Environ. Sci. Technol. **2003**, *37,* 936−943

Effects of Aqueous and Dietary Preexposure and Resulting Body Burden on Silver Elokinetics in the Green Mussel *Perna viridis*

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To determine whether preexposure of green mussel *Perna viridis* to Ag influenced metal uptake kinetics we compared various physiological indicators of metal uptake kinetics between the control mussels and mussels preexposed to Ag in both diet and water at different levels (up to 5 weeks). In all preexposed mussels, the assimilation of Ag increased by 1.1−3.0 times with increasing Ag body concentration (0.651−19.3 μg g^{-1}) as compared with the controls (Ag body concentration of 0.311−0.479 μg g^{-1}), whereas the efflux rate constants decreased by 45−88%. There was no significant increase in Ag associated with the metallothionein-like protein (MTLP) fraction following exposure of the mussels to Ag through either the dissolved or food phase. The clearance rates were little affected or depressed by Ag preexposure, and the relationship between the Ag influx rate from the dissolved phase and the Ag preexposure was somewhat complicated. The influx rate decreased with increasing Ag body burden at <2.5 μg g^{-1}, above which it increased with increasing Ag body burden. Our results indicate that the mussels may modify physiological processes to ambient chronic Ag exposure, consequently accumulating more Ag. Ag body concentration in these mussels may therefore increase disproportionally in response to increasing Ag concentration in the ambient environments. Ag preexposure and resulting body burden should be considered carefully when interpreting the observed Ag concentration in biomonitoring animals to evaluate the Ag pollution in seawater.

Introduction

Marine mussels are able to process a large amount of dissolved and particulate metals because of their extremely high filtration rate (*1*), and thus they accumulate a higher metal concentration in their soft tissue as compared with that in the ambient environment. Because the body metal concentrations generally reflect the ambient bioavailable metal levels, marine mussels have been employed as biological monitors of metal pollution in coastal waters worldwide (*2, 3*). Previous studies have demonstrated that metal bioaccumulation can be affected by a variety of extrinsic conditions (*4*−*7*). Thus, factors influencing the metal accumulation in biomonitors should be carefully considered when interpreting biomonitoring data, especially when comparing the metal body burdens in the same species from different sites. Recently, there has been increasing attention on the effects of metal preexposure on subsequent metal bioaccumulation. For example, the overall Ag and Hg bioaccumulation potential of the clam *Macoma balthica* appears to be largely affected by their long-term exposure in the contaminated environment (*8*). Selck et al. (*9*) reported that Cd assimilation efficiency in the polychaete *Capitella* decreased following Cd preexposure. A recent study found that preexposure to Cd and subsequent induction of metallothionein-like proteins (MTLPs) affected Cd accumulation via ingested food but not Cd uptake from the dissolved phase in the green mussel *Perna viridis* (*10*). These limited studies indicated that metal preexposure may cause potential physiological changes and subsequently affect metal uptake in the animals. However, these previous studies generally only focused on the influence of metal preexposure from the dissolved phase on subsequent metal bioaccumulation, even though it has been demonstrated that food ingestion (i.e., dietary intake) can be a significant pathway for metal accumulation in aquatic animals (*11, 12*).

Silver (Ag) is one of the most toxic metals to aquatic organisms (*11, 13*). Because of the extensive use of Ag in the photographic and imaging industries, Ag discharge from such industrial applications into the aquatic environment may potentially cause contamination and thereby has received increasing attention in recent years (*14, 15*). The high particle-reactivity enables Ag to be easily associated with the particles and, as a result, taken up by aquatic animals (e.g., filter-feeders) through the dietary pathway (*13, 16*). The bioaccumulation of Ag from ingested food is therefore critical in understanding and modeling Ag trophic transfer in the marine food chain and its bioavailability to aquatic organisms. A previous modeling study predicted that 40−70% of Ag in the mussels *Mytilus edulis* from Long Island Sound and San Francisco Bay can indeed be accumulated from the dietary intake (*12*).

The form of metal storage in the animal's tissue is closely related to metal detoxification and bioaccumulation. Blackmore and Wang (*10*) reported that Cd assimilation increased significantly by preexposing the green mussel *Perna viridis* to Cd, as a result of the increase in Cd body burden and the association of Cd with MTLPs. In the same study, Zn was found in the granules of mussels following preexposure to Zn. In addition to metal binding with metallothionein (MT) and granules, metals may also be bound with insoluble compounds such as sulfide (*17, 18*). The subcellular distribution of metals may thus play a critical role in metal accumulation and detoxification processes, but this aspect has received little attention.

Several studies have examined the influences of different Ag uptake pathways (i.e., water and food) on the bioaccumulation and toxicity of Ag as well as the physiology of aquatic invertebrates (*17, 19, 20*). There is, however, limited understanding of the effects of Ag preexposure on subsequent metal bioaccumulation in marine bivalves. This study attempts to illustrate the effects of both aqueous and dietary Ag preexposure on Ag uptake and loss in the green mussel *Perna viridis*. The use of this species as a biomonitor and model organism for bioenergetic-based kinetic modeling study in the subtropical and tropical waters has been well established (*21, 22*). Following exposure of mussels to Ag through either the dissolved or food phase, we then measured the assimilation efficiency from the particulate phase, the clearance rate, the influx rate from the dissolved phase, the efflux rate constant, and the subcellular Ag distribution in the mussels.

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10.1021/es025929t CCC: $25.00
Published on Web 01/29/2003

TABLE 1. Ag Concentrations Used in Different Preexposure Experiments with the Green Mussel *Perna viridis*

expt	preexposure (weeks)	preexposure treatment (μg L^{-1}) dissolved	food[a]
1	2	0.5, 5	0.5, 5
2	1	15	20, 100
3	1, 3, 5	0.5, 5	30

[a] Diatom food *Thalassiosira pseudonana* was exposed to these nominal Ag concentrations in the water before being fed to the mussels.

These physiological parameters are essential for determining the metal body burden in the bivalves. These measurements represented somewhat "short-term" (maximum of 5 weeks exposure) physiological responses to elevated Ag exposure.

Materials and Methods

Green mussels *Perna viridis* with 3.0−3.5 cm shell lengths were collected from Wu Kai Sha, Tolo Harbor, Hong Kong, regarded as relatively uncontaminated by Ag (*23*). They were maintained in aerated artificial seawater (Instant Ocean, 30‰) at 23 °C throughout the experimental period. During the acclimation period, the mussels were fed the diatom *Thalassiosira pseudonana* (clone 3H) at a ration 1−2% of tissue dry weight per day.

Metal Preexposure Treatments. Three different experiments were performed by exposing groups of mussels separately to different concentrations of Ag (as $AgNO_3$) through either the aqueous or dietary phase. The exposure treatments are shown in Table 1. In the dissolved exposure treatments, the mussels were exposed to Ag-spiked seawater for 18 h each day and fed the diatom *T. pseudonana* in unspiked seawater for the remaining 6 h. In the food exposure treatments, the animals were maintained in clean seawater and fed Ag-enriched *T. pseudonana* for 4 h each day. These diatoms had been previously exposed to Ag for 24 h (for concentrations, see Table 1) and then filtered from the growth medium, rinsed with clean seawater and resuspended in clean water before being fed to the mussels. There was minimal desorption of diatom Ag into the dissolved phase within the short feeding period (i.e., >90−100% of the Ag remained in the particle phase, see also reference *24*), thus Ag accumulated in the mussels in these treatments was considered to be derived from the dietary phase. In the control treatments, no Ag was spiked into the seawater or the growth medium of the diatoms. Seawater for all groups was changed every 2 days to keep the nominal metal concentrations relatively constant and the water quality in good condition. In expt 1 and expt 2, the metal assimilation efficiency from the ingested food, the clearance rate, the metal influx rate from the dissolved phase, the efflux rate, the metal subcellular distribution, and the Ag soft tissue concentrations were determined following the preexposure. In expt 3 these measurements (except the efflux rate and metal subcellular distribution) were made after exposing the mussels for 1, 3, and 5 weeks.

Metal Assimilation Efficiency from Diatoms. Metal assimilation efficiency (AE) was determined as the percentage of initial radioactivity retained in the mussels after 60 h of depuration, using an established pulse−chase feeding technique (*25*). The diatom *T. pseudonana* was radiolabeled with the radioisotope ^{110m}Ag for 24 h at a radioactivity addition of 185 kBq L^{-1}. These radiolabeled cells were collected onto a 3-μm polycarbonate membrane and resuspended in 0.22 μm filtered seawater before being added to the feeding beakers.

Mussels from each treatment (including the control) were placed in individual polypropylene beakers containing 500 mL of glass fiber filtered (GF/C) seawater. When the mussels opened and pumped normally, the radiolabeled diatom cells were added to result in a cell density of 4−5 × 10^4 cells mL^{-1}. Further additions were made at 10-min intervals to maintain this density. After 30 min of feeding, the mussels were rinsed with clean seawater and radioassayed. For each treatment, five mussels were then placed into individual polypropylene beakers (180 mL of seawater) held within a 10-L enclosed recirculating flow-through aerated seawater aquarium. Nonradioactive *T. pseudonana* were fed twice daily at a ration of about 2% dry weight per day. The radioactivity retained in the mussels was measured over a 72-h depuration period at intervals from 3 to 12 h. To minimize the possibility that radiotracers may have desorbed from the fecal materials into the ambient water and subsequently be accumulated by the mussels, the pellets were collected frequently during the depuration period.

Clearance Rate of the Mussels. This experiment quantified the influence of Ag body burden on the physiological pumping rate of the mussels. Eight mussels from each treatment were placed individually into 1.5 L of GF/C seawater within a polypropylene beaker. After the mussel opened and pumped normally (usually within 10 min), diatoms of *T. pseudonana* (filtered from their growth medium) were added to each beaker at a concentration of 10^4 cell mL^{-1}. The algal suspension in each beaker was homogenized by a magnetic stirrer. Immediately after adding the algae, a 10-mL water sample was taken and the cell density was counted using a Coulter Counter. Further water samples were taken at 20 and 40 min and the cell density was determined. The clearance rate was calculated using the following equation (*26*)

$$\mathrm{CR} = \mathrm{Vol} \times [\ln(C_1) - \ln(C_2)]/t \qquad (1)$$

where CR is the clearance rate of the mussels (L h^{-1}), C_1 is the cell density (cell mL^{-1}) at time 1, C_2 is the cell density (cell mL^{-1}) at time 2, t is the time interval of measurement ($t_2 - t_1$, in h), and Vol is the volume of water (L). The clearance rate of each individual was finally calculated from the mean of the two consecutive measurements at 20-min intervals.

Trace Metal Influx Rate from the Dissolved Phase. The radiotracer ^{110m}Ag (4.6 kBq L^{-1}, equivalent to 0.4 nM) was spiked into 0. 22 μm of filtered seawater and allowed to equilibrate overnight (*12*). Following this addition, μL amounts of 0.5 N Suprapure NaOH were added to the seawater to maintain the pH. Short-term exposure (1 h) was employed in this study to determine Ag influx rate from the dissolved phase in order to avoid the decline of mussel's ventilating activity due to the absence of food particles, as well as the decrease in Ag ambient concentration. Eight replicated individuals from each treatment were placed individually in 200 mL of seawater for 1 h, after which the mussels were dissected and the radioactivity of the soft tissues was measured. The tissues were then dried at 80 °C overnight and the dry weights were determined. The influx rate was calculated as the amount of metal accumulated by the soft tissue of the mussel and was standardized as per nanogram dry weight per hour (ng g^{-1} h^{-1}). During the 1-h exposure period, any feces egested by the mussels were removed immediately to minimize fecal scavenging of the metal.

Metal Efflux from Mussels. The mussels were radiolabeled with ^{110m}Ag (with the addition of stable Ag to maintain different nominal Ag concentrations) during their preexposure to either the dissolved or the food phase (Table 1). In the control treatments (two controls in expt 1, one for the dissolved exposure, the other for the food exposure), only the radioisotope was used to label the diatom or seawater. Because of the lack of significant efflux difference in the food and dissolved control groups in expt 1 (see results), only one control (for both the dissolved exposure and food exposure)

TABLE 2. Soft Tissue Ag Concentration[a], Ag Assimilation Efficiency (AE)[b], Clearance Rate (CR)[c], Ag Influx Rates, and Ag Efflux Rate Constants in the Green Mussel *Perna viridis* Following Different Preexposure Treatments

expt	preexposure (weeks)	treatment[f] (μg L^{-1})	Ag (μg g^{-1})	AE (%)	CR (L g^{-1} h^{-1})	influx rate (ng g^{-1} h^{-1})	efflux rate constant (d^{-1})
1	2	control	0.479 ± 0.124	21.1 ± 6.4	10.3 ± 2.3	49.6 ± 5.9	0.016 ± 0.005[g] 0.021 ± 0.009[h]
		D0.5	1.46 ± 0.260	29.2 ± 5.9	10.9 ± 2.1	42.6 ± 10.6	0.013 ± 0.006
		D5	2.34 ± 1.60	43.4 ± 10.9	8.4 ± 2.4	33.8 ± 3.8	0.002 ± 0.008
		F0.5	0.857 ± 0.348	25.0 ± 5.1	10.8 ± 1.7	53.0 ± 10.8	0.027 ± 0.013
		F5	0.963 ± 0.199	25.4 ± 8.7	8.0 ± 1.7	52.3 ± 13.2	0.015 ± 0.011
2	1	control	0.581 ± 0.089	18.7 ± 4.3	9.1 ± 1.6	7.1 ± 3.0	0.011 ± 0.004
		D15	3.43 ± 0.199	37.7 ± 5.8	12.6 ± 1.0	6.5 ± 1.6	0.002 ± 0.004
		F20	1.42 ± 0.615	16.3 ± 5.8	8.4 ± 1.8	6.5 ± 1.7	0.011 ± 0.007
		F100	2.63 ± 0.526	20.8 ± 4.6	11.5 ± 2.7	5.6 ± 1.2	0.006 ± 0.008
3	1	control	0.311 ± 0.150	14.5 ± 6.5	10.6 ± 1.9	9.7 ± 3.3	nd[i]
		D0.5	0.651 ± 0.199	18.7 ± 6.5	7.6 ± 2.3	9.4 ± 4.1	nd
		D5	3.14 ± 1.11	24.0 ± 5.2	6.9 ± 1.7	7.6 ± 2.2	nd
		F30	2.26 ± 0.475	15.8 ± 4.5	2.5 ± 1.0	7.5 ± 1.8	nd
	3	control	0.311 ± 0.150	12.4 ± 2.4	17.6 ± 3.0	9.8 ± 2.9	nd
		D0.5	1.13 ± 0.361	14.4 ± 1.4	16.0 ± 1.7	8.6 ± 2.3	nd
		D5	7.46 ± 2.53	28.4 ± 6.2	14.7 ± 3.3	10.1 ± 2.5	nd
		F30	8.12 ± 2.07	24.2 ± 7.1	13.0 ± 3.1	11.7 ± 4.4	nd
	5	control	0.311 ± 0.150	13.4 ± 3.2	18.1 ± 6.4	9.7 ± 1.7	nd
		D0.5	3.92 ± 1.88	29.0 ± 8.9	16.6 ± 4.3	9.0 ± 2.4	nd
		D5	17.2 ± 3.90	39.9 ± 8.7	17.4 ± 5.7	14.0 ± 4.5	nd
		F30	19.3 ± 6.33	25.9 ± 1.4	17.4 ± 5.4	15.3 ± 2.1	nd

[a,b] Data are mean ± SD (n = 5). [c,d,e] Data are mean ± SD (n = 8). [f] D, dissolved exposure; F, food exposure. [g] Control for dissolved exposure. [h] Control for food exposure. [i] nd, not determined.

was used in expt 2. In expt 1, the radioactive addition was 0.74 kBq L^{-1} ^{110m}Ag for both the dissolved and food exposures; in expt 2, the radioactivity addition was 1.48 kBq L^{-1} ^{110m}Ag. Following the exposure, 10 mussels were removed and rinsed with clean seawater, and their radioactivity was counted. They were then placed individually into 180-mL polypropylene beakers within an enclosed recirculating seawater aquarium, as described above, to depurate ^{110m}Ag. The aquarium water was changed twice weekly. Nonradioactive *T. pseudonana* was fed twice daily at a ration of about 2% dry weight per day. Throughout the course of depuration, the radioactivity of each mussel was assayed periodically, and fecal pellets were collected frequently to minimize the desorption of radiotracers from the fecal materials into the water. On day 0 and 16 and the last day of depuration, 3 individuals from each treatment were dissected and the radioactivity in the shell, digestive gland, and other soft tissues was counted.

Stable Ag Tissue Concentration. Following the preexposure, five mussels from each treatment were dissected, and their soft tissues were dried at 60 °C to a constant weight and then digested in concentrated nitric acid (HNO_3, Aristar grade BDH Ltd.). These digests were diluted with Nanopure purified water to make the Ag concentration in an appropriate range for analysis by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) (Perkin-Elmer, Elan 6000). The stable Ag tissue concentration was expressed as μg g^{-1} dry weight.

Subcellular Ag Distribution. Differences in the subcellular Ag distribution among different treatments after the exposure of mussels to stable metal and radioisotope were investigated by homogenizing the soft tissues of the mussels and subjecting the homogenate to differential centrifugation and tissue digestion procedures, using a modified method of Wallace et al. (*27*).

Five mussels from each treatment were dissected and the soft tissues were homogenized in 6 mL of Nanopure purified water. The homogenate was centrifuged at 1450*g* (15 min at 4 °C) and the supernatants were spun at 100 000*g* (1 h at 4 °C) to separate the cytosol and proteins from the intracellular pellets containing nuclear, mitochondrial, and microsomal fractions. The 100 000*g* supernatants containing cytosol and protein were further centrifuged at 50 000*g* (10 min at 4 °C) after being heated (10 min at 80 °C) and further ice-cooled (1 h). This separated the heat-stable proteins or metallothionein-like proteins (MTLPs), which remained in the supernatant, from the heat-sensitive proteins (HSPs) that were denatured by the heat treatment and thus formed the pellet. The 1450*g* pellet contained tissue fragments and other cellular debris (i.e., membranes and metal-rich granules − MRG). MRG was isolated from other cellular debris by resuspending the pellet in 1 mL of Nanopure water, heating at 100 °C for 2 min, and subsequently adding an equal volume of 1 N NaOH, followed by heating at 70 °C for 1 h. The differential centrifugation as well as above treatments resulted in 2 supernatants (i.e., MTLPs and cellular debris) and 3 pellets (i.e., organelles, HSPs, and MRG). All fractions were radioassayed for ^{110m}Ag to elucidate the subcellular Ag distribution.

Radioactivity Measurements and Statistical Analysis. Radioactivity was measured using a Wallac 1480 NaI (T1) gamma detector (Wallac Turku, Finland). All counts were related to standards and radioactive decay was corrected. The gamma emission of ^{110m}Ag was determined at 658 keV. Counting times were adjusted to yield a propagated counting error <5%. Statistical analysis was carried out through analysis of variance (ANOVA) or *t* test. Statistical significant difference was accepted at $p < 0.05$.

Results

Stable Ag Body Concentration. In expt 1, with one exception (food 0.5 μg L^{-1} treatment), the Ag tissue concentrations of all exposed mussels (0.963−2.34 μg g^{-1}) were significantly higher ($p < 0.05$) than those of the controls (0.479 μg g^{-1}) after 14 d exposure (Table 2). A similar trend was observed in expt 2 that body Ag concentrations in exposed mussels (1.42−3.43 μg g^{-1}) increased as compared to those of the controls (0.581 μg g^{-1}) (Table 2). In expt 3, Ag soft tissue concentrations in the mussels from each exposed treatment increased significantly ($p < 0.01$) with increasing exposure time (Table 2). In the 0.5 μg L^{-1} dissolved exposure treatment, soft tissue concentration increased from 0.651 μg g^{-1} in week

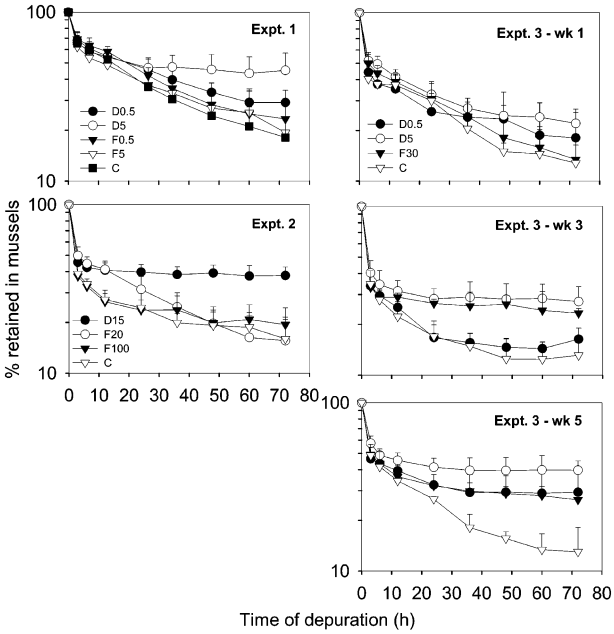

FIGURE 1. Retention of Ag in the green mussel *Perna viridis* preexposed to Ag in the dissolved or food phase for different durations following a pulse ingestion of the radiolabeled diatom *Thalassiosira pseudonana*. Mean + SD (n = 5). D, dissolved exposure; F, food exposure; C, control. Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 1).

1 to 1.13 μg g^{-1} in week 3, and to 3.92 μg g^{-1} in week 5. In the 5 μg L^{-1} dissolved exposure treatment, the soft tissue concentrations in weeks 1, 3, and 5 were 3.14, 7.46, and 17.2 μg g^{-1}, respectively. Following 30 μg L^{-1} food exposure, the Ag body concentrations of the three time points were 2.26, 8.12, and 19.3 μg g^{-1}, respectively. The increase of Ag tissue concentration was the greatest (62×) following the food exposure to 30 μg L^{-1} for 5 weeks.

Ag Assimilation from Ingested Diatoms. Depuration of Ag in the mussels following pulse−chase radioactive feeding is shown in Figure 1. Assimilation efficiencies (AEs) were calculated as the amount of radioactivity left in the mussels at 60 h divided by the amount of radioactivity ingested, as measured following the 30-min pulse radioactive feeding (Table 2). In expt 1, the Ag AEs were 2.1× greater (p < 0.01) in those individuals that had been exposed to dissolved 5 μg L^{-1} for 14 d as compared with those in the controls and other treatments. There was essentially no loss of Ag from the mussel body after 1 d of depuration in this treatment. In expt 2, following 7 d exposure, only the mussels from the 15 μg L^{-1} dissolved exposure treatment assimilated Ag at a significantly higher level (p < 0.001) than the controls. Similarly, there was no apparent loss of Ag after the initial rapid loss (<6 h). In expt 3, the Ag assimilation was gradually affected by increasing the time of exposure. After 1 week of exposure, no major difference (p > 0.05) of Ag AEs between the exposure treatments and the control was observed. Following 2 more weeks of exposure, the AEs in the dissolved 5 μg L^{-1} and the food 30 μg L^{-1} treatments increased significantly (p < 0.01) compared with those of the controls. After another two weeks of exposure, the mussels from all the exposure treatments had significantly higher (p < 0.01) AEs than the control. Generally, the depuration pattern of Ag following a pulse of radioactive feeding was characterized by a rapid initial egestion within the first 12 h, followed by a continuous, but slower, egestion during 24 to 72 h. Mussels that exhibited significantly higher AEs than the controls retained the Ag very efficiently after the initial stage of digestion. The much slower metal egestion replaced the second phase of continuous egestion as found in the control treatments, thereby resulting in the increase of Ag assimilation.

Clearance Rate of Mussels. Following exposure to Ag for 14 d (expt 1), only those mussels from the 5 μg L^{-1} food exposure treatment exhibited a significantly lower clearance rate than the control (p < 0.05) (Table 2). In expt 2, the clearance rates in mussels exposed to dissolved 15 μg L^{-1} and food 100 μg L^{-1} were 26−38% higher compared to those of the controls but these differences were not statistically significant (p > 0.05). In expt 3, after 1 week of exposure, only mussels in the food 30 μg L^{-1} treatment had a significant (p < 0.01) reduction in the clearance rate compared with the controls. Following 3 weeks of exposure, the clearance rates of the exposed mussels were 9−26% lower than the controls, but the differences were not significant (p > 0.05). Additionally, following 2 more weeks of exposure, no significant difference between the exposed mussels and the controls was observed (p > 0.05). Furthermore, with increasing the exposure time (from week 1 to week 5), the clearance rates in the preexposed and control mussels increased gradually and the clearance rates in the preexposed individuals approached those of the controls.

Ag Uptake from the Dissolved Phase. The influx rate of Ag from the dissolved phase in the mussels in the three different experiments is shown in Table 2. In expt 1, the Ag influx into those individuals exposed to dissolved Ag for 14 d was 14−32% lower (33.8−42.6 ng g^{-1} h^{-1}) than that of the controls (49.6 ng g^{-1} h^{-1}). This decrease was significant (p < 0.001) only for the group that had been exposed to dissolved 5 μg Ag L^{-1}. The influx of Ag into the mussels was similar between the food exposure treatment (52.3−53.0 ng g^{-1} h^{-1}) and the controls. After 7 d preexposure (expt 2), the influx rates in the mussels preexposed to dissolved 15 μg L^{-1}, food 20 μg L^{-1}, and food 100 μg L^{-1} were 6.50, 6.46, and 5.58 ng g^{-1} h^{-1}, respectively, and were decreased by 8−21% compared with the control (7.08 ng g^{-1} h^{-1}). In expt 3, the influx rates following 1 week preexposure (7.53−9.35 ng g^{-1} h^{-1}) were somewhat lower than that of the control (9.72 ng g^{-1} h^{-1}) but the difference was not statistically significant (p > 0.05). After 2 more weeks of preexposure, although there was still no significant difference (p > 0.05) between all the preexposed mussels and the control, the influx rates (10.1 and 11.7 ng g^{-1} h^{-1}, respectively) in the mussels from dissolved 5 μg L^{-1} and food 30 μg L^{-1} groups were 3−20% higher than that of the control (9.76 ng g^{-1} h^{-1}). By the end of the preexposure (week 5), the influx rates (14.0 and 15.3 ng g^{-1} h^{-1}, respectively) in these two treatments increased significantly (p < 0.01) as compared with the control (9.68 ng g^{-1} h^{-1}). In contrast, the influx rate in those mussels exposed to 0.5 μg L^{-1} (8.97 ng g^{-1} h^{-1}) was lower than that of the control treatment.

Ag Efflux Rate from Mussels. In expts 1 and 2, the depuration of Ag in the mussels following exposure to both radioisotope and stable metal is shown in Figure 2. The depuration pattern was characterized by an initial rapid loss of Ag during the first 5−6 d followed by a second slower loss for the remaining period (6 d onward). The efflux rate constants, calculated from the slope of the natural log of the percentage of Ag retained in the mussels and the time of depuration (between 6 and 24 d), are shown in Table 2. In expt 1, the Ag efflux rate constant (0.002 d^{-1}) was significantly lower (p < 0.001) following 14 d exposure to dissolved 5 μg L^{-1} than that of the control (0.016 d^{-1}). The efflux rate constant (0.013 d^{-1}) of the mussels exposed to dissolved 0.5 μg L^{-1} was not significantly different (p > 0.05) from that of the control. Compared with the control (0.021 d^{-1}), the two food exposure treatments (0.027 and 0.015 d^{-1}, respectively) were not significantly different (p > 0.05). In expt 2, the efflux rate constants of the mussels exposed to dissolved 15 μg L^{-1} (0.002 d^{-1}) and food 100 μg L^{-1} (0.006 d^{-1}) for 7 d were significantly lower (p < 0.001) than that of the control (0.011 d^{-1}), which

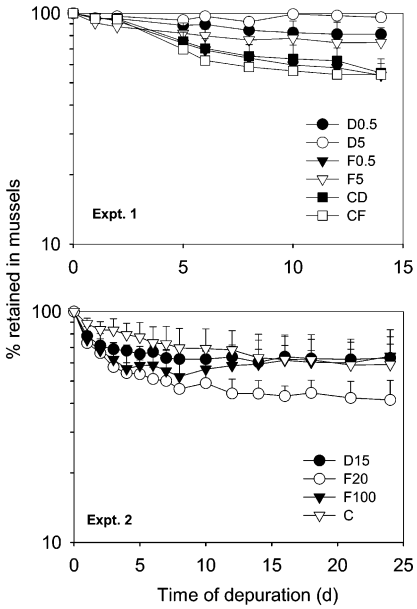

FIGURE 2. Retention of Ag in the green mussel *Perna viridis* preexposed to Ag in the dissolved or food phase for different durations. Mean + SD (n = 8). D, dissolved exposure; F, food exposure; C, control; CD, control after dissolved exposure; CF, control after food exposure. Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 1).

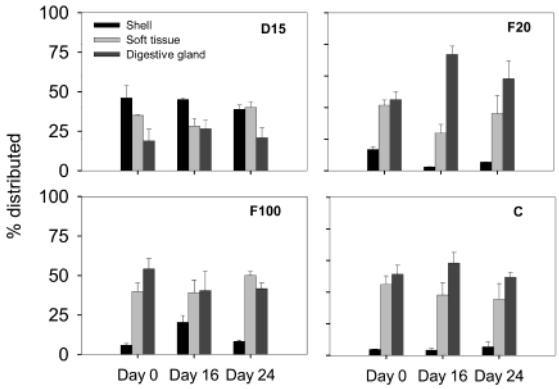

FIGURE 3. Ag distribution in the digestive gland, other soft tissues, and shell of the green mussel *Perna viridis* following a 7-d Ag preexposure (day 0), after 16 d depuration (day 16), and at the end of the depuration (day 24). Mean + SD (n = 3). D15, mussels preexposed to nominal Ag concentration of 15 μg L^{-1}; F20, mussels preexposed to dietary Ag (diatoms loaded at 20 μg L^{-1}); F100, mussels preexposed to dietary Ag (diatoms loaded at 100 μg L^{-1}); C, control.

was not different from that of those individuals exposed to food 20 μg L^{-1} ($p > 0.05$).

In expt 2, the distribution of Ag in the shells (including both surface sorption and incorporation), soft tissues, and digestive glands during the depuration period is shown in Figure 3. The control mussels contained negligible amounts of Ag in the shells (3−5%), and the majority of Ag was distributed in the soft tissues (35−45%) and digestive gland (>49%). In general, following dissolved Ag exposure, a larger fraction (38−46%) of Ag was found in the shell, while most (40−73%) of the Ag was distributed within the digestive gland of the mussels exposed to dietary Ag. The Ag distribution in each part of the mussels throughout the depuration period did not vary greatly.

Subcellular Ag Distribution After Preexposure. Subcellular Ag distributions in the exposed and control mussels of expts 1 and 2 are shown in Figure 4. The control mussels in the two experiments had similar Ag distributions, with about

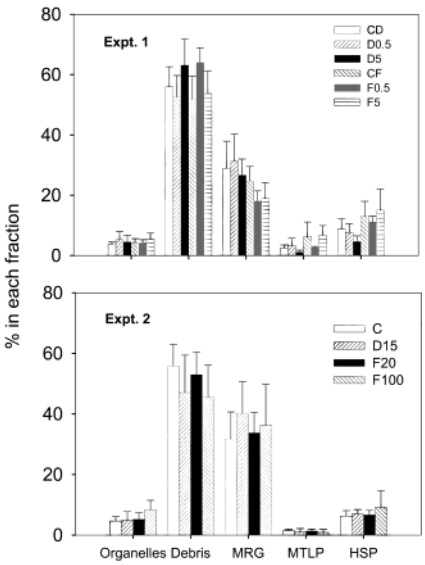

FIGURE 4. Subcellular distribution (%) of Ag in the green mussel *Perna viridis* preexposed to Ag in the dissolved or food phase for different durations. Subcellular fractions (organelles, debris, metal-rich granules (MRG), metallothionein-like protein (MTLP), and heat sensitive protein (HSP)) were obtained through homogenization, differential centrifugation, heat treatment, and tissue digestion procedures. Mean + SD (n = 5). D, dissolved exposure; F, food exposure; C, control; CD, control after dissolved exposure; CF, control after food exposure. Numbers in the symbol legends are the nominal Ag concentrations used in the exposure treatments (see Table 1).

25−32% being distributed in the metal-rich granule fraction (MRG), 52−56% in the other cellular debris (tissue fraction), <5% in the intracellular fraction (organelles), and about 8−19% in the cytosol (100 000g supernatants). For all the exposed mussels in both experiments, most of the Ag was associated with the debris (45−64%) and MRG (18−40%) fractions. When compared with the controls, however, there was no consistent pattern of subcellular Ag distribution in the exposed mussels in two experiments. Ag distribution in the metallothionein-like protein (MTLP) fraction either increased (i.e., dissolved 0.5 μg L^{-1} and food 5 μg L^{-1} groups in expt 1) or decreased (i.e., dissolved 5 μg L^{-1}, food 0.5 μg L^{-1}, and all exposed groups in expt 2) following preexposure, but the difference was not significant ($p > 0.05$). A similar pattern was also observed in the Ag distribution in the MRG, the debris, as well as the heat-sensitive proteins (HSP) and the MTLP when comparing the exposed mussels with the controls.

Discussion

To investigate the effects of elevated body Ag concentrations on subsequent Ag uptake and loss in the mussels, the Ag levels used in the preexposure were much higher than the typical Ag concentrations in coastal seawater. This is to ensure the elevation of Ag body burden in the mussels within a relatively short period (maximum of 5 weeks of exposure). The body Ag concentrations achieved from such preexposure were, however, environmentally realistic. For example, body Ag concentrations in the exposed treatments were lower than the highest concentrations (about 70 μg g^{-1} dry weight) observed in the mussel *Mytilus californianus* (*28*). Moreover, Luoma and Phillips (*29*) reported that Ag concentrations in bivalves living in contaminated environments exceeded 100 μg g^{-1} dry weight, which are much greater than the highest body concentrations resulting from preexposure in our study. It should also be noted that the body burdens of Ag in the mussel tissues were achieved after 1−5 weeks exposure, and it is possible that the Ag biochemical fates in the mussels

may have been different from those found in field-collected individuals that have been exposed to Ag throughout their lifetime. Extrapolation of the laboratory results to the field situation remains to be further tested.

In our study, there were some differences in the measured physiological parameters among the three different experiments in the controlled treatment, especially for expt 1 which had a much higher dissolved uptake rate as compared to the other two experiments. The AE and the clearance rate of the mussels were, however, comparable among the three different experiments. Reason for the abnormally high Ag influx rate from the dissolved phase is unknown, but may be due to the seasonal variation of protein metabolism in the mussels, as the three experiments were conducted at different seasons. In expt 3 which encompassed 5 weeks exposure, however, the quantified Ag AE, influx rate, and clearance rate of the mussels were remarkably comparable in the control treatment following 1, 3, and 5 weeks preexposure, suggesting that these physiological processes were maintained rather constant within this period of exposure. Furthermore, we included a control treatment in each experiment to contrast the biokinetics of Ag in the mussels preexposed to Ag. Any difference from the control treatment within each single experiment was therefore due to the Ag effect.

The present work demonstrated that no significant increase in Ag associated with the MTLP fraction was achieved following exposure to the metal through either the dissolved or food phase in the mussels. Preliminary experiments showed that there was also no significant difference in the metallothionein concentrations in mussels exposed to these concentrations of Ag (Shi et al., unpublished data). This suggests that Ag elevation in the soft tissues following preexposure is likely due to the metal binding with other ligands instead of metalloprotein. Indeed, up to 80% of Ag in marine bivalves may be bound with sulfides while only a small part may be associated with the protein (*24*). Whether the Ag−sulfide complex is associated with any the subcellular fractions (e.g., MRG) remained unknown in this study. Considering the extremely high toxicity of Ag (*11, 13*), the accumulation of Ag mainly as an insoluble sulfide complex, which is very stable, may be regarded as a detoxification mechanism in the mussels. Such strong binding may also have implication for the potential trophic transfer of Ag to higher trophic levels, since earlier experimental studies indicated that metals bound strongly with metal-rich granules may have a lower bioavailability to higher animals (*27*). In agreement, by exposing the mussel *Mytilus edulis* to dissolved 25 μg Ag L^{-1} for 1 year, George et al. (*18*) found that most of the Ag was associated with sulfide and stored along the basement membranes of the digestive gland and kidney.

Data presented herein show that Ag AE generally increased following preexposure to Ag. In expt 3, the AE in each exposed treatment increased with increasing preexposure period and the body Ag concentrations. A possible physiological mechanism underlying the increase of Ag AE may be described as the induction of sulfide complexes in the mussel. As a consequence, compared with the unaffected mussels, the exposed individuals may have more sulfide ligand for Ag binding. Meanwhile, more Ag binding complexes may have been induced when more Ag was accumulated, enabling mussels to further assimilate more Ag from the particulate source. In agreement, Calabrese et al. (*30*) reported that the amount of yellowish-brown to black particulates in the basement membrane and connective tissue of the body organs in the blue mussel *Mytilus edulis* (e.g., digestive diverticula and kidney) increased with an increase in Ag exposure concentration. In a recent study, Blackmore and Wang (*10*) found a similar phenomenon that, due to the sequestration of Cd by MTLPs, Cd preexposure leads to an increase of its AE in the green mussels. These studies thus highlight the complicated mechanisms in metal sequestration that may affect metal subsequent assimilation from the dietary phase.

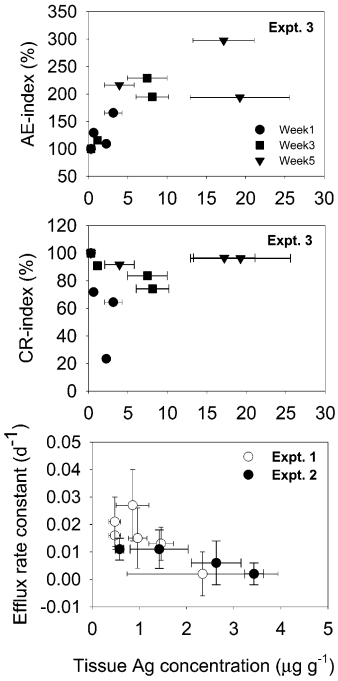

FIGURE 5. Relationships between Ag soft tissue concentrations and Ag assimilation efficiency index, clearance rate (CR) index, and Ag efflux rate constant following Ag preexposure. The index was calculated as the percentage of their respective control value in each treatment. Mean ± SD (n = 5−8).

With a comparable Ag body concentration obtained from different exposure pathways (i.e., dissolved or dietary phase), the effect of different Ag sources on Ag AE can be compared (Figure 5). For example, following the 3 week preexposure (expt 3), the body Ag concentrations in the mussels exposed to the dissolved 5 μg L^{-1} (7.46 μg g^{-1}) were similar to those in the individuals exposed to the food 30 μg L^{-1} (8.12 μg g^{-1}). Concomitantly, there was no significant difference between the Ag AEs of these two treatments (28% and 24%, respectively). This result suggests that the level of accumulated Ag is important rather than the source and, therefore, both the dissolved and food pathways play a role on Ag bioavailability to the mussels.

Clearance rates have been utilized as good indicators reflecting the toxic effects of pollutants in organisms (*31*). For example, Krishnakumar et al. (*32*) found that after a 2-week dissolved exposure to 25 μg L^{-1} Cu and Hg, the clearance rates decreased significantly. In the zebra mussel *Dreissena polymorpha*, the clearance rates were affected by exposure to high concentrations of Pb or Zn (85 and 382 μg L^{-1}, respectively), but not by lower metal levels (*33*). In that study (*33*), a different pattern of variance of the clearance rate with an increase in the metal body concentration was found between Pb and Zn. Our study shows that, in expt 1 and 2, except for the 5 μg L^{-1} (14 d) food exposure treatment, the clearance rates were not significantly affected by Ag preexposure as compared with the control, suggesting that the Ag preexposure treatments had little toxic effect on the mussels. It remains possible, however, that the concentration of the metal accumulated during the preexposure period was not sufficiently high to affect the clearance rate. This was further investigated in expt 3, in which the clearance rate was measured with increasing Ag body concentrations. The results show that although the clearance rates of

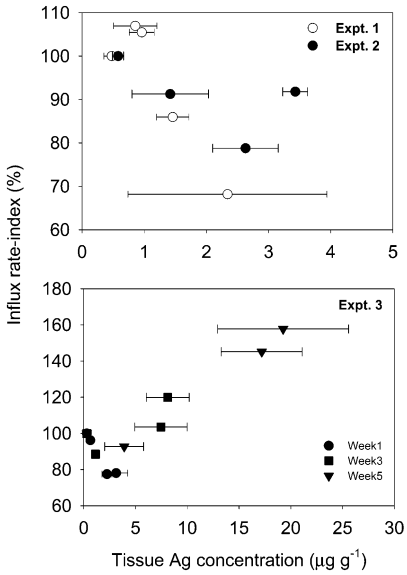

FIGURE 6. Relationship between Ag soft tissue concentration and Ag influx rate index from the dissolved phase following Ag preexposure. The index was calculated as the percentage of their respective control value in each treatment. Mean ± SD ($n = 5$).

preexposed mussels (except for food 30 μg L^{-1} in week 1) were lower (not significant) than those of the controls, which might indicate some toxic effects of Ag preexposure, there was no significant relationship between the increased Ag accumulation in the soft tissues and the clearance rate. Moreover, the clearance rates of all mussels (both preexposed and the control) increased gradually over the experimental period. This phenomenon may be a reflection of the acclimatization of mussels to the laboratory conditions, and furthermore suggests that over the concentrations measured in the present study, Ag body burdens have little effect on the clearance rate.

Our results indicate that the relationship between Ag preexposure and dissolved uptake rate was rather complex (Figure 6). With increasing body Ag concentrations following preexposure, the influx rate either decreased or increased. In three independent experiments, the influx rate decreased with increasing body Ag concentration until it reached around 2.5 μg g^{-1}, above which this pattern was then reversed and the influx rate began to increase. It may be likely that the binding ligand such as sulfides had not yet been induced when the body Ag concentration was at a low level (<2.5 μg g^{-1}), and the decrease in the influx rate may reflect a protection mechanism for the ambient toxic metal. With a continual accumulation of Ag to a high soft tissue concentration (>2.5 μg g^{-1}), Ag complexing ligands may have been induced, and the uptake may therefore increase because of the strong affinity of Ag with these complexing ligands (*24*). Thus, mussels with higher body Ag concentrations may have more potential sulfide binding sites, resulting in a higher efficiency of Ag uptake from the dissolved phase. This is also consistent with our results of AE (see above).

The reduction of the Ag influx rate agreed with the phenomenon observed in the clam *Macoma balthica* by Boisson et al. (*8*). Clams subjected to chronic Ag contamination accumulated Ag at a significantly lower rate than those originating from a clean estuary. In the mussel *P. viridis*, the Cd and Zn dissolved uptake reduced following Zn preexposure, although this pattern was not achieved following Cd preexposure (*10*). Rainbow et al. (*34*), however, reported that the mean metal uptake rates of amphipods and crabs from a metal-enriched site were not lower than those of the same crustaceans from a control site. Further study is therefore needed to fully understand the influence of Ag preexposure and resulting body burden on the dissolved uptake rate and to better illustrate the function of sulfides in this process.

It should be noted that we preexposed the mussels to different Ag concentrations for a maximum of 5 weeks, and thus our measurements represented relatively short-term physiological responses to Ag exposure (e.g., physiological acclimatization) and the influences of different Ag tissue body burdens on the biokinetics of Ag in the mussels. In the natural environments, the bivalve populations have been exposed to different metal levels throughout their lifetimes, and it is likely that they may have developed adaptations to metal-enriched environments, as shown by Boisson et al. (*8*). Thus, it is possible that the metal accumulation in the metal-enriched environments may be due to the selective forces where offsprings tolerant of a metal-enriched environment have been selected for, resulting in a metal-tolerant population. In a recent study, we compared the Cd biokinetics in mussels and clams following physiological acclimatization (e.g., 5 weeks exposure) and from a Cd contaminated bay. We found that the physiological responses following 5 weeks preexposure were consistent with those collected from the contaminated bay where the bivalves were exposed to Cd throughout their lifetimes (Shi and Wang, unpublished data).

Following preexposure, the efflux rate constants of Ag decreased with increasing body Ag concentrations. This effect can be similarly explained by the binding of Ag with sulfides induced by the chronic Ag preexposure. Such complexes are very stable and not easily depurated by the mussels, thus leading to a smaller efflux rate constant in those individuals with higher body Ag concentration. Our studies were also designed to compare the efflux rate constants in mussels preexposed to Ag through different routes. Generally, Ag efflux was slower in the mussels preexposed to dissolved Ag than in those preexposed to dietary Ag (e.g., expt 1). Furthermore, the results show that Ag uptake from seawater (the dissolved phase) is mainly distributed in shells, whereas following the food exposure Ag is found mostly in the digestive glands. These results agree well with results from the mussel *Mytilus galloprovincialis* (*35*). In contrast, Berthet et al. (*17*) found that the oyster *Crassostrea gigas* lost Ag accumulated from food slower than from the dissolved phase.

This study shows that Ag preexposure substantially affects its bioavailability to the green mussel *P. viridis*. Additionally, in contrast to Cd accumulation in the mussel *Mytilus edulis* (*36*) and Ag accumulation in the oyster *Crassostrea gigas* (*16, 17, 19*), the present study indicates that Ag uptake from the dietary phase can be important to its bioaccumulation in the mussel *P. viridis* and thus contribute to bioavailability. Following chronic exposure to Ag, mussels increased AE and the dissolved uptake rate (when the metal body burden was above certain level, see above) and decreased the efflux rate, resulting in accumulating more Ag as compared with the control individuals. One point to highlight is that Ag−sulfide complexes may play a significant role in the mechanism of Ag uptake and loss by the mussels. These results indicate that the body Ag concentration may not simply reflect the real bioavailable metal level in the environment.

Mussels from Ag-enriched environments may have somewhat higher Ag body burden than anticipated due to the disproportionally higher uptake from both the aqueous and dietary pathways. In this case, the physiological process of acclimatization to ambient conditions appears to be of importance when interpreting Ag body concentrations in mussels used as biomonitors for metal pollution. Further study is, therefore, needed to investigate the detailed process of intracellular metal detoxification in order to better understand the acclimatization of the marine mussels to chronic exposure to Ag from different pathways.

Acknowledgments

We thank the three anonymous reviewers for their very constructive and helpful comments on this work. Support by a Competitive Earmarked Research Grant from the Hong Kong Research Grants Council (HKUST6097/02M) to W.-X.W. is gratefully acknowledged.

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Received for review July 1, 2002. Revised manuscript received December 6, 2002. Accepted December 18, 2002.

ES025929T