

Mechanism of acute silver toxicity in marine invertebrates

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Abstract

In freshwater crustaceans and in both freshwater and marine fish, the key mechanism of acute silver toxicity involves ionoregulatory impairment. An inhibition of the Na^+, K^+ -ATPase located at the basolateral membrane of the gill epithelium seems to be the key site for silver toxicity. However, studies to determine if the same mechanism of toxicity is occurring in marine invertebrates, which also are ionoregulators, had not been done. Thus, the present study was carried out to determine acute silver effects on hemolymph osmo- and ionoregulation in three marine invertebrates: the shrimp *Penaeus duorarum*, the sea hare *Aplysia californica*, and the sea urchin *Diadema antillarum*. Animals were exposed to silver (1 or 10 $\mu\text{g/L}$), as silver nitrate, in seawater for 48 h. Results show that acute silver exposure did not affect hemolymph osmolality or ion concentration (Na^+ , Cl^- , K^+ , Ca^{2+} and Mg^{2+}) in the three species studied. However, silver induced significant changes in the water content in shrimp gill and sea hare gill and hepatopancreas. Silver also caused significant changes in Na^+, K^+ -ATPase activity and in both total and intracellular ion (Cl^- , Na^+ , K^+ , Mg^{2+} , and Ca^{2+}) concentrations in different tissues of the three species studied. Overall, these results show that the key mechanism of acute silver toxicity in marine invertebrates is not associated with an osmotic or ionoregulatory impairment at the hemolymph level, as observed in freshwater fish and crustaceans and in seawater fish. However, they indicate that acute waterborne silver induces significant changes in Na^+, K^+ -ATPase activity and probably affects other mechanisms involved in water and ion transport at the cell membrane level, inducing impairments in water and ion regulation at the cellular level in different tissues of marine invertebrates. These results indicate the need to consider other “toxic sites” than gills in any future extension of the biotic ligand model (BLM) for seawater.

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1. Introduction

Although most silver in surface waters originates from natural leaching, elevated concentrations are usu-

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ally associated with anthropogenic activities such as mining and photographic processing. Several different geochemical or biological modeling approaches have been developed in the last few years in an attempt to predict acute silver toxicity in aquatic invertebrate and vertebrate species (Wood et al., 1999). The “biotic ligand model” (BLM) is a modeling approach to predict silver toxicity to freshwater organisms, which takes into account the geochemistry of a biological ligand, the gill (Janes and Playle, 1995; Paquin et al., 1999, 2002). According to this model, the gill is considered a negatively charged ligand to which Ag^+ can bind. Toxic effects are considered a function of the degree of saturation of “toxic sites” on the biotic ligand by Ag^+ . Current versions of the BLM consider the competition between other cations and Ag^+ for binding sites on the gills as well as the influence of different complexing agents on silver speciation and availability (Paquin et al., 1999; McGeer et al., 2000).

Parallel to the development of the BLM, evidence elucidating the physiological mechanism of acute silver toxicity in freshwater fish and crustaceans has been accumulating. In rainbow trout, the most studied freshwater fish species, the Na^+, K^+ -ATPase located at the basolateral membrane of the gill epithelium seems to be the key site for Ag^+ toxicity (Morgan et al., 1997; McGeer and Wood, 1998). Inhibition of Na^+, K^+ -ATPase induced by silver exposure causes an inhibition of active Na^+ and Cl^- uptake, resulting in a net loss of ions from the animal, with death probably occurring due to an associated cardiovascular collapse (Hogstrand and Wood, 1998). Recently, the same toxic mechanism (ionoregulatory failure associated with the inhibition of branchial Na^+, K^+ -ATPase) has been demonstrated in silver tolerant (crayfish; Grosell et al., 2002a) and silver sensitive freshwater crustaceans (daphnids; Bianchini and Wood, 2003). However, despite the important differences in the concentration of possible silver ligands (Cl^- , SO_4^{2-} , NOM, $\text{S}_2\text{O}_3^{2-}$, sulfide, Br^- , and $\text{B}(\text{OH})_4^-$) or competitors (Na^+ , Mg^{2+} , Ca^{2+} , K^+ , and Sr^{2+}) among freshwater, brackish, and seawater that certainly modify silver toxicity to aquatic animals, most of the information and knowledge on silver toxicity has been restricted to freshwater species (reviewed by Ratte, 1999; Wood et al., 1999). Because the primary site of toxic action for waterborne silver in freshwater fish and crustaceans is the gill, this organ is the correct “target” for the BLM

for freshwater. However, the situation may be very different in seawater because fish in seawater drink to replace water lost by osmosis across the gills. This water is absorbed in the gut, so this tissue also seems to be an important “target” for Ag^+ interactions. In fact, it has been reported that both the gut and the gills are sites of ionoregulatory toxicity, with ~50% of the toxic response being attributable to each site (Grosell and Wood, 2001). Furthermore, both in vivo and in vitro experiments have shown that silver induces ionoregulatory impairment characterized by a gain of ions and dehydration in brackish and marine fish (Grosell et al., 1999; Hogstrand et al., 1999; Webb et al., 2001).

Based on the background described above, it is clear that the effect of silver involves ionoregulatory impairment in freshwater and marine fish, as well as in freshwater crustaceans (Wood et al., 1999; Grosell et al., 2002b; for review). However, attempts to describe the physiological mechanism(s) of acute silver toxicity in marine invertebrates are completely lacking from the literature. In this case, the situation is different from seawater fish, because marine invertebrates in general are osmoconformers, but are still ionoregulators (Barnes et al., 1993). This condition suggests that the gill may become the main site of toxicity for silver in marine invertebrates as opposed to fish, where both gills and gut seem to be involved in silver accumulation and toxicity. So, an understanding of the key mechanism of toxicity in marine invertebrates is imperative for future extension of the BLM to seawater environments. Therefore, the main goal of the present study was to characterize, for the first time, the acute toxic mechanism of silver in three different marine invertebrate species.

2. Material and methods

Marine shrimp *Penaeus duorarum* (3.6 ± 1.1 g) were collected in central Biscayne Bay (Miami, FL). Sea hares *Aplysia californica* (35.7 ± 6.1 g) and sea urchins *Diadema antillarum* (28.5 ± 8.2 g) were provided by the NIH/University of Miami National Resource for *Aplysia* (Miami, FL). After arrival at the National Institute of Environmental Health Sciences (NIEHS) Marine and Freshwater Biomedical Science Center at the Rosenstiel School of Marine and Atmospheric Sciences of the University of

Miami (Miami, FL), invertebrates were transferred to glass aquaria containing 100 L of local seawater (33‰ salinity) pumped from Biscayne Bay. Water was constantly aerated and was renewed daily. Temperature was held at 20 °C. Animals were not fed during both the acclimation and the experimentation periods.

Three groups of shrimps, sea hares and sea urchins ($n = 6$ for each species in each group) were maintained for 96 h, under the same acclimation conditions described above. After acclimation, one group of each species was maintained under control conditions (no addition of silver to the water). The other two groups from each species were maintained under the same acclimation conditions, but exposed to two different concentrations of waterborne silver for 48 h. A stock solution (1 mg/L) of AgNO_3 (SigmaUltra, Sigma Co., St. Louis, MO, USA) acidified with 1% HNO_3 was added into the test solution 3 h prior to introduction of animals. Total and filtered silver concentrations were followed over the experiment. Silver concentration in filtered (Acrodisc 0.45 μM polyethersulfone in-line filters; Pall, Ann Arbor, MI, USA) and non-filtered water samples (2 mL) was measured using a graphite furnace atomic absorption spectrophotometry (Zeiss GFAAS-5; Carl Zeiss Jena GmbH, Germany). Mean total measured silver concentrations over the experiment were 1.1 and 9.9 $\mu\text{g Ag/L}$. Mean measured filtered silver concentrations were 0.9 and 8.5 $\mu\text{g Ag/L}$, respectively. These concentrations will be referred hereafter as 1 and 10 $\mu\text{g Ag/L}$ of waterborne silver. These concentrations were selected to bracket the marine water quality criteria for silver recommended by the United States Environmental Protection Agency (USEPA, 1980) and the Canadian Ministry of Environment (Warrington, 1996). The acute criterion recommended by the USEPA is 3.2 and 2.7 $\mu\text{g Ag/L}$ as total and dissolved silver, respectively. The Canadian criterion for open coast and estuaries is 1.5 $\mu\text{g Ag/L}$ as a 30-day mean, and 3.0 $\mu\text{g Ag/L}$ as a maximum.

Before and after the 48 h exposure period, water samples from the different media were also collected and stored for osmolality and ion (Na^+ , Cl^- , K^+ , Mg^{2+} , and Ca^{2+}) concentration measurements as described below.

After 46 h of exposure, control or silver exposed animals were weighed and injected with 10 μL (1 μCi) of radiolabeled PEG-4000 (polyethylene glycol, PEG,

[1,2- ^3H]; 57.7 MBq/g—1.56 mCi/g; Perkin-Elmer Life Sciences, Boston, MA). Injections were given into one of the hemolymph sinus using a 50 μL Hamilton-type syringe. After injection, animals were gently rinsed in clean sea water and isolated in 500 mL beakers (sea hare and sea urchin) or 100 mL beakers (shrimp) containing aerated sea water, for 1 h. At time 0, 30, and 60 min, a sample (3 mL) of the experimental media was collected and stored for radioactivity counting, as described below. The effect of acute silver exposure (48 h) on whole body permeability was then determined using the flux of PEG over the 1 h period, considering the mean value obtained in control animals as 100% of the whole body permeability.

After water collection for PEG flux determination (i.e., 2 h after PEG injection), two hemolymph samples from each animal were collected by puncture of hemolymph seins. One sample was stored in a pre-weighed scintillation vial, which was immediately re-weighed to determine the tissue wet weight. This sample was used to determine the radioactivity present in the hemolymph. The other sample was stored in an Eppendorf type tube. After hemolymph coagulation in the Eppendorf tube, the samples were homogenized with a micro ultrasonic cell disrupter (Kontes Brinkmann homogenizer; Brinkmann Instruments, Westbury, NY) and centrifuged (Eppendorf centrifuge 5415C; Jouan CR 412; Jouan S.A.; Cedex, France) for 5 min at 10,000 rpm. Collected plasma was used for ions (Na^+ , Cl^- , K^+ , Mg^{2+} , and Ca^{2+}) and osmolality determinations, as described below.

After hemolymph collection, different tissues were dissected from each shrimp (gills, hepatopancreas, muscle and eyestalk), sea hare (gills, hepatopancreas, red muscle and abdominal ganglia) and sea urchin (eggs, gonads, oral muscle and tube feet). Immediately after dissection, each tissue sample was split into three sub-samples. The first sub-sample was placed in an Eppendorf-type tube and immediately stored in liquid nitrogen for Na^+ , K^+ -ATPase determination as described below. The second tissue sub-sample was stored in a pre-weighed scintillation vial, which was immediately re-weighed to determine the tissue-wet weight. This sample was used for radioactivity determination as described below. The third sub-sample was stored in a pre-weighed Eppendorf-type tube, which was immediately re-weighed to determine the tissue-wet weight. This sample was kept for determination

of tissue ion composition. It was dried at 60 °C until constant weight was obtained (tissue-dry weight), rehydrated with 1 mL of ion-free (Milli-Q) water and then homogenized using a micro ultrasonic cell disrupter (Kontes Brinkmann homogenizer; Brinkmann Instruments, Westbury, NY). After homogenization, the sample was centrifuged (Eppendorf centrifuge 5415C; Jouan CR 412; Jouan S.A.; Cedex, France) for 5 min at 10,000 rpm. The supernatant was collected and stored for ion (Na^+ , Cl^- , K^+ , Mg^{2+} , and Ca^{2+}) concentration measurements as described below.

For tissue Na^+ , K^+ -ATPase activity measurement, samples were thawed and kept on ice throughout the analysis. They were then homogenized in 0.5 mL of ice-cold buffer solution (150 mM sucrose 10 mM ethylenediaminetetraacetic acid—EDTA, 50 mM imidazole, 11.5 mM sodium deoxycholate and 1 mM phenylmethylsulfonyl fluoride—PMSF—Sigma; pH adjusted to 7.3 with HCl), and centrifuged at $5000 \times g$ for 30 s at 4 °C. Enzyme activity was measured in the supernatant using the method described by McCormick (1993) with modifications according to Wheatly and Henry (1987). Two reaction mixtures were assayed. Reaction mixture A consisted of 20 μL of sample, 50 μL of salt solution A, and 150 μL of working solution A. Reaction mixture B consisted of 20 μL of sample, 50 μL of salt solution B, and 150 μL of working solution B. Salt solution A contained 100 mM NaCl, 10.5 mM MgCl_2 , 30 mM KCl, and 50 mM imidazole, pH adjusted to 7.5. In salt solution B, NaCl replaced KCl at the same concentration. Working solution A contained 4 U/mL lactate dehydrogenase, 5 U/mL pyruvate kinase, 2.8 mM phosphoenolpyruvate, 3.5 mM adenosine triphosphate (ATP), 0.22 mM nicotinamide adenine dinucleotide phosphate (NADH), and 50 mM imidazole, pH adjusted to 7.5. To obtain the working solution B, 1 mM ouabain was added to working solution A. Kinetic assays were then run in duplicate at 25 °C in a temperature-controlled microplate reader (Molecular Devices, Menlo Park, CA, USA), for 10 min. An adenosine diphosphate (ADP) standard curve (0–20 nmol/10 μL) was also run. Na^+ , K^+ -ATPase activity was then calculated considering the difference in ADP production between the two reaction mixtures (A and B). Protein content in the homogenate was measured using Bradford reagent (Bio-Rad, Richmond, CA, USA). Enzyme activity was expressed as $\mu\text{mol ADP/mg protein/h}$.

For radioactivity determination in water samples, 15 mL of a liquid scintillation cocktail (ECOLUMETM, ICN, Costa Mesa, CA) was added to each scintillation vial. For radioactivity determination in tissues, samples were solubilized at 42 °C using the NCSTM solubilizer for liquid scintillation counting (0.5 mL of 0.65N solution per sample; Amersham Canada Limited, Oakville, ON, Canada). After solubilization, sample was cooled down, neutralized with glacial acetic acid, and 5 mL of scintillation liquid (CYTOSCINTTM, ICN, Costa Mesa, CA) was added to each scintillation vial. All radioactivity measurements were done using the TM Analytic 6895 BetaTrac Liquid Scintillation System (Elk Grove Village, IL). A quench curve for each tissue analyzed was built using different amounts of hemolymph or tissue sample from animals subjected to the same control conditions used in the experiment. These samples were treated using the same protocol described for tissues from the experimental animals.

Hemolymph and water osmolality were determined using a vapor pressure osmometer (WESCOR 5100C; Wescor, Logan, UT). Hemolymph, tissues and water cation concentrations (Na^+ , K^+ , Mg^{2+} and Ca^{2+}) were measured using an atomic absorption spectrophotometer (Perkin-Elmer Mod. 2380; Wellesley, MA). Hemolymph and water Cl^- concentrations were determined using a chloride titrator (CMT10 Chloride Titrator; Radiometer; Copenhagen, Denmark).

Total extracellular volume was estimated as a percentage of the whole body wet weight considering the dilution of the 10 μL of PEG injected, which was calculated based on the volume and radioactivity of the hemolymph collected 2 h after PEG injection. Tissue intracellular volume (TIV; in μL) was estimated based on the water tissue content (WTC; in μL) and the tissue extracellular volume (TEV; in μL), according to the following equation: $\text{TIV} = \text{WTC} - \text{TEV}$. The TEV was determined considering tissue (TR; in cpm) and hemolymph radioactivity (HR; in cpm), as well as the volume (wet weight) of the hemolymph sample (HV; in μL), according to the following equation: $\text{TEV} = \text{TR} \times \text{HV}/\text{HR}$. Changes in cellular volume after silver exposure were then expressed as percentage of the volume determined in the control animals. Intracellular ion concentration (IIC; in mM) was calculated using the following equation: $\text{IIC} = ((\text{TIC} \times \text{WTC}) - (\text{HIC} \times \text{TEV}))/\text{TIV}$, where TIC and HIC are expressed in mM and correspond to the

tissue and hemolymph ion concentration, respectively. WTC and TIV are expressed in L and correspond to the water tissue content and the tissue intracellular volume, respectively. TIV was calculated as described above.

All data obtained were expressed as mean (± 1 standard deviation; $n = 6$) and were subjected to one-way analysis of variance (ANOVA) followed by the Tukey's test using the software STATISTICA Version 5.1 (StatSoft Inc., Tulsa, OK). ANOVA assumptions, i.e., data normality and homogeneity of variances, were previously checked. If these tests failed, data were mathe-

matically transformed using the logarithmic function. The significance level adopted was 95% ($\alpha = 0.05$).

3. Results

Seawater employed in the present study had a mean osmolality of 1103 ± 11 mOsmol/Kg H_2O . Ionic composition of this water was in mM: $Cl^- = 565 \pm 20$; $Na^+ = 490 \pm 5$; $K^+ = 10.3 \pm 0.4$; $Mg^{2+} = 57.5 \pm 1.5$; and $Ca^{2+} = 9.8 \pm 0.2$.

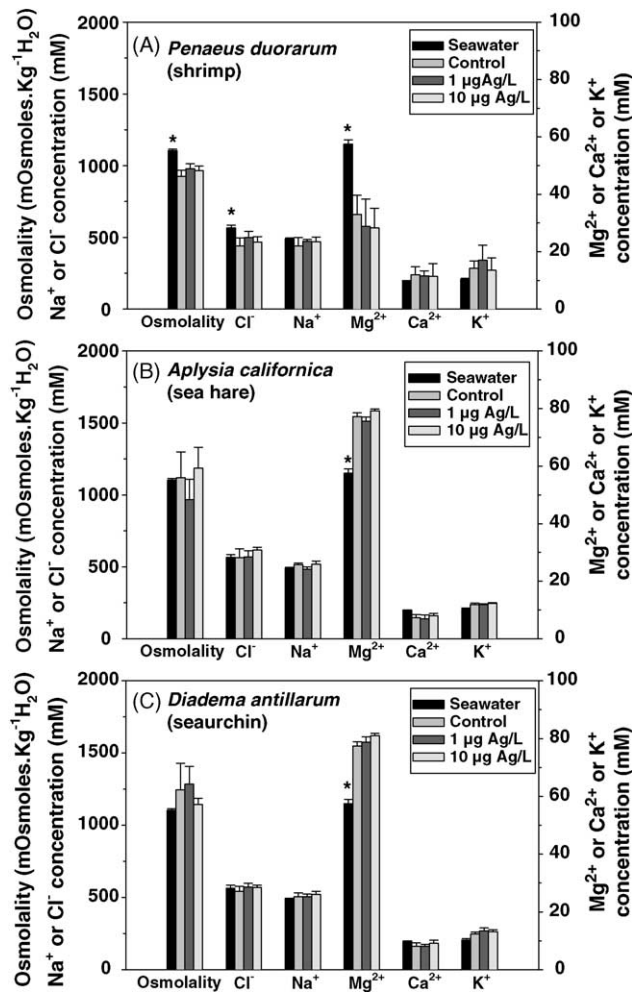


Fig. 1. Hemolymph osmolality and ion concentrations in the shrimp *Penaeus duorarum* (A), the sea hare *Aplysia californica* (B), and the sea urchin *Diadema antillarum* (C), non-exposed or exposed for 48 h to waterborne silver (1 or 10 µg Ag/L) in seawater. Data are mean \pm S.D. ($n = 6$). (*) Indicates significantly different mean values between the seawater and the control animals ($P < 0.05$). There were no significant differences in means values between the control and silver-exposed animals ($P > 0.05$).

The marine shrimp *P. duorarum* (Fig. 1A) showed the hemolymph osmolality slightly lower, but significantly different from that of the seawater used in the experiment. However, both the sea hare *A. californica* (Fig. 1B) and the sea urchin *D. antillarum* (Fig. 1C) had no significant differences in osmolality between the hemolymph and the seawater. The shrimp had slightly, but significantly, lower hemolymph levels of Cl^- than the seawater. Also, the shrimp had a markedly lower Mg^{2+} concentration ($\sim 50\%$) in the hemolymph compared to that measured in the seawater. Hemolymph K^+ and Ca^{2+} concentrations were not significantly different from those measured in seawater (Fig. 1A). In the sea hare, a higher Mg^{2+} concentration was observed in hemolymph than in seawater. However, hemolymph Cl^- , Na^+ , K^+ , and Ca^{2+} concentrations were similar to those in the seawater (Fig. 1B). In the sea urchin, also a higher concentration of Mg^{2+} was observed in hemolymph than in seawater. However, Cl^- , Na^+ , K^+ and Ca^{2+} concentrations were similar to those observed in the seawater (Fig. 1C). In the three species tested, acute exposure to either 1 or 10 $\mu\text{g Ag/L}$ for 48 h did not affect the osmolality and the ionic composition of the hemolymph (Fig. 1).

Whole body permeability measured using polyethylene glycol was not affected in either sea hare or sea urchin acutely exposed to silver. However, it was

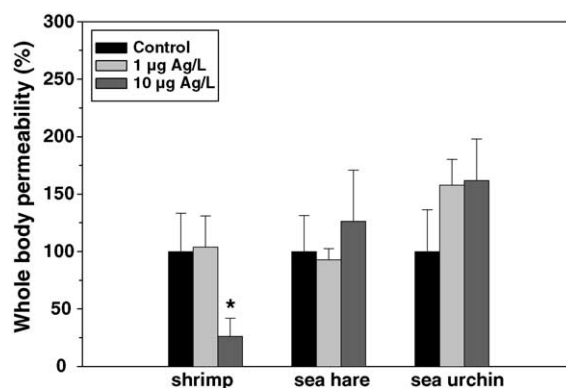


Fig. 2. Effect of acute exposure (48 h) to sub-lethal concentrations of waterborne silver (1 or 10 $\mu\text{g Ag/L}$) on whole body permeability in the shrimp *Penaeus duorarum*, the sea hare *Aplysia californica* and the sea urchin *Diadema antillarum*. Whole body permeability was measured using radiolabeled polyethylene glycol (PEG- ^3H). Data are means \pm S.D. ($n=6$). (*) Indicates significantly different mean values between the control and silver-exposed animals ($P < 0.05$).

significantly reduced ($\sim 60\%$) in the shrimp exposed to 10 $\mu\text{g Ag/L}$ for 48 h (Fig. 2).

Acute exposure to silver (10 $\mu\text{g Ag/L}$) for 48 h significantly reduced water content in the gills of the shrimp *P. duorarum* and the sea hare *A. californica*. In the latter, a significant decrease in the hepatopancreas water content was also observed. In contrast, silver did

Table 1

Whole body extracellular volume (WBEV) and intracellular volume (ICV) in different tissues of the shrimp *Penaeus duorarum*, the sea hare *Aplysia californica*, and the sea urchin *Diadema antillarum* non-exposed (control; 0 $\mu\text{g Ag/L}$) or exposed for 48 h to waterborne silver (1 or 10 $\mu\text{g Ag/L}$) in seawater

	<i>Penaeus duorarum</i>			<i>Aplysia californica</i>			<i>Diadema antillarum</i>		
	Silver ($\mu\text{g Ag/L}$)			Silver ($\mu\text{g Ag/L}$)			Silver ($\mu\text{g Ag/L}$)		
	0	1	10	0	1	10	0	1	10
WBEV	8 \pm 3	6 \pm 2	10 \pm 4	28 \pm 9	29 \pm 7	40 \pm 8	18 \pm 6	18 \pm 7	25 \pm 8
ICV									
Gill	100 \pm 5	92 \pm 9	93 \pm 8	100 \pm 6	105 \pm 6	93 \pm 8	nd	nd	nd
Hepatopancreas	100 \pm 4	102 \pm 7	96 \pm 8	100 \pm 4	103 \pm 6	104 \pm 6	nd	nd	nd
Muscle	100 \pm 7	97 \pm 4	102 \pm 8	100 \pm 5	94 \pm 5	94 \pm 7	100 \pm 4	97 \pm 8	96 \pm 7
Eyestalk	100 \pm 6	94 \pm 8	98 \pm 4	nd	nd	nd	nd	nd	nd
Ganglia	nd	nd	nd	100 \pm 6	98 \pm 7	93 \pm 5	nd	nd	nd
Eggs	nd	nd	nd	nd	nd	nd	100 \pm 6	102 \pm 7	98 \pm 3
Gonads	nd	nd	nd	nd	nd	nd	100 \pm 2	103 \pm 7	93 \pm 5
Tube feet	nd	nd	nd	nd	nd	nd	100 \pm 5	103 \pm 5	102 \pm 6

WBEV is expressed as a percentage of body wet weight. ICV is expressed as percentage of the ICV observed in tissues of control animals. Data are means \pm S.D. ($n=6$). (*) No significant differences in mean values between control and silver-exposed animals were detected ($P < 0.05$); nd = no data.

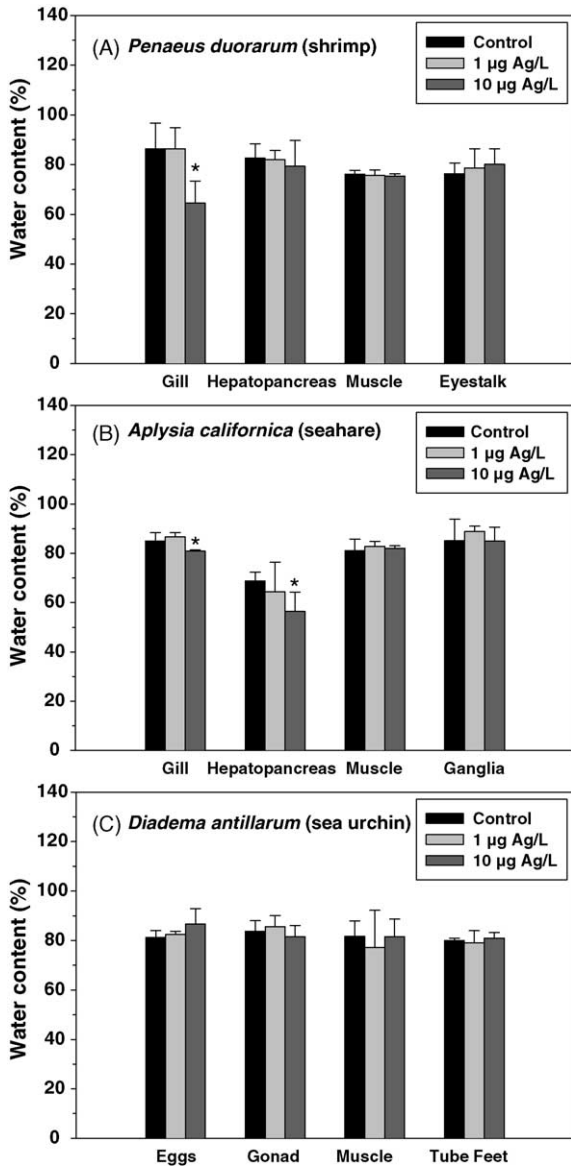


Fig. 3. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 µg Ag/L) of waterborne silver on water content of different tissues from the shrimp *Penaeus duorarum* (A), the sea hare *Aplysia californica* (B), and the sea urchin *Diadema antillarum* (C). Data are means ± S.D. (n = 6). (*) Indicates significantly different mean values between the control and silver-exposed animals (P < 0.05).

not affect the water content in the tissues of sea urchin *D. antillarum* (Fig. 3).

Whole body extracellular volume (WBEV) corresponded to about 10, 20 and 30% of the wet body

weight in the shrimp, sea urchin and sea hare, respectively. In the three species, waterborne acute silver exposure did not significantly affect the whole body extracellular volume (Table 1). Acute silver exposure also did not significantly affect the cellular volume in all tissues analyzed in the three species (Table 1).

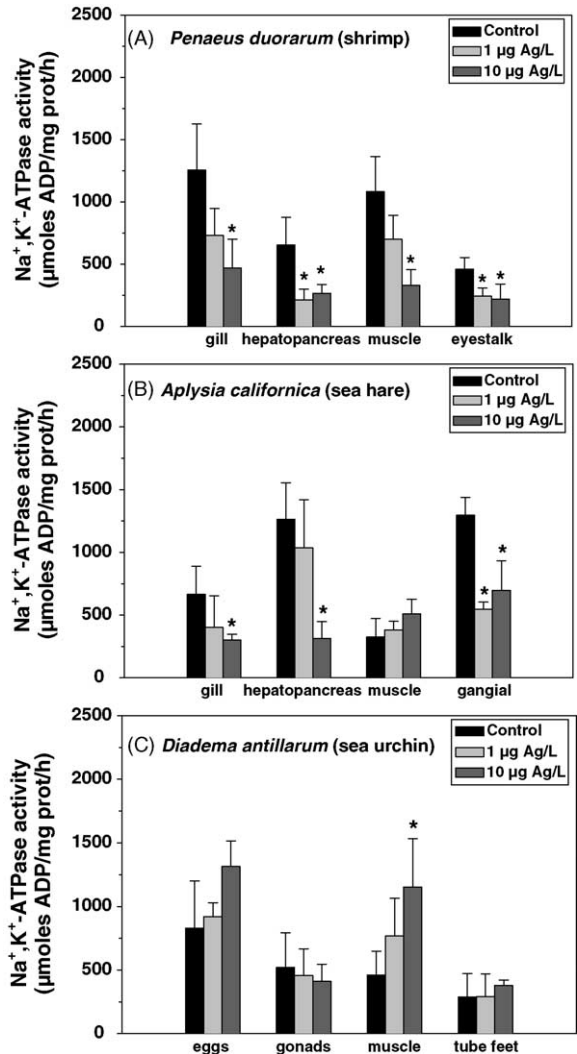


Fig. 4. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 µg Ag/L) of waterborne silver on Na⁺,K⁺-ATPase activity in different tissues of the marine shrimp *Penaeus duorarum* (A), the sea hare *Aplysia californica* (B), and the sea urchin *Diadema antillarum* (C). Data are means ± S.D. (n = 6). (*) Indicates significantly different mean values between the control and silver-exposed animals (P < 0.05).

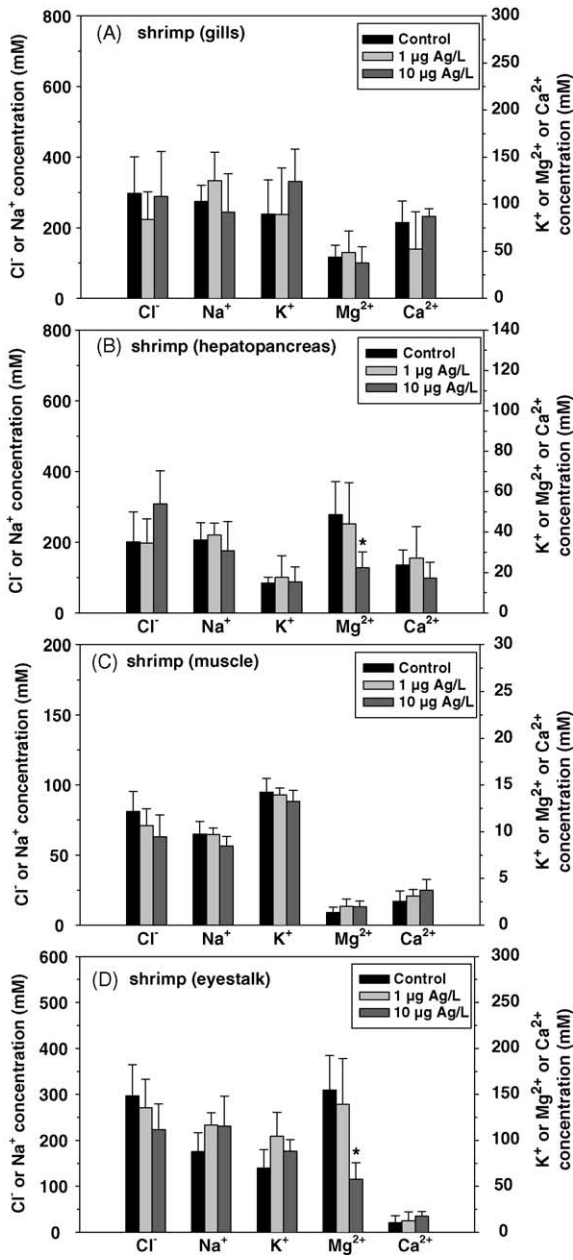


Fig. 5. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 μg Ag/L) of waterborne silver on ion content in different tissues of the shrimp *Penaeus duorarum*: (A) gills; (B) hepatopancreas; (C) muscle; and (D) eyestalk. Data are means ± S.D. ($n = 6$). (*) Indicates significantly different mean values between the control and silver-exposed animals ($P < 0.05$).

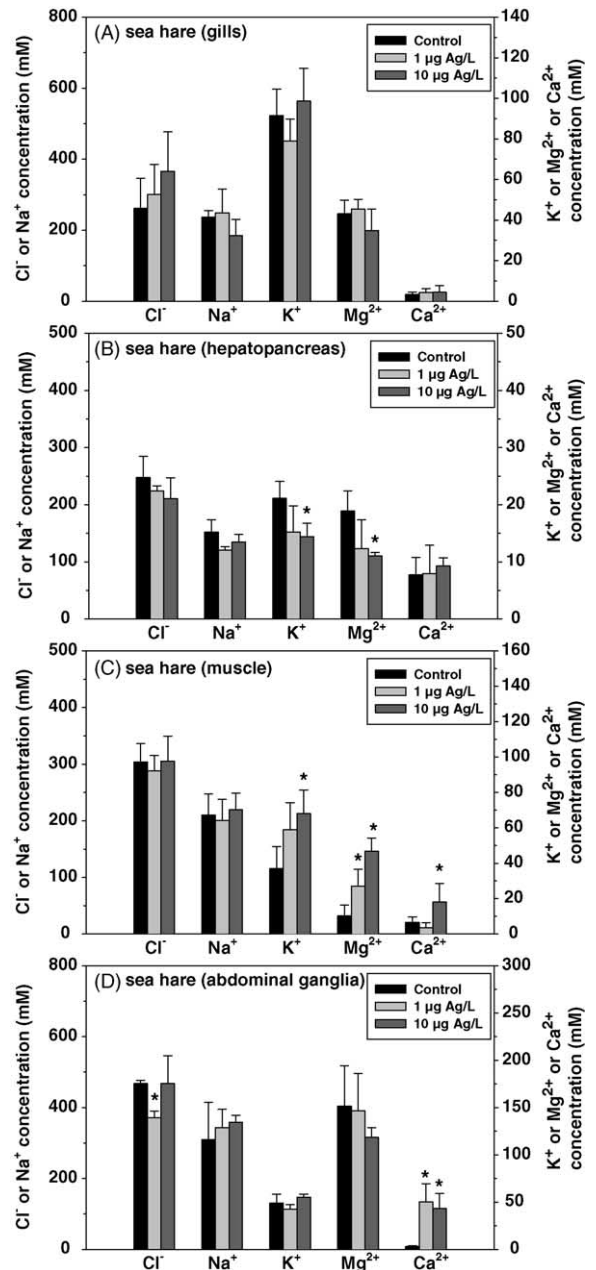


Fig. 6. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 μg Ag/L) of waterborne silver on ion content in different tissues of the sea hare *Aplysia californica*: (A) gills; (B) hepatopancreas; (C) red muscle; and (D) abdominal ganglia. Data are means ± S.D. ($n = 6$). (*) Indicates significantly different mean values between the control and silver-exposed animals ($P < 0.05$).

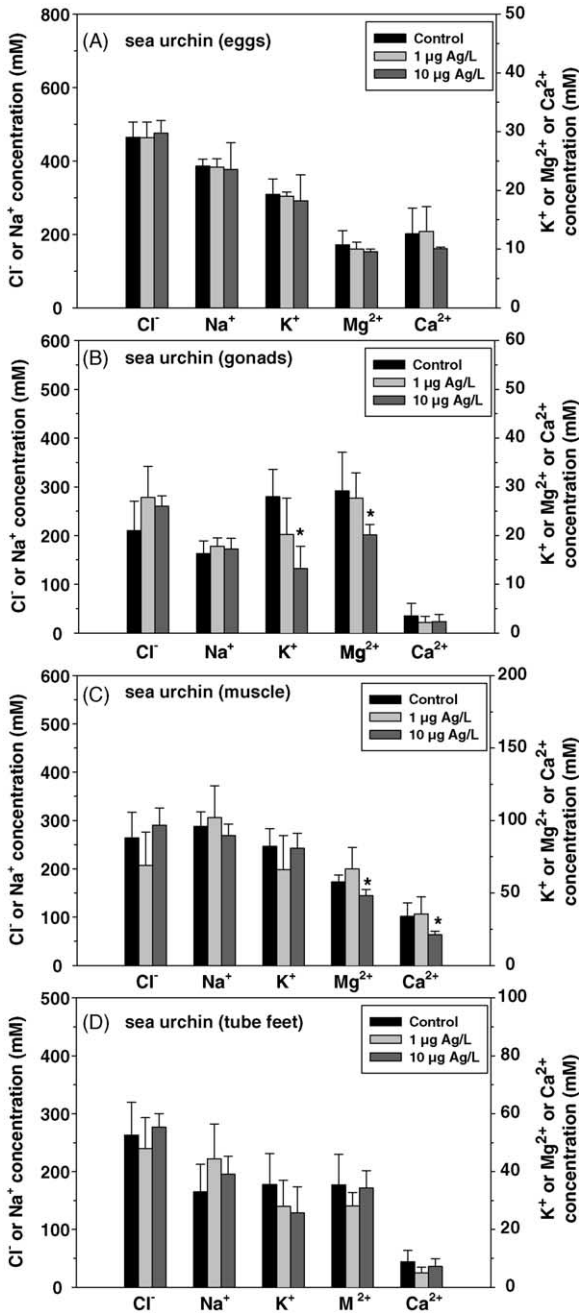


Fig. 7. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 µg Ag/L) of waterborne silver on ion content in different tissues of the sea urchin *Diadema antillarum*: (A) eggs; (B) gonads; (C) muscle; and (D) tube feet. Data are means ± S.D. (n = 6). (*) Indicates significantly different mean values between the control and silver-exposed animals (P < 0.05).

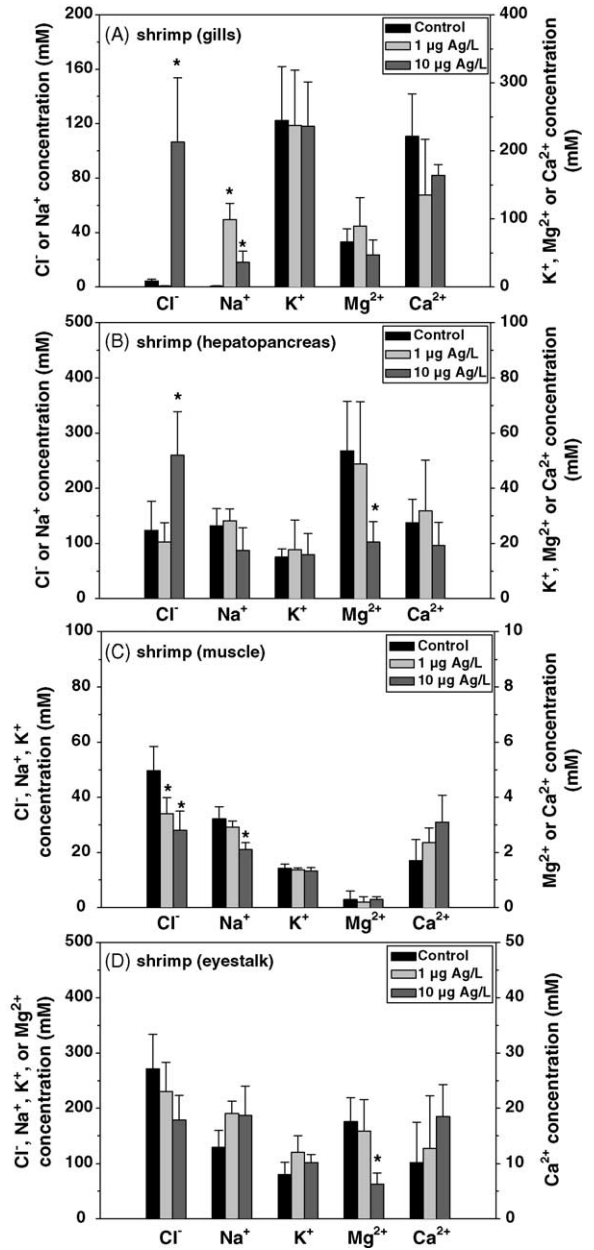


Fig. 8. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 µg Ag/L) of waterborne silver on intracellular ion concentration in different tissues of the shrimp *Penaeus duorarum*: (A) gills; (B) hepatopancreas; (C) muscle; and (D) eyestalk. Data are means ± S.D. (n = 6). (*) Indicates significantly different mean values between the control and silver-exposed animals (P < 0.05).

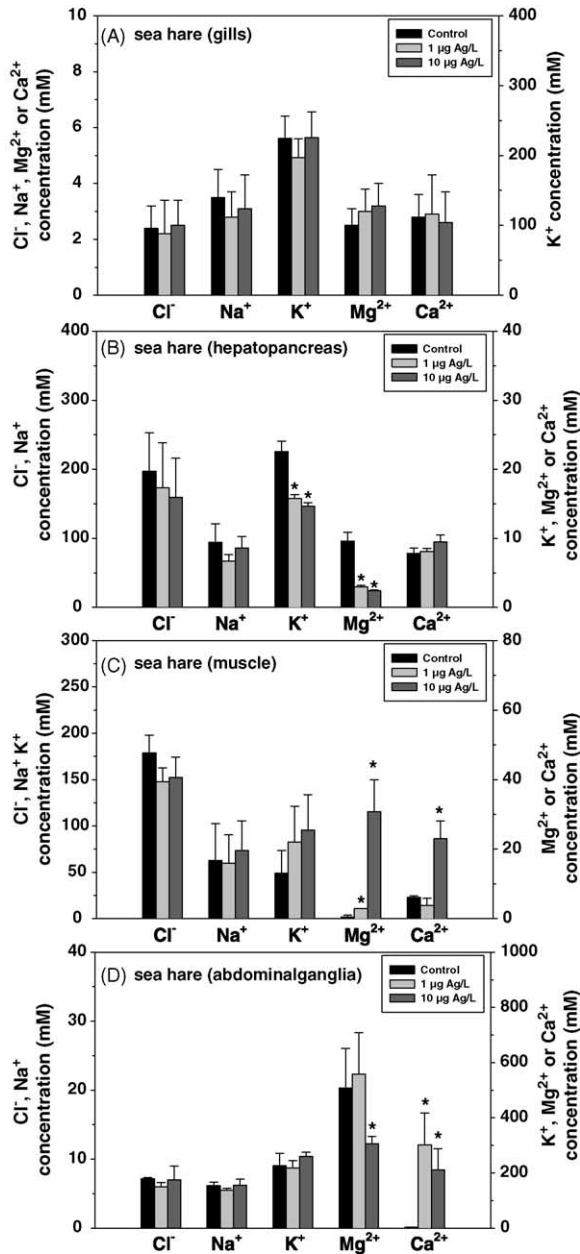


Fig. 9. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 µg Ag/L) of waterborne silver on intracellular ion concentration in different tissues of the sea hare *Aplysia californica*: (A) gills; (B) hepatopancreas; (C) red muscle; and (D) abdominal ganglia. Data are means ± S.D. ($n=6$). (*) Indicates significantly different mean values between the control and silver-exposed animals ($P < 0.05$).

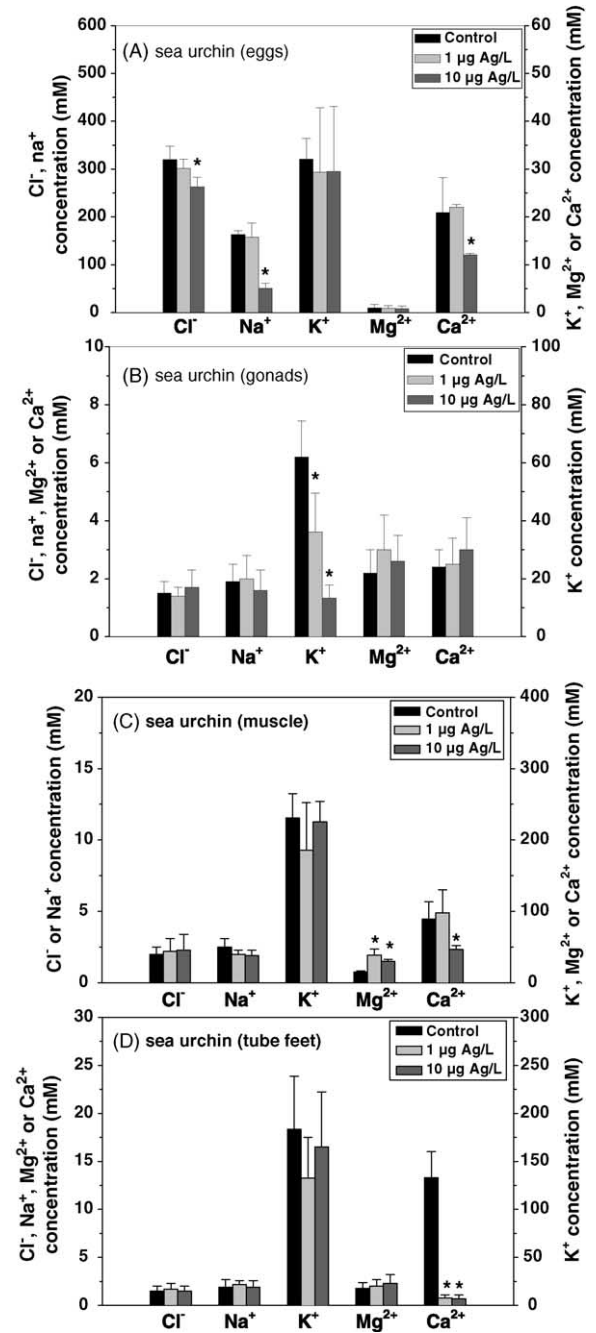


Fig. 10. Effect of acute exposure (48 h) to sub-lethal concentrations (1 or 10 µg Ag/L) of waterborne silver on intracellular ion concentration in different tissues of the sea urchin *Diadema antillarum*: (A) eggs; (B) gonads; (C) muscle; and (D) tube feet. Data are means ± S.D. ($n=6$). (*) Indicates significantly different mean values between the control and silver-exposed animals ($P < 0.05$).

In both shrimp (Fig. 4A) and sea hare (Fig. 4B), acute exposure to waterborne silver at 10 $\mu\text{g Ag/L}$ for 48 h induced significant inhibition of Na^+, K^+ -ATPase activity in all tissues analyzed, except in the sea hare muscle. On percentage basis, inhibition ranged from 52% in the eyestalk to 70% in the muscle of shrimp, and from 46% in the abdominal ganglia to 75% in the hepatopancreas of sea hare. Significant enzyme inhibition was also observed in hepatopancreas and eyestalk of shrimp (Fig. 4A), as well as in abdominal ganglia of sea hare (Fig. 4B) exposed to 1 $\mu\text{g Ag/L}$. In the sea urchin muscle, an increased Na^+, K^+ -ATPase activity was observed after exposure to 10 $\mu\text{g Ag/L}$ (Fig. 4C).

In the shrimp, silver did not induce significant changes in gill (Fig. 5A) and muscle (Fig. 5C) ion concentration. However, acute exposure to waterborne silver (10 $\mu\text{g Ag/L}$) caused a significant decrease ($\sim 60\%$) in hepatopancreas (Fig. 5B) and eyestalk (Fig. 5D) Mg^{2+} concentration. In the sea hare, silver did not induce significant changes in gill (Fig. 6A) ion concentration. However, acute exposure to 10 $\mu\text{g Ag/L}$ significantly reduced K^+ ($\sim 25\%$) and Mg^{2+} content ($\sim 40\%$) in the hepatopancreas (Fig. 6B). In the sea hare muscle, approximately 1.4-fold, five-fold, and three-fold increases in K^+ , Mg^{2+} and Ca^{2+} concentration were observed after exposure to 10 $\mu\text{g Ag/L}$, respectively (Fig. 6C). Magnesium ion concentration in muscle also increased in the sea hare after exposure to 1 $\mu\text{g Ag/L}$. An ~ 10 -fold increase in Ca^{2+} concentration was registered in the abdominal ganglia of sea hare exposed to 1 or 10 $\mu\text{g Ag/L}$ (Fig. 6D). In the sea urchin, no significant changes in ion concentration were observed in eggs (Fig. 7A) and tube feet (Fig. 7D) after acute exposure to silver. However, silver exposure significantly decreased K^+ ($\sim 50\%$) and Mg^{2+} ($\sim 30\%$) concentrations in gonads (Fig. 7B) and Mg^{2+} ($\sim 10\%$) and Ca^{2+} ($\sim 30\%$) concentrations in muscle (Fig. 7C) of sea hare exposed to 10 $\mu\text{g Ag/L}$.

Regarding intracellular ion concentration, acute exposure to silver induced significant changes in all tissues and species tested, except in the sea hare gill. In the shrimp, significant increases in intracellular Cl^- and Na^+ concentrations were observed in gills after exposure to 10 $\mu\text{g Ag/L}$. A significant increase in intracellular Na^+ concentration was also observed at 1 $\mu\text{g Ag/L}$ (Fig. 8A). In the hepatopancreas, silver (10 $\mu\text{g Ag/L}$) induced an increase in intracellular Cl^-

and a decrease in intracellular Mg^{2+} concentration (Fig. 8B). In muscle, significant decreases in intracellular Cl^- and Na^+ concentrations were observed after acute exposure to 10 $\mu\text{g Ag/L}$. Also, a significant decrease in intracellular Cl^- concentration was observed at 1 $\mu\text{g Ag/L}$ (Fig. 8C). Silver (10 $\mu\text{g Ag/L}$) induced a decrease in Mg^{2+} intracellular concentration in shrimp eyestalk (Fig. 8D).

In the sea hare, silver exposure did not affect gill intracellular ion concentration (Fig. 9A). However, it caused decreases in intracellular K^+ and Mg^{2+} concentrations in hepatopancreas (Fig. 9B) and increases in intracellular Mg^{2+} concentration in muscle of sea hare exposed to 1 or 10 $\mu\text{g Ag/L}$ (Fig. 9C). A significant increase in intracellular Ca^{2+} concentration in muscle of sea hare exposed to 10 $\mu\text{g Ag/L}$ was also observed (Fig. 9C). In abdominal ganglia, silver at 10 $\mu\text{g Ag/L}$ decreased intracellular Mg^{2+} and increased intracellular Ca^{2+} concentrations (Fig. 9D). A significant increase in intracellular Ca^{2+} concentration was also observed in abdominal ganglia of sea hare exposed to 1 $\mu\text{g Ag/L}$ (Fig. 9D).

In the sea urchin, acute exposure to waterborne silver (10 $\mu\text{g Ag/L}$) decreased intracellular Cl^- , Na^+ , and Ca^{2+} concentrations in eggs (Fig. 10A). On the other hand, it decreased intracellular K^+ concentration in gonads (Fig. 10B) and intracellular Ca^{2+} concentration in muscle (Fig. 10C) and tube feet (Fig. 10D). Significant decreases in intracellular K^+ and Ca^{2+} concentrations were also observed in gonads (Fig. 10B) and tube feet (Fig. 10D) of sea urchin exposed to 1 $\mu\text{g Ag/L}$. A significant increase in Mg^{2+} concentration was also observed in muscle of sea urchin exposed to 1 or 10 $\mu\text{g Ag/L}$ (Fig. 10C).

4. Discussion

Recent studies have demonstrated that “key toxic sites” for acute Ag^+ toxicity are the Na^+, K^+ -ATPase molecules located at the basolateral membrane of the gill epithelium in both freshwater fish (rainbow trout; Morgan et al., 1997; McGeer and Wood, 1998; Bury et al., 1999) and invertebrates (crayfish and daphnids; Grosell et al., 2002a; Bianchini and Wood, 2003). In these animals, the gills are the main organ involved in the active transport of Na^+ and Cl^- from the water into the extracellular fluid to compensate the dif-

fusive loss of salts taking place at gill and excretory organs. Branchial Na^+ , K^+ -ATPase activity plays a direct role in this transport (Péqueux, 1995; Flik et al., 1997; Perry, 1997; Castilho et al., 2001). Thus, the inhibition of branchial Na^+ , K^+ -ATPase activity induced by acute Ag^+ exposure leads to lower sodium uptake across the gills and causes ionoregulatory failure and death in these animals (Grosell et al., 2002b, for review; Bianchini and Wood, 2003).

In marine fish, *in vivo* experiments showed that drinking rate and intestinal NaCl absorption is reduced after fish exposure to waterborne silver. Furthermore, *in vitro* experiments showed that Na^+ , Cl^- , and H_2O absorption across the intestinal epithelium is reduced by silver exposure. So, these effects indicated that silver caused ionoregulatory impairments and dehydration in marine fish after acute exposure to waterborne silver (Grosell et al., 1999; Hogstrand et al., 1999; Wood et al., 1999, for review).

Thus, it is clear from the studies reported above that acute waterborne silver exposure is inducing an osmo- and ionoregulatory disturbance at the extracellular fluid level in freshwater fish and invertebrates, as well as in marine fish. Despite the fact that most marine invertebrates are osmoconformers, they are still ionoregulators (Barnes et al., 1993). In fact, one of the marine invertebrates species analyzed in the present study, the shrimp *P. duorarum*, actually slightly hypo-osmoregulates relative to seawater, but the other two species, the sea hare *A. californica* and the sea urchin *D. antillarum*, are true osmoconformers in seawater (Fig. 1). However, all these three marine invertebrate species are, to various extents, hyporegulating the concentration of some ions in the hemolymph. For example, shrimp hyporegulates Cl^- and both sea hare and sea urchin hyper-regulate the Mg^{2+} concentration. In contrast, the shrimp strongly hyporegulates the hemolymph Mg^{2+} concentration (Fig. 1). All these results are in complete agreement with data reported in the literature for invertebrates (Barnes et al., 1993; Schmidt-Nielsen, 1996). Thus, if the key mechanism of acute silver toxicity in ionoregulating marine invertebrates is similar to that observed in marine and freshwater fish and freshwater invertebrates, acute exposure to waterborne silver should induce ionoregulatory disturbances at the hemolymph level in the three species tested in the present study. However, results from the present study clearly indicate that acute exposure to wa-

terborne silver (1 and 10 $\mu\text{g Ag/L}$) did not induce any significant effect on hemolymph osmo and ionoregulation (Cl^- , Na^+ , K^+ , Mg^{2+} and Ca^{2+}) in those species (Fig. 1).

Results from the present study using radiolabeled PEG (^3H polyethylene glycol-4000) are also in agreement with the lack of silver effect on the osmolality and composition of the hemolymph. They showed that silver exposure did not significantly increase the whole body permeability to PEG in the three species studied (Fig. 2), indicating that epithelia are not becoming leaky under acute silver exposure. In freshwater fish, it seems that silver also does not increase Na^+ efflux (Grosell et al., 2002b). However, increased Na^+ efflux has been reported in the freshwater crayfish *Cambarus diogenes diogenes* after exposure to waterborne silver (Grosell et al., 2002a). In the present study, the only change induced by silver on whole body permeability was a significant decrease of this parameter in the hypo-osmoregulating shrimp *P. duorarum* exposed to the higher concentration of silver tested (10 $\mu\text{g Ag/L}$). This decreased permeability could be a response to the dehydration that the shrimp gill is suffering at 10 $\mu\text{g Ag/L}$ (Fig. 3A). Notably gill and hepatopancreas dehydration were also observed in the sea hare (Fig. 3B), but without any significant change in whole body permeability in this species (Fig. 2). This is probably due to the fact that crustaceans possess a greater ability to control gill permeability, especially because the gill cuticle plays an important role in this process (Péqueux, 1995). In any case, tissue dehydration observed in both shrimp and sea hare exposed to silver was not associated with any significant change in hemolymph ion concentration (Fig. 1), extracellular fluid volume (Table 1), and cell volume maintenance (Table 1). Thus, it seems clear from these findings that the key mechanism of acute silver toxicity in marine invertebrates is definitely not associated with osmotic or ionoregulatory impairments at the hemolymph level, as observed in seawater fish and both freshwater fish and crustaceans.

Despite the lack of silver effect on hemolymph osmo- and ionoregulation in the marine invertebrates studied here, it is important to consider the three following facts: (1) all marine invertebrates have been reported to accumulate silver during exposures to levels of waterborne silver similar to those employed in the present study (Nelson et al., 1983; Calabrese et al., 1984; Cain and Luoma, 1985; Majorta et al., 1988;

Metayer et al., 1990; Whyte and Boutillier, 1991); (2) once inside the animal, silver is differentially distributed among tissues (Whyte and Boutillier, 1991) and causes toxicity in marine invertebrates (Lussier et al., 1985; Metayer et al., 1990); and (3) silver is a potent ionoregulatory toxicant in fish and invertebrates due to its inhibitory effect on enzymes such as the Na^+, K^+ -ATPase and carbonic anhydrase (Grosell et al., 2002a; Bianchini and Wood, 2002, 2003; Bianchini et al., 2002; Mann et al., 2004; Morgan et al., 2004). Thus, silver accumulated in marine invertebrates could be causing toxicity by primarily acting on the same key enzymes, i.e., Na^+, K^+ -ATPase (and perhaps carbonic anhydrase), at the gill level, but causing effects other than disturbances in hemolymph osmo- and ionoregulation. In fact, results from the present study show that Na^+, K^+ -ATPase activity is significantly inhibited by silver exposure in all tissues from both of the gill-breathing animals tested, i.e., the shrimp *P. duorarum* and the sea hare *A. californica*, except in the sea hare muscle (Fig. 4). Na^+, K^+ -ATPase inhibition was not observed in tissues of the sea urchin exposed to silver, though there was a significant increase in enzyme activity in the tube feet of this species (Fig. 4C). At this point, it is interesting to note that the picture regarding silver effects on Na^+, K^+ -ATPase is very similar in both marine fish and invertebrates. This statement is based on the fact that inhibition, lack of effect, and even increase in enzyme activity, thought to be a compensatory effect has been reported in different marine fish acutely exposed to silver (Wood et al., 1999; Webb et al., 2001).

Inhibition of Na^+, K^+ -ATPase in euryhaline invertebrate tissues could lead to disturbances in volume regulation, intracellular ion concentration and acid–base balance at the cellular level (Gilles and Gilles-Baillien, 1985; Gilles et al., 1987). In the present study, we did not evaluate the effect of silver on the latter parameter. However, our results show that silver exposure did not induce any significant effect on cell volume maintenance in any of the tissues tested. This lack of silver effect could be explained by the great ability that marine invertebrate cells have to regulate their volume. This ability is not only linked to the pump-and-leak system, i.e., the Na^+, K^+ -pump and the K^+ channels which regulates the intracellular levels of both Na^+ and K^+ , but also to the control of the intracellular level of “peptides”, amino acids and other ninhydrin-positive

substances (Gilles and Gilles-Baillien, 1985; Yin et al., 2000; Gómez-Angelats et al., 2000). Thus, tissue Na^+, K^+ -ATPase inhibition with consequent changes in tissue and intracellular concentration of inorganic ions does not necessarily lead to significant changes in cellular volume. It is then necessary to consider the contribution of the variety of leaks and secondary pumps to the cell volume maintenance under isoosmotic conditions. For example, any pump producing a net extrusion of Na^+ (or any other solute) leaking in bulk into the cells may be operative (Gilles and Gilles-Baillien, 1985). However, the absence of changed cell volume after silver exposure does not negate a possible effect of silver on the processes involved in the cellular volume maintenance and the regulation of intracellular ion concentrations. In fact, silver exposure induced several changes in tissue and intracellular ion concentrations in the three marine invertebrates tested in the present study.

Effects of silver on tissue ion concentrations were dependent on the ion, tissue, or species considered (Figs. 5–7). Overall, acute exposure to waterborne silver did not induce significant changes in tissue Cl^- and Na^+ concentrations in the three species analyzed. However, it did cause marked changes in the concentration of univalent (K^+) and divalent cations (Mg^{2+} and Ca^{2+}) in several tissues. Effects of silver on the intracellular distribution of all univalent ions (Cl^- , Na^+ , and K^+) analyzed were also observed (Figs. 8–10). These silver effects on tissue or intracellular ion concentrations cannot be explained simply by the observed changes in tissue water content, which were minimal (Fig. 3). Thus, silver effects on mechanisms involved in ion transport at the cellular membrane level must be considered.

It is expected that an inhibition of the Na^+, K^+ -ATPase activity by acute exposure to silver would lead to increases in intracellular concentrations of Cl^- and Na^+ parallel to a decrease in intracellular K^+ concentration. Such effects could explain, at least in part, the following observed variations in the intracellular ion distribution: (1) the increases in Cl^- and Na^+ concentration in shrimp gill (Fig. 8A), (2) the increase in intracellular Cl^- concentration in shrimp hepatopancreas (Fig. 8B), and (3) the decrease in K^+ concentration in the sea hare hepatopancreas (Fig. 9B). However, decreases in both Cl^- and Na^{2+} concentration in the shrimp muscle and the sea urchin eggs are inconsistent with the observed inhibition of the Na^+, K^+ -ATPase

activity. Furthermore, enzyme activity was not significantly affected in the sea urchin gonads, thus not explaining the observed decrease in intracellular K^+ concentration in this tissue. At this point, it is interesting to note that, as observed in sea urchin eggs, decrease in both Na^+ and Cl^- concentrations in developing rainbow trout eggs were also reported after an acute silver challenge (Guadagnolo et al., 2000). The ionoregulatory disturbances observed in both egg and gonads of the sea urchin after exposure to silver deserves further attention, because they could constitute the key mechanism not only for acute but also for chronic silver toxicity in marine invertebrates. In fact, chronic silver effects on marine invertebrates, including reproduction and larval development, have been reported extensively in the literature (Calabrese and Nelson, 1974; Calabrese et al., 1977; Coglianesi and Martin, 1981; Nelson et al., 1983; Eyster and Morse, 1984; Lussier et al., 1985; Martin et al., 1981; Metayer et al., 1990; Ward and Kramer, 2002; Hellou et al., 2003).

Briefly, the effects induced by silver on univalent and divalent ions distribution inside the cells of the three marine invertebrates tested cannot be explained solely by silver binding on Na^+, K^+ -ATPase and the consequent inhibition of this enzyme. These effects seem to be related also to silver binding and action on sites other than the Na^+, K^+ -ATPase in marine invertebrates. Taking into account the results obtained in the present study regarding silver effects on intracellular ion concentrations, candidate molecules could be other ATPases (Mg^{2+} and Ca^{2+} -ATPase), Cl^- channels and carbonic anhydrase. In fact, these molecules are known to be associated with cellular volume maintenance and intracellular ion distribution in both vertebrates and euryhaline invertebrates (Gilles and Gilles-Baillien, 1985; Gilles et al., 1987; Gelband et al., 1996; Gómez-Angelats et al., 2000; Henry and Watts, 2001; Bloomquist, 2003; Sardini et al., 2003).

Because the primary site of toxic action for waterborne silver in freshwater fish and crustaceans is the gill, this organ is the correct “target” for the biotic ligand model for freshwater. However, the situation may be different in seawater because cationic Ag^+ is much less prevalent (Ward and Kramer, 2002) and cationic competition from Na^+ , Ca^{2+} , Mg^{2+} , K^+ , and Sr^{2+} at Ag^+ -gill binding sites will also be stronger in seawater. Furthermore, the situation is complicated for fish in seawater because they drink to replace water lost by

osmosis across the gills. This water is absorbed in the gut, so this tissue seems to be an important “target” for Ag^+ interactions in brackish and marine fish (Grosell et al., 1999; Hogstrand et al., 1999; Webb and Wood, 2000; Webb et al., 2001). In fact, it has been demonstrated that silver accumulates to comparable levels in gill and gut tissues and that both are sites of ionoregulatory toxicity, with ~50% of the toxic response being attributable to each site (Grosell and Wood, 2001).

Regarding marine invertebrates, results obtained in the present study clearly indicate that the toxic response to acute silver exposure is not linked to osmotic or ionoregulatory disturbances at the hemolymph level, even in the hypo-osmoregulating shrimp *P. duorarum*. However, they suggest that acute silver toxicity could be associated with changes in the intracellular distribution of several univalent (Cl^- , Na^+ , and K^+) or divalent (Mg^{2+} and Ca^{2+}) ions in different marine invertebrate tissues, including gills. Furthermore, they indicate that these changes cannot only be ascribed to silver binding and subsequent effect on Na^+, K^+ -ATPase, except in the shrimp gill. Thus, it seems that the gill of marine crustaceans could be considered as a good “target” for the extension of the BLM from freshwater to seawater. However, the possibility of considering other additional “sites of toxicity” in other groups of marine invertebrates should be taken into consideration in the development of a marine BLM version for silver.

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