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Contribution of dietary bacteria to metal accumulation in the slipper limpet

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ABSTRACT: Recent studies have shown that dietary ingestion can be an important source for metal accumulation in aquatic animals. Whether bacteria can contribute significantly to the overall metal accumulation in these animals remains little known. In this study, we used the kinetic modeling approach to examine the relative contribution of the aqueous phase, bacteria, and phytoplankton, as sources for Cd, Cr, and Zn accumulation by the slipper limpet *Crepidula onyx*, a suspension feeder that can capture bacteria efficiently. Metal uptake rate constants measured in *C. onyx* were 0.200, 1.232, and 1.294 l g^{-1} d^{-1} for Cd, Cr, and Zn, respectively. The assimilation efficiency (*AE*) of metals from ingested phytoplankton (11 to 44 % for Cd, and 31 to 41 % for Zn) was comparable to *AE*s of metals from ingested bacteria (21 to 42 % for Cd, 35 to 47 % for Zn), whereas the *AE*s of Cr from ingested phytoplankton (10 to 22 %) were lower than from ingested bacteria (44 to 59 %). The *AE*s of Cr from different phytoplankton and bacterial diets were related to its distribution in the cytoplasm of cells and its passage time across the animal's guts. The limpets filtered the bacteria at rates (17 l h^{-1} g^{-1}) 1.3 to 1.9× lower than the filtration rate on different phytoplankton diets (22 to 33 l h^{-1} g^{-1}). The estimated average bioconcentration factors of metals by 2 bacterial strains were 2 to 6×10^5 for Cd, 1 to 7×10^5 for Cr, and 1.5 to 7×10^5 for Zn. The kinetic model predicted that uptake from dietary phase dominated metal accumulation in the slipper limpets (87 % for Cd, 72 % for Cr, and 83 % for Zn). Ingestion of bacteria contributed 23 % for Cd, 27 % for Cr, and 17 % for Zn accumulation in the limpets under typical conditions for the limpets. Our study therefore highlights bacteria as a potentially important source of metal accumulation in this filter-feeding mollusc.

KEY WORDS: Limpets · Metals · Bacteria · Trophic transfer

INTRODUCTION

In aquatic ecosystems, bacteria production amounts up to 30 % of the total primary production (Fenchel 1987). Bacteria are an important component of the microbial loop (Azam et al. 1983). A variety of aquatic animals, common or dominant in their communities, have been shown to effectively capture bacteria in addition to other planktonic food (e.g., acleractinian coral, Bak et al. 1998; reef sponges, Reiswig 1974, Pile 1997; polychaete larvae and juveniles, Gosselin & Qian 1997, 2000; mussels, Seiderer & Newell 1985, Langdon & Newell 1990, Kreeger & Newell 1996; clams, Decho & Luoma 1991, 1996; oyster larvae and adults, Langdon & Newell 1990, Douillet 1993, Douillet & Langdon 1993; asteroid larvae, Rivkin et al. 1986; and ascidians, Bak et al. 1998). These studies have pointed out the significance of bacteria as a carbon source for the animal nutrition. However, no study has considered the potential contribution of bacteria as source for metal accumulation in these herbivores. There is essentially no knowledge of the quantitative significance of bacteria as potential contributors to metal accumulation in suspension-feeding macroinvertebrates, although several studies have emphasized the transfer of metals in the microbial loop (e.g., from bacteria to flagellates and protozoans, Barbeau et al. 1996, Twiss et al. 1996).

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Dietary exposure has been increasingly recognized as a major source for metal accumulation in many aquatic invertebrates (Wang & Fisher 1999a). Recent laboratory and field studies have shown that they acquire a significant proportion of metals from their ingested food, rather than solely from the ambient water (Reinfelder et al. 1998, Munger et al. 1999, Wang & Fisher 1999a). Both experimental manipulation and kinetic modeling approaches have generally been used to delineate the exposure pathways of metals in these aquatic invertebrates (Wang & Fisher 1999a). The kinetic modeling approach requires the measurement of physiological parameters including ingestion, assimilation, depuration, and growth. The accuracy of such modeling depends on how representative the selected food items are of the natural diet, because the organism's physiological processes vary dramatically with food type (Reinfelder et al. 1998). Most previous studies were concerned with phytoplankton diets as food sources for metal uptake by marine herbivores. Very few studies have considered the uptake of dietary bacteria by aquatic macroinvertebrates (e.g., Decho & Luoma 1991, 1996, Roditi & Fisher 1999).

The slipper limpet *Crepidula onyx* Sowerby is a mollusc common in Hong Kong waters. Its maximum density can reach up to 900 limpets m^{-2} in the sublittoral zone (Huang et al. 1984). This species has been found to effectively remove bacteria from the water column, suggesting that bacteria may be a potential food source for the mollucs (J.-W.Q. pers. obs.). This study attempted to quantify the relative contribution of the aqueous phase, bacteria, and phytoplankton to the total metal accumulation by this species. The objectives are to better understand the pathways through which the limpets acquire metals and the physiological processes responsible for metal intake. The results will be helpful for understanding the role of bacteria in transferring trace metals through the food web and whether it is appropriate to ignore bacteria when identifying the sources and predicting the fate of trace metals in aquatic ecosystems.

MATERIALS AND METHODS

Slipper limpets. Adult *Crepidula onyx* were collected from the pilings at the ferry terminal of Tsim Sha Tsui, Hong Kong, at low tide. Upon arrival in the laboratory, the adults were allowed to attach onto glass plates (15 × 15 cm) and maintained in a 30 l aquarium and fed with algal food *Isochrysis galbana*. Individual limpets carrying brooding pouches were transferred into beakers containing 2 l of seawater where veliger larvae were released. At 25°C and a food concentration of 2×10^5 cells ml^{-1} (*I. galbana*), the larvae developed to the competent stage in 10 d, settled and metamorphosed into juveniles. Under the same culture conditions, juveniles reached 1.2 to 1.5 cm shell length within 2 to 3 mo and were used in the experiments. All experimental seawater was collected from Clear Water Bay, Hong Kong (28 psu).

Metals. Three metals (Cd, Cr, and Zn), which are of major environmental concern in Hong Kong waters (Blackmore 1998), were examined in this study. Among the 3 metals, Zn is essential whereas Cd and Cr can be toxic to aquatic organisms at relatively low concentrations. A radiotracer technique using ^{109}Cd, ^{51}Cr, and ^{65}Zn was employed to study the accumulation of Cd, Cr, and Zn, respectively. Radioactivity of ^{109}Cd, ^{51}Cr(III), and ^{65}Zn in the samples was measured using a Wallac 1480 NaI(T1) gamma detector (Turku, Finland). The gamma emissions of ^{109}Cd, ^{51}Cr, and ^{65}Zn were detected at 88, 320, 1115 keV, respectively. The radioactivity was calibrated for radioactive decay, spillover, and counting efficiency using appropriate standards. Counting time was adjusted to yield a propagated counting error < 5%.

Uptake of metals from the dissolved phase. Metal uptake was determined at different concentrations (4.5, 17.8, 89.3, and 446 nM for Cd, 9.6, 38.5, 192, and 962 nM for Cr(III), and 15.4, 76.9, 308, and 1538 nM for Zn). The lowest concentrations used in the experiments were about 5 to 50× higher than the lowest metal concentrations recorded in Hong Kong coastal waters (Wang & Dei 1999a). A radioisotope of each metal was added as a tracer and was equilibrated with stable metals for 12 h prior to the experiments. Radioisotope addition was 17.5 kBq l^{-1} for each isotope. Individual limpets were placed in 100 ml of 0.2 µm filtered seawater containing both isotopes and stable metals. Eight replicate individuals were used at each concentration treatment. At time intervals of 2, 4, 6 and 8 h, the limpets were removed from the radioactive media, rinsed with non-radioactive seawater, and their radioactivity was immediately counted (for 2 min). Following counting, the limpets were returned to the exposure beakers. At the end of experiment (8 h), they were dissected and the dry weights of their tissues and shells were measured following drying at 80°C for 1 d.

Assimilation of metals from ingested food. The assimilation by the limpets of metals from ingested food including different algal diets and bacteria was quantified. Four algal species (diatom *Thalassiosira pseudonana*, green alga *Chlorella autotrophica*, prasinophyte *Tetraselmis levis*, and dinoflagellate *Prorocentrum minimum*) and 2 heterotrophic bacteria strains were used as food for the limpets (Table 1). The algae were obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton (Maine, USA), while the bacteria were isolated from Port

Table 1. *Crepidula onyx*. Phytoplankton and bacteria used as diets for the measurement of metal assimilation efficiency in the slipper limpets. Data in parentheses are the standard deviations of 10 replicate measurements. Bacteria strain numbers correspond to the strains and properties described in Gosselin & Qian (1997)

Food item	Phylum	Cell shape	Cell dry weight (pg cell^{-1})	Cell dimensions (µm)
Prorocentrum minimum	Pyrrohophyta	Oval	250	17.8 (0.6) × 20.1 (1.1)
Tetraselmis levis	Chlorophyta	Oval	144	8.2 (1.3) × 14.0 (1.2)
Thalassiosira pseudonana	Chrysophyta	Cylinder	24	5.0 (0.4) × 6.5 (1.0)
Chlorella autotrophica	Chlorophyta	Sphere	15	3.9 (0.3)
Bacteria strain M11	Schizomycophyta	Rod	0.019	0.8 (0.3) × 1.5 (0.2)
Bacteria strain M4	Schizomycophyta	Cocci	0.015	1.22 (0.15)

Shelter, Hong Kong. The algae were cultured using f/2 enriched seawater (Guillard 1975) at 18°C and 30 psu. The bacteria were cultured using Nutrient Broth No. 2 (Oxoid Inc., Hampshire, UK) in the dark at 30°C. The dry weights of algae and bacteria were determined after filtering the cells onto pre-combusted GF/F membranes, rinsing with 0.5 M ammonia formate, and drying at 60°C for 24 h. Algal cells in the exponential growth phase were filtered and re-suspended into 150 ml of 0.22 µm filtered seawater enriched with the f/2 levels of N, P, Si, and vitamins, and f/20 levels of trace metals minus EDTA, Cu, and Zn (Wang & Fisher 1996). The initial cell density ranged from 10 000 to 50 000 cells ml^{-1}. Radioisotopes (30 nM ^{109}Cd, 0.6 nM ^{51}Cr, and 30 nM ^{65}Zn) were added into the medium to co-incubate with the algae. The cells were cultured for 3 to 5 d to allow for several divisions. The cells considered uniformly radiolabeled were collected onto 1 to 3 µm polycarbonate membranes and resuspended into 0.22 µm filtered seawater. This procedure was repeated twice to remove the radioisotopes weakly bound onto the cell surfaces. After cell density determination, the algae were added into the feeding beakers at 4.8×10^4 cells ml^{-1} for *P. minimum*, 8.3×10^4 cells ml^{-1} for *T. levis*, 5×10^5 cells ml^{-1} for *T. pseudonana*, and 8×10^5 cells ml^{-1} for *C. autotrophica* before adding the slipper limpets. The cell biomass in the feeding beakers was comparable among the different algal diet treatments. Metal distribution in the algal cytoplasm and cell walls was determined by the method described by Fisher et al. (1983) and Reinfelder & Fisher (1991).

Bacteria were cultured using Nutrient Broth No. 2 (Oxoid Inc.) in the dark for 2 d at 30°C and 28 psu in a shaking table and were harvested by centrifugation. Since our preliminary experiments showed that, in the presence of the broth, bacteria cells were not able to take up sufficient radioactive metals for experimentation, radiolabeling was conducted in suspension after the harvest. Bacterial concentrations were determined by fluorescence microscopic counting following staining the cells with 4′, 6′-diamidino-2-phenylin-dole (DAPI) (Porter & Feig 1980). After 1 d of radiolabeling (with initial cell densities of 8.7×10^6 cells ml^{-1} for strain M11 and 1.6×10^7 cells ml^{-1} for strain M4), the cells were collected onto 0.7 µm polycarbonate membranes and re-suspended in 0.22 µm filtered seawater. This procedure was repeated to remove weakly bounded radioisotopes. The bacteria were then diluted to 4.8×10^6 cells ml^{-1} for strain M11 and 6.5×10^6 cells ml^{-1} for strain M4 before being fed to the slipper limpets. To determine metal distribution in the cytoplasm and cell surface of each strain, the cells were collected by filtration, rinsed with EDTA and re-suspended in seawater. The samples were frozen at –20°C for 2 d, thawed, ultrasonicated for 10 min, and centrifuged at 10 000 rpm for 10 min to separate the supernatant and pellets before the radioactivity measurement.

A pulse-chase feeding technique was employed to determine the metal assimilation efficiency for each algal species and the bacterial strain (Wang & Fisher 1999b). There were 5 to 6 replicate exposure beakers per treatment, each with 1 slipper limpet. After 0.5 to 1 h feeding on radiolabeled algae or bacteria, the slipper limpets were retrieved, rinsed with seawater, and their radioactivity was immediately determined. The slipper limpets were then allowed to depurate for 46 h in the presence of non-radiolabelled *Isochrysis galbana*. During the depuration period, the radioactivity of the juveniles and their egested fecal pellets were determined at time intervals. Seawater and food were renewed each time the animals were taken for radioassay.

Assimilation efficiency (*AE*) was calculated as the *y*-intercept of the linear regression between the ln % of the metal retained in the animal and the time of depuration (during the slower phase of depuration, 21 to 46 h):

$$\%A = A_o e^{-kt} \quad (1)$$

where, $\%A$ is the percentage of radioactivity retained in slipper limpets at time t, A_o is the *y*-intercept of the

percentage of radioactivity retained in the slipper limpets, which is defined as the *AE* of the metals, k is the depuration rate constant, and *t* is time of depuration. This model assumes a 2-compartment depuration pattern for ingested metal, with the first compartment representing the digestive and assimilative process, and the second compartment representing the physiological turnover of metals.

Filtration rate. Filtration rate of the limpets was determined by monitoring the decrease of algal or bacterial density over time using established methods (Widdows 1985, Wang & Dei 1999b). The algal cell density was determined using a Coulter Multisizer II (Coulter Co., FL, USA). The concentration of bacteria was determined by fluorescence microscopy after staining the cells with DAPI. For each food item, there were 3 replicates and 3 controls. Individual slipper limpets were placed in 500 ml of filtered seawater containing the algal or bacterial suspension. The control contained the same concentration of algae or bacteria without the slipper limpet. The water was gently mixed by a magnetic stirrer. Algal and bacterial concentrations were adjusted such that the slipper limpets had access to similar algal and bacterial biomass at the beginning of the experiment, which was run for 2 h. Triplicates of 15 ml of seawater were taken for cell density determination at the beginning and at the end of the exposure. The filtration rate was calculated from the exponential decrease in particle concentration during the 2 h feeding period, after calibrating for the change in cell density in the control treatments.

Kinetic separation of metal uptake pathways. According to a bioenergetic-based kinetic model (Thomann 1981, Landrum et al. 1992, Wang et al. 1996), metal accumulation in the organism can be described as:

$$dC/dt = k_u C_w + AE \times IR \times C_f - k_e C \quad (2)$$

where C is the metal concentration in the animal at time t, k_u is the uptake rate constant from the dissolved phase, C_w is the metal concentration in the dissolved phase, AE is the metal assimilation efficiency from ingested food, IR is the ingestion rate, C_f is the metal concentration in the food source, k_e is the organism's metal efflux rate constant. Influx rate from the dietary phase ($AE \times IR \times C_f$) can be further separated into 2 components, namely, the seston (s) (e.g., phytoplankton) and the bacteria (b):

$$dC/dt = k_u C_w + AE_b \times IR_b \times C_{fb} + AE_s \times IR_s \times C_{fs} - k_e C \quad (3)$$

In the slipper limpets, the relative importance of dissolved uptake versus seston ingestion and bacteria ingestion can be estimated by comparing the relative influx rate of metal from each source. Thus, the ratio of metal uptake from dissolved phase (R_w), seston ingestion (R_s), and ingestion of bacteria (R_b), can be calculated as:

$$R_w = k_u C_w/(k_u C_w + AE_b \times IR_b \times C_{fb} + AE_s \times IR_s \times C_{fs}) \quad (4)$$

$$R_s = (AE_s \times IR_s \times C_{fs})/(k_u C_w + AE_b \times IR_b \times C_{fb} + AE_s \times IR_s \times C_{fs}) \quad (5)$$

$$R_b = (AE_b \times IR_b \times C_{fb})/(k_u C_w + AE_b \times IR_b \times C_{fb} + AE_s \times IR_s \times C_{fs}) \quad (6)$$

Assuming that the metal concentration in ingested seston (C_{fs}) and in bacteria (C_{fb}) can be predicted based on measurements of the metal partitioning coefficients in seston (K_{ds}) and in bacteria (K_{db}):

$$C_{fb} = K_{db} \times C_w \quad (7)$$

$$C_{fs} = K_{ds} \times C_w \quad (8)$$

Thus, Eqs (3) & (4) can be rewritten as:

$$R_w = k_u/(k_u + AE_b \times IR_b \times K_{db} + AE_p \times IR_p \times K_{ds}) \quad (9)$$

$$R_b = (AE_b \times IR_b \times K_{db})/(k_u + AE_b \times IR_b \times K_{db} + AE_p \times IR_p \times K_{ds}) \quad (10)$$

$$R_s = (AE_s \times IR_s \times K_{ds})/(k_u + AE_b \times IR_b \times K_{db} + AE_p \times IR_p \times K_{ds}) \quad (11)$$

RESULTS

Metal uptake from the dissolved phase

Uptake of Cd, Cr, and Zn from the dissolved phase by whole individual limpet followed a linear pattern over the exposure time (data not shown). Although the tissue accounted for only 11.2 ± 0.2% (mean ± SD, n = 32) of the total body weight, >75% of metals were associated with the tissues during the uptake period (Cd: 76.1 ± 15.0%, Cr: 77.1 ± 22.8%, Zn: 78.2 ± 9.8%). It was therefore not possible to calculate the influx rate of metals into the soft tissues based on the measurements of radioactivity in whole individual limpets. We calculated the influx rate of metals into the soft tissues based on the measurements of radioactivity in the soft tissues at the end of the exposure. With this method, it was assumed that the rate of uptake by soft tissues was constant over time. A log-log linear relationship between metal influx rate into the limpet tissues and metal concentration in the dissolved phase was evident for the 3 metals (Fig. 1). The dissolved uptake constant (k_u), calculated as the slope of the linear regression, was 0.200 l g^{-1} d^{-1} for Cd, 1.232 l g^{-1} d^{-1} for Cr, and 1.294 l g^{-1} d^{-1} for Zn. Such calculation assumed that the power coefficient of the relationship between metal

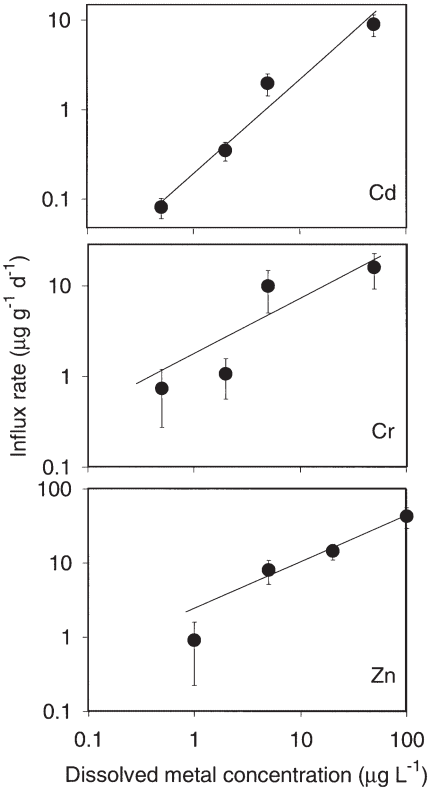

Fig. 1. *Crepidula onyx*. Metal influx rate (I_w) into the limpets' soft tissue as a function of metal concentration in the dissolved phase (C_w). Mean ± SD (n = 8). Regressions describing such relationships are: for Cd: $I_w = 0.200C_w^{1.036 \pm 0.150}$ ($r^2 = 0.960$); for Cr: $I_w = 1.232C_w^{0.723 \pm 0.244}$ ($r^2 = 0.815$); for Zn: $I_w = 1.294C_w^{0.802 \pm 0.140}$ ($r^2 = 0.943$)

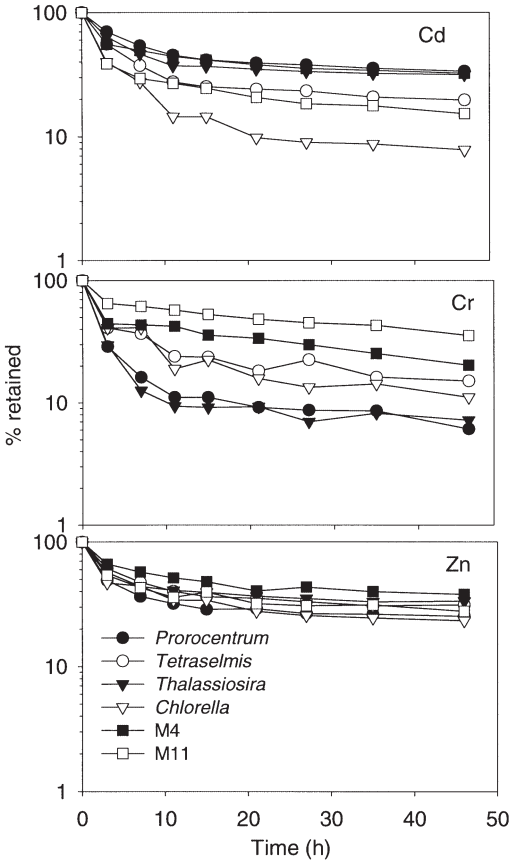

Fig. 2. *Crepidula onyx*. Metal retention in the limpets following a pulse feeding on different radiolabeled phytoplankton and bacteria (Strains M4 and M11). Only means are presented. The standard deviation of the calculated metal assimilation efficiency is shown in Table 2

influx rate and metal concentration in the dissolved phase was close to 1.

Metal assimilation from ingested food particles

The retention of Cd, Cr, and Zn in the slipper limpets following a pulse feeding is shown in Fig. 2. The pattern of metal depuration was characterized by a rapid release within the first few hours, followed by a gradual decline. We calculated the assimilation efficiencies (*AE*s) of the metals by regressing the natural log of the % of metal retention with time of depuration during the second phase of depuration (21 to 48 h) (Table 2). There were considerable intra-elemental variations among the metal *AE*s, ranging from 10 to 59% for Cr, 11 to 44% for Cd, and 31 to 47% for Zn. For Cd, *Chlorella autotrophica* had the lowest *AE* (11%), whereas *Prorocentrum minimum* had the highest *AE* (44%). Difference in algal diets significantly affected the *AE*s of Cd ($p < 0.001$, 1-way ANOVA), but not the *AE*s of Cr and Zn. The *AE* varied by about 2× for the 2 bacterial strains tested, and the difference was significant for Cd ($p < 0.01$, *t*-test) and Cr ($p < 0.05$, *t*-test). For Cr, the *AE*s from the 2 bacteria strains (44 to 59%) were substantially higher than those from algae (10 to 21%). There was less variation

Table 2. *Crepidula onyx*. The measured assimilation efficiency (%) of Cd, Cr, and Zn in the slipper limpets feeding on different phytoplankton and bacterial diets. Values are mean ± SD (n = 5)

Food item	Cd	Cr	Zn
Prorocentrum minimum	43.8 ± 6.4	11.9 ± 7.2	31.2 ± 9.6
Tetraselmis levis	28.0 ± 11.9	21.3 ± 7.4	37.8 ± 3.9
Thalassiosira pseudonana	37.2 ± 7.6	9.7 ± 3.6	42.4 ± 8.0
Chlorella autotrophica	11.3 ± 4.5	14.8 ± 7.8	30.7 ± 20.2
Bacteria strain M11	20.9 ± 1.0	59.0 ± 8.7	35.3 ± 1.7
Bacteria strain M4	41.6 ± 8.0	44.5 ± 6.5	46.8 ± 8.0

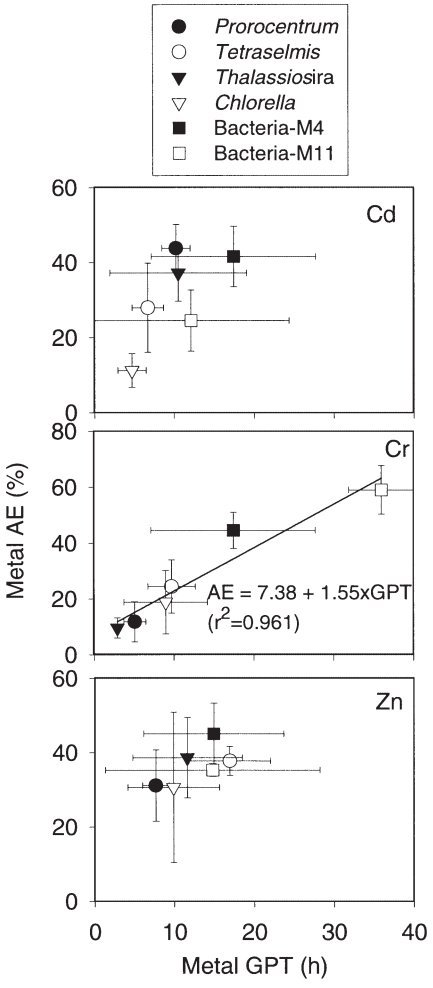

Fig. 3. *Crepidula onyx.* Metal assimilation efficiency (*AE*) as a function of metal gut passage time (GPT) in the limpets feeding on different phytoplankton and bacterial (Strains M4 and M11) diets. Mean ± SD (n = 5)

of Zn *AE* among the different diets tested than the variation of Cd and Cr. Within the 4 algal diets, *AE* was not correlated with the cell size.

Metal gut passage time (GPT) was defined as the time at which 90% of unassimilated metals were recovered in the feces, assuming a 100% recovery of feces during the 46 h depuration period. Overall, metal passage was completed within 35 h (Fig. 3). The GPTs of metals associated with bacteria exhibited greater variations than the GPTs of metals associated with the algae. For example, the GPTs of Cd and Cr for the algae were <10 h, whereas the GPTs of the same metals for the bacteria ranged between 12 and 36 h. When all algae were considered, there was a significant positive correlation between the Cr *AE* and its GPT.

There were considerable differences in the metal distribution in the cytoplasm between the algae and the bacteria and among the elements (Fig. 4). Less than 10% of Cr was found in the algal cytoplasm, whereas a significantly higher fraction of Cr was found in the bacterial cytoplasm (41 to 54%). In general, a higher percentage of metal penetrated into the bacterial cytoplasm than into the algal cytoplasm. When all food items were considered, there was a significant positive relationship between the *AE* and the metal distribution in the cytoplasm for Cr, whereas no significant relationship was evident for the other 2 metals (Fig. 4).

Filtration rate of the limpets

After 2 h feeding, about 56 to 76% of phytoplankton and 48 to 50% of bacteria had been cleared by the

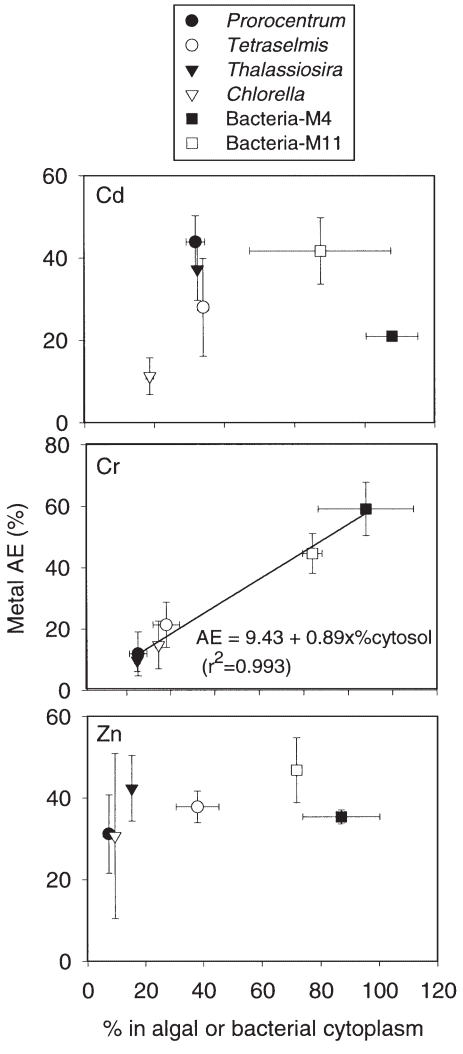

Fig. 4. *Crepidula onyx.* Metal assimilation efficiency (*AE*) in the limpets feeding on different phytoplankton and bacterial diets as a function of metal distribution in the cytoplasm of phytoplankton and bacteria (% cytosol). Mean ± SD (n = 5)

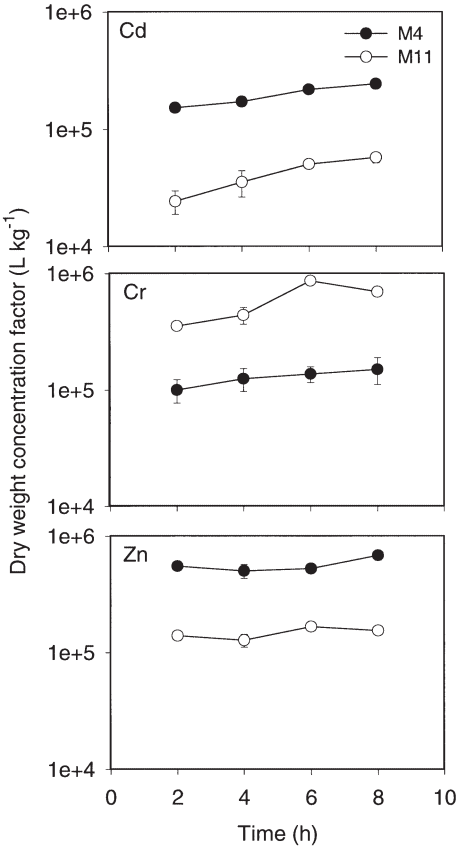

Fig. 5. *Crepidula onyx*. The calculated dry weight concentration factor of metals in 2 bacterial strains (M4 and M11) over 8 h exposure period. Mean ± SD (n = 3)

limpets. The calculated weight specific filtration rate was 21.9 ± 2.5, 26.0 ± 0.3, 32.7 ± 9.2, 30.9 ± 3.4 l h^{-1} g^{-1} (mean ± SD, n = 3) for limpets feeding on *Prorocentrum minimum*, *Tetraselmis levis*, *Thalassiosira pseudonana*, and *Chlorella autotrophica*, respectively, and was 16.9 ± 0.9, and 17.8 ± 3.3 l h^{-1} g^{-1}, for limpets feeding on bacteria strains M4 and M11, respectively.

Uptake of metals by bacteria

The calculated dry weight concentration factors (DCF) of metals in the 2 bacterial strains over the exposure time are shown in Fig. 5. Following the initial accumulation (2 h), there was a small increase in Cd and Cr accumulation by the bacteria, whereas bacteria did not show appreciable accumulation of Zn following the initial sorption. The calculated DCFs after 8 h of incubation in the M4 strain were 2.4×10^5 for Cd, 1.5×10^5 for Cr, and 6.8×10^5 for Zn, and in the M11 strain were 5.7×10^4 for Cd, 7.0×10^5 for Cr, and 1.5×10^5 for Zn. Accumulation of Cd and Zn by the M4 strains was 4.2 to 4.4× higher than the accumulation by the M11 strain, whereas the accumulation of Cr by the M11 strain was 4.6× higher than by the M4 strain.

Kinetic separation of metal uptake pathways

Parameters required in the kinetic model include *AE*, k_u, *IR*, and K_d. *AE* and k_u were determined in this study (Table 3). The mean K_d values for phytoplankton were taken from Fisher & Reinfelder (1995), whereas

Table 3. *Crepidula onyx*. Mean numeric values of metal physiological and geochemical parameters used in the kinetic modeling of metal uptake from different sources. Numbers in parentheses reflect the likely variability of each parameter. k_u: uptake rate constant from the dissolved phase; *AE*: assimilation efficiency; K_d: partition coefficient of metals in food particles; *IR*: ingestion rate of the limpets; % uptake: the predicted percentage of metal uptake from each source

Food/Metal	k_u (l g^{-1} d^{-1})	*AE* (%)	K_d (l kg^{-1})	*IR* (g g^{-1} d^{-1})	% uptake
Phytoplankton					
Cd	0.200	30 (10–45)	5000 (1000–10 000)	0.67	64
Cr	1.232	15 (10–20)	20 000 (10 000–100 000)	0.67	45
Zn	1.294	35 (30–45)	20 000 (10 000–100 000)	0.67	63
Bacteria					
Cd	0.200	30 (20–40)	100 000 (30 000–300 000)	0.0061	23
Cr	1.232	50 (45–60)	200 000 (100 000–800 000)	0.0061	27
Zn	1.294	40 (35–50)	300 000 (100 000–700 000)	0.0061	19

those of bacteria were measured in this study. The mean filtration rates of *Crepidula onyx* were 28 l h^{-1} g^{-1} and 17 l h^{-1} g^{-1} when the animals fed on algal cells and bacteria, respectively. The typical bacterial concentration in Hong Kong waters is approximately 1 to 3 × 10^6 cells ml^{-1} (Gosselin & Qian 1997), and the typical seston concentration is about 1 mg l^{-1} (Chiu et al. 1994). In modeling, we therefore used 2 × 10^6 cells ml^{-1} and 1 mg l^{-1} as representative bacterial and seston concentrations for Hong Kong waters. The calculated ingestion rate of slipper limpets on bacteria was therefore 12 mg g^{-1} d^{-1}, assuming a dry weight of 0.015 pg $cell^{-1}$ for bacteria (Table 1). The calculated ingestion rate of these limpets on seston was about 0.67 g g^{-1} d^{-1}.

Using the mean numeric value of each physiological and geochemical parameter, the model shows that uptake from the dissolved phase accounts for only 13, 28, and 17% of Cd, Cr, and Zn accumulation in the slipper limpets, whereas the particulate phase accounts for 87, 72, and 83% of Cd, Cr, and Zn accumulation (Table 3). When the particle ingestion was further divided into bacterial and seston components, a larger fraction of metals is derived from ingestion of natural seston (64% for Cd, 45% for Cr, and 63% for Zn). However, a measurable fraction of metals is indeed derived from the ingestion of bacteria. For example, the model predicts that 23% of Cd, 27% of Cr, and 17% of Zn in the slipper limpets is derived from bacteria due to their efficient filtration of bacteria.

DISCUSSION

In this study, metal uptake from the dissolved phase by the slipper limpet *Crepidula onyx* proceeded linearly over time, consistent with many previous studies of aquatic invertebrates (George & Coombs 1977, Bjerregaard et al. 1985, Wang & Dei 1999b). Because there was substantial accumulation of metals in the shells, measurements of radioactivity in the soft tissues after 8 h of exposure were used to calculate the metal influx rate into the soft tissues, assuming that metal accumulation by the soft tissues also proceeded linearly over time of exposure. The uptake rate constant was then calculated by regressing the log of the metal influx rate against the metal concentration in the dissolved phase. The calculated uptake rate constants (0.200, 1.232, and 1.294 l g^{-1} d^{-1} for Cd, Cr, and Zn, respectively) were generally comparable to the metal uptake rate constants measured with suspension-feeding mussels such as *Mytilus edulis*, with the exception of Cr (Wang et al. 1996). The uptake of Cr(III) by the slipper limpets was much higher than its uptake by other suspension-feeding invertebrates. For example, the uptake rate constant of Cr(III) measured in the mussel *M. edulis* was about 0.034 l g^{-1} d^{-1} (Wang et al. 1997). This study did not measure the uptake of Cr(VI), which generally has a higher uptake rate than Cr(III) and is more toxic than Cr(III) (Wang et al. 1997).

Among different species of marine bivalves, Wang (2001) has recently demonstrated that the difference in the uptake rate constants of metals is positively related to the filtration rate of the bivalves. The high rate of uptake from the dissolved phase by the slipper limpets was thus due, at least in part, to the high filtration rate of the animals, as a result of the well-developed gill filaments which integrate into a mesh covering the back of the mantle cavity (Ruppert & Barnes 1994). The extended gill surface area may facilitate metal diffusion. In *Crepidula onyx*, the water current enters the mantle cavity from the right side of the body and exits from the left. Such a well developed structure may have facilitated the capture of phytoplankton and bacteria, although the efficiency of food capturing is somewhat higher for phytoplankton. The gill may retain a lower percentage of particles when feeding on bacteria-size particles, as is the case in the bivalves *Mytilus edulis* and *Geukensia demissa* (Jörgensen 1990). However, this study demonstrates that bacteria can be very efficiently retained by the limpets. The filtration rate for bacteria was only 1.3 to 1.9× lower than the filtration rate for phytoplankton particles.

Metal assimilation by *Crepidula onyx* followed a biphasic pattern, with a faster release rate within the first few hours and a slower release rate afterwards. The *AE*s measured for Cd, Cr, and Zn from ingested phytoplankton were generally comparable to the *AE*s measured in other suspension feeding invertebrates (Wang & Fisher 1999b). However, there was great variation in the metal *AE*s among different phytoplankton diets tested (Cd: 10 to 45%, Cr: 10 to 20%, and Zn: 30 to 45%). Previously, different mechanisms have been proposed to explain the variation of metal *AE*s observed among different metals and different diets, including metal distribution in the phytoplankton cytoplasm (Reinfelder & Fisher 1991, Wang & Fisher 1996), metal GPT across the digestive tract (Wang & Fisher 1996, Chong & Wang 2000), and the partitioning of digestive systems (Decho & Luoma 1991, 1996). In this study, there was no clear trend to indicate that metal *AE* was directly correlated with the metal distribution in the phytoplankton, presumably because only 4 algal diets were tested. When its distribution of metals in the bacteria is also considered, there appears to be a significant correlation between metal *AE* and metal distribution in the cytoplasm for Cr, whereas no significant correlation is evident for Cd and Zn. There was also a significant correlation between the *AE* of Cr and its GPT. These results suggest that both metal partition-

ing in the diet and the animal's digestive processes are critical in Cr assimilation by the limpets.

The metal *AE*s measured from bacteria were somewhat comparable to the *AE*s measured from phytoplankton for Cd and Zn, whereas for Cr the *AE*s from bacteria were much higher than the *AE*s from phytoplankton. The higher *AE* of Cr from bacteria was due at least in part to its greater distribution in the bacterial cytoplasm and the longer GPT. Decho & Luoma (1991, 1996) similarly found that the *AE*s of Cr from bacteria were generally much higher than its *AE* from phytoplankton ingested by the clams *Potamorbula amurensis* and *Macoma balthica*. For example, an *AE* as high as 90% was found in the clams feeding on bacteria. They attributed such a high *AE* to the efficient partitioning of Cr in the second phase of digestion, namely, the intracellular digestion, in which the digestive cells in the digestive diverticula phagocytized the cells. In *Crepidula onyx*, whether the digestive system was bi-phasic (e.g., extracellular and intracellular digestion) as observed in marine bivalves (Widdows et al. 1979) is unknown.

There are few studies quantifying the accumulation of metals by the marine bacteria. This study demonstrated that the accumulation of metals by the bacteria was controlled mainly by passive sorption. Following an initial sorption of metals onto the bacteria, there was little further increase in metal accumulation over the exposure time. The study quantified the dry weight concentration factors of metals over the 8 h exposure period. Because metals showed little accumulation following the initial sorption, the bioconcentration factor (assuming equilibrium conditions) was probably close to the concentration factor (which is a kinetic parameter) measured in this study. The measured concentration factor for bacteria was generally much greater than the bioconcentration factor measured for marine phytoplankton (Fisher & Reinfelder 1995), largely due to the small size of bacteria, and thus the higher ratio of surface area to volume. However, metals may then be rapidly transported intracellularly by the bacteria, as reflected by the high percentage of metal distribution in the cytoplasm.

In the kinetic model employed in this study, the sources of metal accumulation in *Crepidula onyx* were broken down into the aqueous phase, bacteria, and phytoplankton. We assumed that metal uptake from each source was additive, i.e., metal uptake from 1 particulate source was independent of the influx from another source. Metal accumulation from the aqueous phase was quantified after exposing the animal to different metal concentrations. The influx of metals from bacteria and phytoplankton was further broken down into separate quantifiable parameters such as the metal's partition coefficient, filtration rate, and assimilation efficiency, each of which can be subjected to sensitive analysis to determine the most important parameter in the overall bioaccumulation.

Calculation using the kinetic model suggests that bacteria may be an important source for metal accumulation in the slipper limpet. Using the mean numeric value for each physiological and geochemical parameter, our model predicts that about 19 to 27% of dietary metals are indeed derived from ingested bacteria. Such an important contribution of metals from bacteria is primarily due to the high metal concentrations in the bacteria and the high assimilation of metals from ingested bacteria, despite the fact that the total biomass of bacteria was much lower than the natural seston. In addition, the slipper limpets have an efficient filtration system to retain the bacteria from the ambient water. These calculations considered only the free-living bacteria. When the attached bacteria are also considered as a potential source for metal accumulation, it is likely that the contribution of bacteria as a source for metal accumulation may be much greater than this model predicts.

These calculations used the average bacterial abundance to calculate the ingestion by limpets of bacteria. In Hong Kong waters, bacterial abundance tends to be relatively high and stable (1 to 3×10^6 cells ml^{-1}, Gosselin & Qian 1997) throughout most of the year. Similar bacteria concentrations have also been reported in other coastal areas (Rivkins et al. 1986, Douillet 1993). At the lowest bacterial concentration (10^6 cells ml^{-1}, Gosselin & Qian 1997), the ingestion rate of bacteria by limpets is predicted to be 6 mg g^{-1} d^{-1}, and the contribution of bacteria as source for metal accumulation will be about 13% for Cd, 16% for Cr, and 11% for Zn. At the upper level of bacterial abundance (e.g., 3×10^6 cells ml^{-1}), the contribution of bacterial metals would be 26 to 36% for the 3 metals considered in this study.

Several processes reflecting natural variability may complicate prediction of the quantitative importance of bacterial metals. This study used 2 bacterial strains originally isolated from Hong Kong coastal waters that are relatively large in size (0.8 to 1.2 µm). The calculated ingestion rate of bacteria based on measurements of the filtration rate and the dry weight of the 2 bacterial strains (M4 and M11) may have overestimated the actual ingestion rate under natural conditions. Both bacteria are free-living strains and in natural environments bacteria attached to algae or detritus may also be ingested by the limpets. These calculations assumed an average seston concentration of 1 mg l^{-1} to predict the relative importance of natural seston contributing to metal accumulation. The concentration of natural seston in Hong Kong, however, fluctuates with the seasons (Chiu et al. 1994). It is thus

expected that the influx rate from the ingested phytoplankton can vary substantially under natural conditions. Furthermore, it is relatively well known that the concentration factors for metals in natural seston and bacteria are not single values and may vary greatly in a natural environment. Such variability can also substantially affect predictions of the exposure pathways of metals in the limpets.

The size of the food items used in this study ranged from 0.56 to 3750 μm^3 (calculated from data in Table 1). This size spectrum may cover bacteria, flagellates, protozoa, phytoplankton, small zooplankton, and detritus. Because the flagellates and protozoa graze on the bacteria (Fenchel 1987), metals associated with bacteria may be transferred to flagellates and protozoa (e.g., microbial loop) before they are finally transferred to the limpets. Despite the complicated picture in applying these modeling results to the real field situation, the study does indicate that bacteria are a potentially important source for metal accumulation by the limpets as a result of efficient filtering of bacteria and the high metal concentration in bacteria. With the emerging picture that filter feeders are able to capture bacteria in locations where these limpets are abundant, the role of bacteria in transferring metals should be assessed when predicting the source and fate of metals in coastal ecosystems.

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