs of any of the 3 metals was raised significantly when the experimental food source was also *T. weissflogii*, although the AE of each metal when an invertebrate was feeding on *T. weissflogii* was the highest AE in all but 1 case.

This study set out to seek generalities in the relationships between metal partitioning in phytoplankton and their subsequent assimilation in filter-feeding herbivores. Reinfelder & Fisher (1991) concluded that metal assimilation in copepods is directly related to the metal bound in the cytoplasm of a diatom (*Thalassiosira pseudonana*). Wang & Rainbow (2000) and Rainbow & Wang (2001) provided some support for this conclusion as a general principle in the barnacles *Balanus trigonus* and *Elminius modestus*, respectively. In *B. trigonus* the AEs of Cd and Zn (but not Ag) were correlated with the percentage of metal present in the cytoplasm of 4 dietary phytoplankton species (Wang & Rainbow 2000), while in *E. modestus* the AEs of Cd and Se (but not Zn or Cr) showed the same correlation, in this case for 5 phytoplankton species as diets. There was also a significant correlation between the AEs of Am, Co and Se in the mussel *Mytilus edulis* and the cytosolic distribution in the natural seston (Wang et al. 1996) or the diatom *T. pseudonana* (Wang & Fisher 1996), but this did not apply to Ag, Cd and Zn (Wang &

Fisher 1996, Wang et al. 1996). In addition, the percentages of Ag, Am, Cd, Co, Se and Zn assimilated by *M. edulis* were positively related to the cytoplasmic fraction in ingested *Isochrysis galbana* cells, as they were for Ag and Cd in the oyster *Crassostrea virginica* and the clams *Macoma balthica* and *Mercenaria mercenaria* (Reinfelder et al. 1997).

Before we discuss the relationships found in this study, it is necessary to compare our fractionation scheme with ones previously used. Conventional studies have often focused on the bioavailability of intracellular/cytoplasmic metals. Our study has also investigated the bioavailability of metals adsorbed onto the cell walls, as identified by extraction with a metal-complexing agent. Among the different metal-complexing agents that have been used to discriminate between intra- and extracellular metals, 8-hydroxyquinoline-5-sulfonic acid has been regarded to have the smallest effect on membrane permeability (Hassler et al. 2004). This ensures that no initially intracellular labelled metal will be lost during the first step of fractionation, and 8-hydroxyquinoline-5-sulfonic acid was, therefore, chosen as the complexing agent to be used here. Furthermore, the phytoplankton fractions were classified in this study into groups that differ from those in other studies. Reinfelder & Fisher (1991) separated the phytoplankton into 3 fractions—a cell-wall fraction, a heavy organelles fraction and cytoplasm. Other studies have separated the cells into 2 fractions—a cell-wall fraction and the cytoplasm (including heavy organelles) (Reinfelder & Fisher 1991, Wang & Fisher 1996, Chong & Wang 2000). By comparing the different speeds of centrifugation used, it remains possible that the combined cell-wall fraction and heavy organelles used by Reinfelder & Fisher (1991) may be equivalent to our insoluble fraction, and their cytoplasm may be equivalent to our soluble fraction.

The choice of 7 phytoplankton species from a wide systematic range has provided a wide range of percentage accumulated metal contents in the different fractions separated, but the study has failed to establish the generalities hinted at in earlier, more restricted studies. As perhaps is inevitable in such a large study, a few significant relationships were demonstrated, but these were inconsistent and sometimes contradictory (Table 4). Xu & Wang (2004) reported that a few important processes are responsible for Ag assimilation, including its distribution in different subcellular fractions (e.g. cytoplasmic), concentration factor in the phytoplankton and particle reactivity (e.g. tendency to bind with particles), and gut passage time. In general, soluble metal is important for determining metal assimilation in the herbivores, as also shown for the cadmium uptake in *Ruditapes philippinarium* and the zinc uptake in *Balanus amphitrite* in our study. There is, however, no support for a generalised conclusion that any of the 3 fractions isolated represents that form of phytoplankton metal that is bioavailable for trophic transfer to a herbivore.

This study has also shown that even trace metals bound to the insoluble fraction in phytoplankton can be bioavailable to herbivores. Similarly, some of the accumulated trace metal bound to the insoluble fraction in animal prey has been shown to be bioavailable to predators. The decapod crustacean *Palaemonetes pugio* fed Cd-contaminated oligochaete worms assimilated 48.6% of the Cd bound to a subcellular debris fraction that included granules and tissue fractions, although this AE was not as high as the AE of 84.8% from the cytosolic fraction (Wallace & Lopez 1997). Wallace et al. (2003) have also indicated that, in addition to any metal bound to protein, metal bound to organelles in the bivalves *Macoma balthica* and *Potamocorbula amurensis* may also be trophically available. Furthermore, Cheung & Wang (2005) have shown that metals in metal-rich granules that are in the insoluble fraction of prey can be bioavailable to the gastropod *Thais clavigera*. In oysters, a significant fraction of the trace metals stored in insoluble form may be released during *in vitro* digestion (silver: 35 to 63%; cadmium: 30 to 48%; zinc: 70 to 88%) (Bragigand et al. 2004). Even sediment-bound metals may be partly available to filter or deposit feeders using incubation of sediments with digestive fluids from different species of marine benthic invertebrates (Mayer et al. 2001, Fan et al. 2002, Yan & Wang 2002, Weston et al. 2004). In mussels (*Mytilus edulis*) fed various sediments, silver AE varied from 4.6 to 23%, cadmium AE from 9.5 to 35% and zinc AE from 21 to 36% (Griscom et al. 2000).

The opportunity was taken to compare the AEs of cadmium and zinc in *Perna viridis* at 2 diet concentrations—at 0.5 mg l^{-1}, representing typical phytoplankton concentrations in Hong Kong coastal waters, and at 3 mg l^{-1}, below the phytoplankton concentration at which green mussels produce pseudofaeces. Essentially there was no effect of diet on cadmium or zinc AE. The 2 exceptions (Table 3c) were contradictory in the diet concentration that was associated with the higher AE. The AEs of cadmium and zinc have been found to be dependent on the food composition and food concentration, respectively, in the mussels. The AE of Cd in the mussel *Mytilus trossulus* from silt particles increased from 36% at 50 mg l^{-1} of silt to 92% with the addition of 20 000 cells ml^{-1} of the diatom *Thalassiosira pseudonana* (Arifin & Bendell-Young 2000). Chong & Wang (2000) found that the presence of sediment reduced Cd assimilation from ingested diatoms by green mussels *Perna viridis*. In addition, an increase in food concentration from 1 to 15 mg l^{-1} during diges-

tion of *P. viridis* resulted in a significant decrease in the AE of zinc bound in either sediments or diatoms (Wong & Wang 2003). In our study, the food concentration may not have been high enough for such effects to be observed on the AE of zinc.

In conclusion, therefore, the physico-chemical form of trace metals accumulated by phytoplankton does not control their assimilation by filter-feeding invertebrates, at the relatively crude level of distinction into exchangeable, soluble and insoluble fractions. Differential bioavailabilities of trace metals in the diet are therefore controlled by more subtle differences in the form of chemical binding of the accumulated metals in the phytoplankton. It is not possible to draw a generalised conclusion that any of the 3 fractions isolated in our study represents that form of phytoplankton metal that is bioavailable for trophic transfer to a herbivore.

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