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Behavioural response to the bioavailability of inorganic mercury in the hydrothermal mussel *Bathymodiolus azoricus*

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Summary

The hydrothermal vent bivalve *Bathymodiolus azoricus* is naturally exposed to putatively elevated levels of mercury (Hg), exposure that dates back to the geological occurrence of vent ecosystems, and thus may have evolved evolutionary detoxification mechanisms. Therefore, it was used as a model organism in the present investigation to study the Hg–animal interaction. Mussels were exposed to inorganic Hg by daily administration of 20 μg l^{-1} Hg for 21 days (cumulative added concentration was 420 μg l^{-1}, i.e. ~2 mmol l^{-1}) under controlled laboratory conditions, and consequent bioaccumulation and detoxification patterns were investigated, while shell gaping behaviour indicative of filtering activity was monitored.

As a result of Hg exposure, significant increase in duration, as well as decline in frequency of shell gaping occurred, which did not recover to pre-exposure levels following 21 days of Hg-free treatment. An increase in the duration of open-shelled status may indicate the absence of an avoidance reaction in the vent mussel coming in contact with Hg, unlike other bivalves that normally close their shells in response to stress compounds. Alternatively, it may suggest that Hg had an inhibitory effect on the adductor muscle function that is responsible for closing the shells. As a result, elevated Hg levels were measured in the soft tissues (270±71 μg g^{-1} in gills, 245±52 μg g^{-1} in digestive glands, 93±25 μg g^{-1} in the mantle and 46±9 μg g^{-1} in the foot), in byssus threads (peak levels of 442±89 μg g^{-1}) and in pseudofaeces (reaching levels as high as 1000 μg g^{-1}). Overall, gills contributed 75% to the total Hg body burden followed by mantle (13%), digestive gland (7%), byssus (3%) and foot (2%). Tissue Hg levels remained elevated in mussels transferred to Hg-free seawater even after 21 days, despite the high concentrations persistently eliminated with pseudofaeces both, during and after, exposure.

This potential for bioaccumulation of inorganic Hg (concentration factors reached the order of magnitude of 10^4) by the vent mussel, which does not seem to prevent uptake by shell closure, suggests that the main Hg-handling strategy is elimination via mucus.

Key words: mercury, shell gaping, hydrothermal vents, bivalve, *Bathymodiolus azoricus.*

Introduction

Hydrothermal environments are characterised by high metal concentrations related to interactions of the convective seawater circulation with basaltic rocks inside the ocean crust. Thus, hydrothermal fluids are enriched with, among other toxic metals, mercury (Hg) that becomes bioavailable to vent macro invertebrates and that may determine development of specialised defence mechanisms. Unusually high Hg concentrations in many deep sea hydrothermal vents have suggested that invertebrate communities inhabiting these extreme environments are naturally exposed to elevated levels of the metal (Prol-Ledesma et al., 2002; Ando et al., 2002; Martins et al., 2001; Stoffers et al., 1999; Costa et al., 1988; Falkner et al., 1997). Even though our recent investigations failed to detect markedly high Hg levels in the water column of mixing zones at vent sites of the Mid Atlantic Ridge (MAR), concentrations in the tissues of the vent bivalve *Bathymodiolus azoricus* exceed many fold those in mussels from industrially polluted areas (E. Kadar, J. J. Powell, V. Costa and R. S. Santos, unpublished). However, for a clear demonstration of organism–habitat interaction in hydrothermal vents, an in-depth characterisation of this mixing zone is needed that is lacking to date, given the dynamic nature of the system with such large spatial and temporal variations of environmental conditions. Since vent chemistry is complex and, as yet, poorly understood even after two decades of intense research, post-capture experiments under controlled laboratory conditions designed to simulate the natural environment may be the way to find answers to the paradigm of densely populated hydrothermal vents in spite of the harsh conditions.

Mercury is a ubiquitous element, which is highly toxic (Florentine, 1991), and is present in the environment under three main forms: elemental Hg, divalent ‘inorganic’ Hg and organo-Hg (methyl-, dimethyl-, aril-Hg, etc). Organic forms of Hg have been previously considered as having a greater lipid

solubility than the inorganic forms, which allows them to more readily cross cell membranes and be absorbed. However, the work over a decade conducted by Mason and co-workers (Mason et al., 1993, 1994, 1996, 1997, 2000; Andres et al., 2002) contradicts the above reason for the bioaccumulation of methyl Hg (MMHg) over inorganic Hg, and concludes that both MMHg and inorganic Hg uptake involves a number of mechanisms, both passive and active (energy dependent), and the importance of each is highly dependent on the particular organism and on the specific tissue membrane. Thus the toxicity of Hg to aquatic organisms is widely recognized (Hassett-Sipple et al., 1997) and occurs even at low concentrations (Amiardtriquet et al., 1993; Bellas et al., 2001; Hill and Soares, 1987; Sheuhammer, 1987; Simas et al., 2001). The general mechanism of action of both Hg and MMHg are based on their relatively non-specific ATPase inhibition that disturbs osmotic and ionic regulation (Barradas and Pequeux, 1996; Pequeux et al., 1996; Pagliarani et al., 1996; Jagoe et al., 1996). Furthermore, while the presence of Hg in the external medium appears to influence ion regulation, the Hg, once taken up, is sequestered throughout the gill tissues and not in specific cells (Jagoe et al., 1996). Histochemical examination of tissues after exposure showed localization of Hg in the gill of the green crab *Carcinus maenas* and suggested that it was bound to the cuticle/membrane complex whereas MMHg was distributed evenly in the cell/cytoplasm (Laporte et al., 2002). Domouhtsidou and Dimitriadis (2000) found, in mussels, that Hg was concentrated in the abfrontal part of the gill filament and in the lysosomes and residual bodies of the gut tissue. Similarly, Jensen and Baatrup (1988) found that while Hg was widely distributed, it was concentrated in the apical part of intestinal epithelial cells and was mostly within cell lysosomes that are probably responsible for the sequestration and elimination of the metal.

Hg levels increase as it is passed up the aquatic food chain, resulting in relatively high levels of mercury in fish, where it is mainly (>80%) found in the methylated form (Fergusson, 1990) and ultimately consumed by humans. Furthermore, there is recent evidence suggesting that the high levels of methyl mercury in some predators in the Atlantic most probably originate from hydrothermal vents and deep ocean sediments, and not from increased pollution as previously thought (Kraepiel et al., 2003). Under 'hydrothermal' conditions, when the hot water interacts with the volcanic rocks, mercury and other elements are leached out and remain in solution in the thermal fluid until it reaches the sea floor (Prol-Ledesma et al., 2002) giving rise to cinnabar deposits (Stoffers et al., 1999) or alternatively, undergo methylation being transformed into organic forms by micro-organisms. However, in the water surrounding mussel communities, because of the extreme reducing conditions and sulphide load (Sarradin et al., 1999), formation of the stable HgS would prevent much of the mercury from being methylated (Fergusson, 1990) that may indicate preponderant presence of the metal in its inorganic form. Acknowledging the problems associated with prediction of chemical species of trace metals present under such dynamic ecosystems, we cautiously make assumptions on putatively elevated levels of inorganic Hg. At any rate, invertebrates here are exposed to the metal for periods with geological time-scale, and thus may have developed effective detoxification mechanisms that may have deeply rooted evolutionary significance. Typical mechanisms of handling high levels of toxic elements in species inhabiting this mixing zone at hydrothermal vents are unresolved to date.

The genus *Bathymodiolus* has 11 species known to date, having world-wide distribution (Von Cosel et al., 1999); *Bathymodiolus azoricus* is endemic at many of the hydrothermal vents of the MAR. Its ecological success under the extreme environment of the vent is due to simultaneous nutrient uptake from several sources: firstly by harbouring endosymbiotic chemoautotrophic bacteria (both methanotrophs and thiotrophs) within specialised cells of the gills (Pond et al., 1998), secondly, by filter-feeding (Page et al., 1991; Le Pennec and Bejaoui, 2001) and finally it can also absorb and incorporate dissolved amino acids (Fiala-Medioni et al., 1986; Fiala-Medioni et al., 1994; Fiala-Medioni et al., 2002; Fisher et al., 1988).

B. azoricus has been chosen as a model organism for this study, primarily because it naturally accumulates high levels of heavy metals, including Hg. The main organs accumulating heavy metals are the byssus followed by the gill and the digestive gland (E. Kadar, J. J. Powell, V. Costa and R. S. Santos, unpublished) in agreement with results of other authors (Rousse, 1998; Fiala-Medioni et al., 2000). Mantle levels are lower, this organ being mostly devoted to storage and secretion of the shell. We measured Hg levels in tissues of *B. azoricus* from the MAR vent sites and highest total Hg was recorded in gills of mussels collected from Rainbow (average 12 $\mu g\ g^{-1}$ dry weight) followed by those from Lucky Strike (8 $\mu g\ g^{-1}$). Mussels collected from Menez Gwen (MG) had lowest concentrations among vent sites of the MAR (2.85±0.3 $\mu g\ g^{-1}$ in the digestive gland, 2.4±0.7 $\mu g\ g^{-1}$ in the gill, 1.6±0.4 $\mu g\ g^{-1}$ in byssus threads and 0.4±0.2 $\mu g\ g^{-1}$ in the mantle), possibly because of the local exposure levels linked to the undiluted fluid chemistry (Charlou et al., 2000) and/or metal speciation. In spite of these relatively low Hg concentrations in mussels, MG was chosen for experimental sampling because it is the shallowest (–870 m), and thus long-term laboratory maintenance of mussels is possible without specialised pressure gear (Kadar et al., 2005). Another reason for selecting this particular organism for our experiment was its feeding flexibility that enables contact with both soluble and particulate forms of the metal that would impose highly efficient detoxification mechanisms. The extent of exposure and thus the potentially toxic effect of a metal in a bivalve will depend primarily on the duration of direct contact with the toxic agent, i.e. when the shell is open. The present study therefore, examines duration and frequency of shell gaping activity, indicative of filter feeding in other bivalves (Kádár et al., 2001; Riisgård, 2003), in order to assess the response of the vent bivalve *B. azoricus* to inorganic Hg. In a filter feeder bivalve, under optimal conditions of feeding, the adductor muscle is

relaxed and the valves are open. Extended periods of shell gape are interrupted by rest periods, giving a continuous pattern of periodicity that may, however, be perturbed by changes in environmental conditions, thus providing a measure of the impact of pollutants (Salánki, 1992; Kontreczky et al., 1997). In our experimental conditions, and based on our previous finding on the total loss of endosymbiont bacteria in *B. azoricus* following transfer to sulphide/methane-free seawater for up to 30 days, the assumption is made that mussels were filter-feeding. Thus, shell gaping as proportional to the time engaged in filtering of *B. azoricus* was monitored following 21 days exposure to inorganic Hg followed by an additional 21 days in Hg-free seawater to look into recovery processes. Exposure concentrations, i.e. daily addition of 100 nmol l^{-1} (20 μg l^{-1}) for 21 days, were above those encountered in its natural habitat (an average of 0.18 nmol l^{-1} total Hg was measured in discrete water samples from above mussel clumps at Menez Gwen; data not shown) in order to induce a well assessable behavioural response. Total Hg in tissues (gill, digestive gland, mantle, foot and byssus threads) were measured during and after exposure in order to determine biological half life and uptake/release dynamics. Water Hg concentrations were also monitored for dosage evaluation. Specific Hg-handling strategies that enable these vent bivalves to survive under the hostile hydrothermal environment are discussed with emphasis on the metal–organism interactions in this unique habitat.

Materials and methods

Animal collection and maintenance

Animals were obtained from Menez Gwen (MG) hydrothermal vent site (31°31′W, 37°50′N) by recovering acoustically retrievable mussel cages. These cages were placed close to venting exits where natural mussel clumps are present at MG, during the SEAHMA I cruise in August, 2002, using the telemanipulated arm of the Remotely Operated Vehicle (ROV) Victor 6000. Cages were recovered in February 2003, using the small R/V *Arquipelago*. Fitness of mussels was satisfactory as indicated by their gill appearance, foot-waving and by absence of mortality during acclimatisation to laboratory conditions in the LabHorta set-up specifically designed for the long-term maintenance of the vent mussel for post-capture analysis (for detailed information see http://www.soc.soton.ac.uk/ventox/files/focuson/labhorta.html).

Experimental design

The experiments carried out for this study complied with the current pertinent laws in Portugal. A total of 80 adult *Bathymodiolus azoricus* (Von Cosel) (shell length 7–11 cm) were used in the experiment carried out between February and May 2003. Twenty individuals were placed in each of the three replicate, 8 l volume experimental tanks (HDP containers) containing sand-filtered seawater that was pumped into a reservoir from an unpolluted bay in Horta, Azores (38.5°N 28.7°W). The experiment lasted for 63 days and consisted of 21 days of control in Hg-free seawater followed by exposure to Hg by the daily addition of 160 μl $HgCl_2$ from a commercial standard solution of 1000 mg l^{-1} at pH 2, yielding a final added concentration of 20 μg l^{-1}, followed by 21 days of recovery in Hg-free seawater. Full water changes were completed at the end of each week. One additional aquarium hosted twenty individuals in seawater with no Hg addition and served as control. Prior to water changes, i.e. on a weekly basis, water samples were taken for total Hg, both prior to and after filtration, and pseudofaeces was collected using decontaminated Pasteur pipettes. We termed ‘pseudofaeces’ the gelatinous, mucus-bound material and care was taken to separate it from true faeces that is darker and has a more consistent texture. On days 7, 15 and 21 (control, exposure and post-exposure, respectively) five specimens from each replicate tank (including the untreated control) were measured and dissected into the main organs of suspected Hg metal storage (i.e. gill, digestive gland, mantle, foot and byssus) and kept frozen until dehydration, acid digestion and analysis. Animals removed for dissection were replaced by marked mussels, with similar shell length in order to keep variations in the system’s equilibrium to a minimum.

Water parameters (pH, dissolved oxygen and temperature) were measured daily using portable sensors, and were maintained constant during the experiment: dissolved oxygen ranged between 65 and 80%, pH was 8±0.4 and temperature ranged between 7 and 9°C (same as the mussels’ natural habitat at Menez Gwen). Average total Hg concentrations were measured both in the filterable and the filter passing fractions, prior to each weekly water change and were as follows: (a) during the 21 days of the pre-exposure period, referred to as control: not detectable (detection limit of the method employed was 6 ng l^{-1}); (b) during 21 days exposure, water Hg ranged between 25 and 35 μg l^{-1} with a constant total/filter-passing Hg ratio around 1.5; (c) during recovery, while animals were kept in Hg-free seawater, total Hg decreased to values below 2 μg l^{-1}.

Shell gape monitoring

The shell gaping activity of three animals (one in each replicate aquarium) was recorded using a device called ‘mussel actograph’ (Veró and Salánki, 1969) which is shown schematically in Fig. 1. This device permits continuous monitoring of adductor muscle activity, without disturbing the free movement of the mussels, using the magnetic field built up between the shells while opening and closing. Duration of shell opening and closing was recorded continuously over the 63-day experiment (21 days control, 21 days exposure and 21 days recovery). Using the signal analysis system Signalview (1994 Real Time Devices USA, Inc.) calculation of average duration and frequency of shell gaping was possible simultaneously for three animals. Based on the positive correlation between shell gape and filtration rate [reviewed by Riisgård (2001) and experimentally supported by Riisgård (2003)] when the shell is widely open, we only considered the duration of shell gapes when shells were open

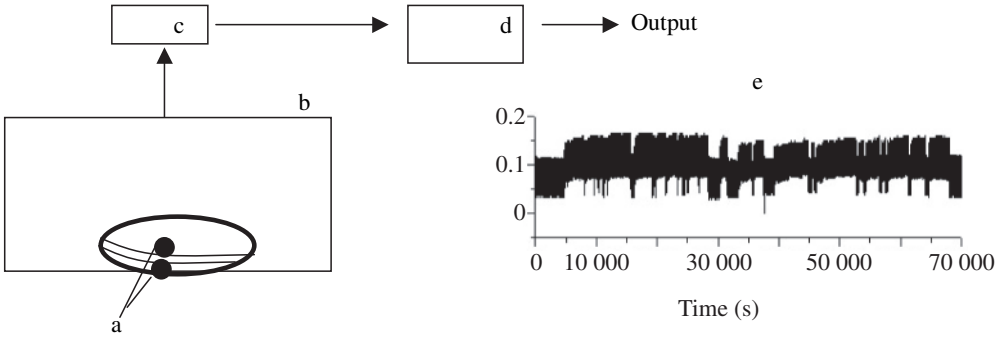

Fig. 1. Schematic diagram of the apparatus used for monitoring shell gaping of the vent mussel *B. azoricus* consisting of (a) a pair of electric sensors attached to the edge of the shell that permits free movements of the animal; (b) experimental closed-system aquarium housing 20 animals; (c) signal amplifier and (d) computer interface that uses the signal analysis system Signalview (Real Time Devices USA, Inc., 1994) to produce the output (e), the 'mussel actograph'.

to their maximum. In order to see possible exposure-time-dependent effect of Hg, duration of consecutive shell gaping was averaged for 7-day sub periods within each treatment. This experimental design made it possible to compare the nine time periods as well as the three treatments using analysis of variance in order to demonstrate potential behavioural changes induced by Hg as well as its exposure-time-dependent effect.

Water sampling for total and filter-passing Hg analysis

Aliquots of 10 ml were taken prior to each water change, on day 7, 15 and 21 (of the control, exposure and recovery periods respectively), from the middle of the aquarium, 10 cm below the water surface. Samples were acidified to pH 2 by adding concentrated HNO_3 and kept refrigerated in HDP vials until analysis. Identical samples were taken and filtered through 0.45 μm pore size Teflon membranes (Millipore, Iberica, Spain) and stored as above for total Hg analysis in the filter-passing fraction that would represent chiefly dissolved forms of the metal. Water samples were post-digested by bromination and total Hg was analysed by Cold Vapour Atomic Fluorescence Spectrometry on the PSA Millenium Merlin System (PSA Analytical, Orpington, Kent, UK) using a tin (II) reductant (Millenium Merlin 2001 method for total mercury in drinking, surface, ground, industrial and domestic waste waters and saline waters). Standards were prepared in filtered and acidified seawater using a commercially available 1000 mg l^{-1} Hg standard solution (Merck), and ranged between 0 and 100 μg l^{-1}. Accuracy of the analytical method was monitored by analysing sample spikes and blank spikes. Spike recoveries were between 86 and 115%.

Tissue partitioning of Hg

Whole tissues (gill, mantle, digestive gland, foot and byssus threads) and pseudofaeces samples were acid digested prior to analysis as previously described (Jugdaohsingh et al., 1998). Briefly, 0.5–1 g of dry tissue (previously lyophilised using a Savant refrigerated vapour trap system, overnight) was digested with equal volumes (1 ml added to 0.1 g dry weight) of Aristar grade concentrated (69% v/v) nitric acid and 30% hydrogen peroxide. Digested samples were diluted 12× with high purity deionised water prior to analysis on the previously described PSA for total Hg using a tin (II) reductant (Millenium Merlin method for determination of total mercury in mussel homogenate – *Mytilus edulis*, Application note 019). Standards were prepared in 4% HNO_3 using 1000 mg l^{-1} Hg standard solution, and ranged between 0 and 20 ppb (parts per billion) for control animals, and between 0 and 200 ppb for mussels dissected during and after exposure. The accuracy of the analytical method was monitored by analysing two different certified reference materials: CRM 414 (plankton) and CRM 278R (mussel tissue), sample spikes and blank spikes. For both reference materials measured, Hg concentrations were within 10% variation from the certified values. Spike recoveries were between 86 and 115%.

Statistical analysis

Factorial analysis of variance was used to test statistical significance of differences in Hg concentration in tissues as compared to control values, as well as differences in duration of shell gaping periods. Specific differences between treatments were examined *a posteriori* using the Student–Neuman–Keuls (S-N-K) multiple range test (Steel and Torrie, 1980). Normal distribution of data was confirmed by the One-Sample Kolmogorov–Smirnov test. Calculations were made using SPSS (SPSS Inc. 1989).

Results

Total Hg partitioning in soft tissues, byssus threads and pseudofaeces

Mercury levels increased significantly ($P<0.05$) in all organs after only 1-week exposure as compared to control levels (Fig. 2). Levels in the gill and digestive gland continued to increase with exposure time, reaching highest levels (in dry weights) of 270±70 μg g^{-1} and 245±52 μg g^{-1}, respectively, on day 21 of exposure. The exceptions were the mantle and foot where levels remained similar over the entire exposure period ($P<0.05$). Byssus threads accumulated highest concentrations among all organs, reaching peak levels of 442±89 μg g^{-1} on day 15 followed by decrease by day 21 of exposure. These levels correspond to bioconcentration factors (calculated as the tissue:water Hg ratio) with an order of magnitude of 10^4, considering an average 25 μg l^{-1} total Hg in the water column. As a result of placing animals in Hg-free seawater for 21 days, metal levels in all organs investigated decreased, but remained

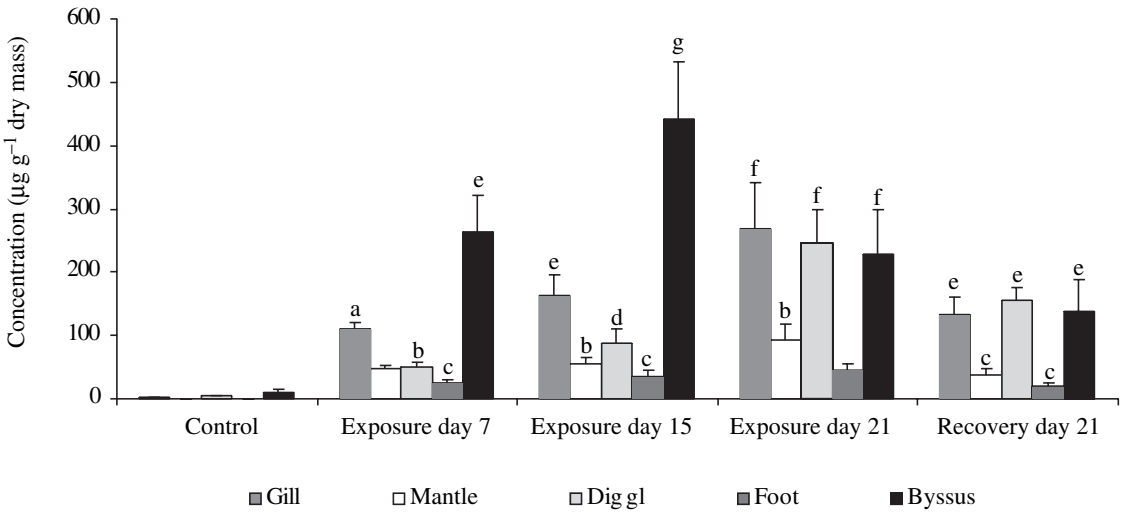

Fig. 2. Total Hg concentration in the soft tissues (gill, digestive gland, mantle and foot) and byssus threads of the vent mussel *B. azoricus* kept in seawater for 3 weeks (Control), followed by daily exposure to 20 µg l^{-1} inorganic Hg for 3 weeks (Exposure) followed by transfer to Hg-free seawater for an additional 3 weeks (Recovery). Vertical bars represent mean concentrations ± S.E.M. (*N*=15) in animals dissected at the end of each week of Control, Exposure and Recovery. Statistical differences between tissue levels at different experimental times was tested by one-way analysis of variance. Columns with the same letters are not significantly different according to a S-N-K multiple range test ($P<0.05$).

significantly above pre-exposure levels: byssus, gill and digestive gland 137±50, 132±28 and 156±18 µg g^{-1}, respectively.

Concentration of Hg in pseudofaeces (samples pooled for all animals in individual aquaria) were one order of magnitude higher than in soft tissues and peaked on the second week of exposure with over 1000 µg g^{-1} that dropped to 800 µg g^{-1} by the end of the 21-days exposure and remained elevated (around 600 µg g^{-1}) on day 21 of recovery (Fig. 3).

The effect of Hg on shell gaping activity

Duration of shell opening and closing was continuously monitored during the 63-day experiment – 21 days under control with no Hg addition, followed by 21 days exposure to Hg and a further 21 days of recovery in Hg-free seawater. Sum of the consecutive shell gapes over 1-week sub-periods of the control, exposure and recovery are shown in Fig. 4A together with the frequency of shell openings (Fig. 4B). The full statistical details are given in Table 1. Under control conditions mussels spent 54% of total time with their shells widely open (average 5500 min per week), the average duration of consecutive shell gapes being 37 min, i.e. frequency of 150 times/week. Exposure to Hg caused a significant increase ($P=0.001$) in the total time that animals spend with open shells as compared to pre-exposure. The frequency of consecutive shell openings decreased significantly ($P<0.001$) as shown in Fig. 4. Following placement of animals in Hg-free seawater the time that mussels spent with open shells further increased ($P<0.001$) as compared to during exposure, but no significant difference was detected ($P<0.05$ level), in the frequency of shell gaping, i.e. the consecutive shell gapes became longer. Time of exposure as an influencing factor, *per se*, did not have significant impact ($P<0.05$) on shell gaping as the 7-day sub periods of the three treatments (control, exposure and recovery) were similar.

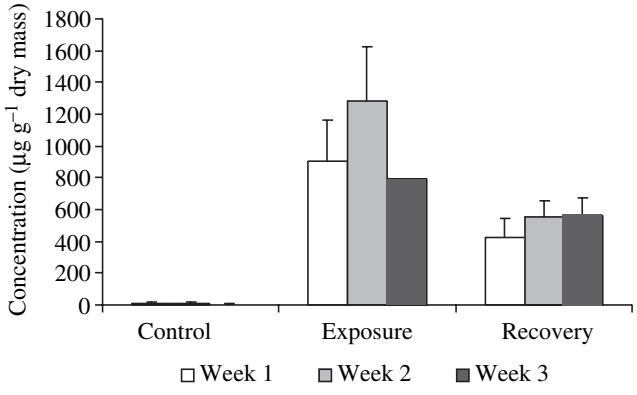

Fig. 3. Total Hg concentration in the pooled pseudofaeces of 20 mussels that were kept in seawater for 3 weeks (Control), then exposed daily to 20 µg l^{-1} inorganic Hg for 3 weeks (Exposure), followed by transfer to Hg-free seawater for a further 3 weeks (Recovery). Vertical bars represent means ± S.E.M. (*N*=3).

Discussion

These results demonstrate a clear effect of Hg on shell gaping of *B. azoricus*, consisting of the increase in duration and reduction in frequency of consecutive shell opening periods, which did not seem to recover to pre-exposure levels following transfer of mussels to Hg-free seawater for periods as long as those of exposure. This behaviour may indicate that *B. azoricus* does not respond to Hg exposure by closing its shell and thus reducing uptake, as observed in other bivalves exposed to toxic compounds (Salanki et al., 2003; Kadar et al., 2002, 2001; Kontreczky et al., 1997). The mechanism of avoidance is explained by the ability of bivalves to respire anaerobically for prolonged periods, keeping their shells closed under stress, such as Hg-exposure (Devi, 1996), achieved in

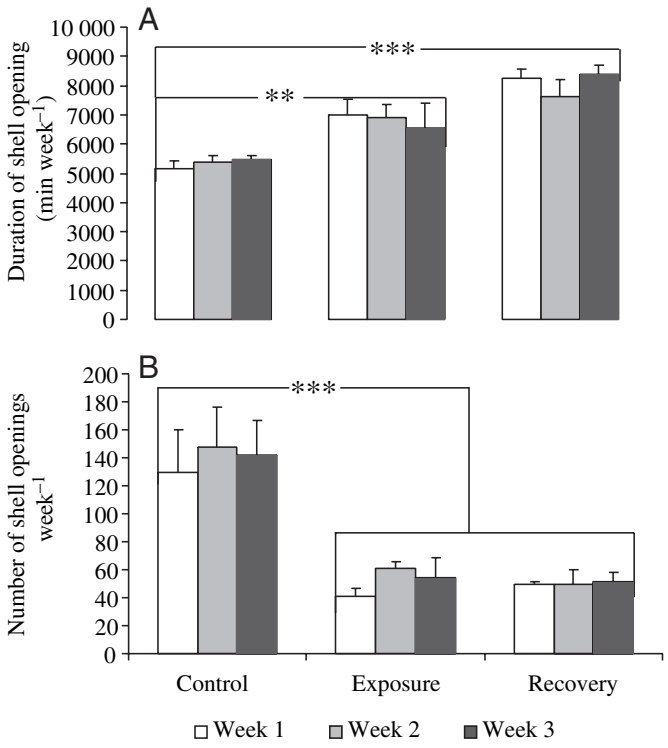

Fig. 4. Effect of inorganic Hg on (A) the duration and (B) frequency of shell gaping activity of the vent mussel *B. azoricus* during 3 weeks in seawater (Control) followed by 3 weeks of daily exposure to 20 μg g^{-1} inorganic Hg, and an additional 3 weeks in Hg-free seawater (Recovery). Vertical bars represent means of replicate mussels ± S.E.M. (N=3). Statistical significance of Hg on shell gaping was tested by one-way analysis of variance, and *post-hoc* tests were performed to detect differences at different experimental moments, indicated by asterisks (**P=0.001 and ***P<0.001). See Table 1 for further details.

spite of the energy-expensive contraction of the adductor muscle responsible for shell closure. In fact, such 'shell closing response' was observed in *B. azoricus* as a result of disturbance during shipment and laboratory manipulations. By contrast, the animals responded to Hg exposure by increased shell gapes. A similar increase in shell gaping was documented in the freshwater bivalve *Anodonta cygnea* as a response to aluminium exposure, but the effect was followed shortly by inhibition of filtration (prolonged shell closure), and was termed a biphasic effect (Kádár et al., 2002). The vent bivalve does not seem to prevent Hg toxicity by closing its shell, and thus significant increase in tissue concentrations would be expected. However, tissue concentrations in the main target organs – gills and digestive glands – reaching bioconcentration factors with the order of magnitude 10^4 on day 21 of exposure, closely matched concentrations reported in *Crassostera gigas* and *Mytilus edulis* under similar laboratory-exposure conditions. The fact that these levels generally exceed levels reported for invertebrates inhabiting Hg polluted areas (data compilation in Jorgensen et al., 2000), together with the lack of shell closure, may be an indication that an efficient mechanism for the elimination of the metal in *B. azoricus* is operating, most probably via mucus that may prevent the inhibitory effect of the metal. The extremely high pseudofaeces Hg concentrations, both during and after exposure, are of significance and may indicate the important role of mucus in detoxification of Hg. Biogenic mucus produced by other vent invertebrates was previously reported to bind trace elements including Hg, and was proposed as a dual internal and external detoxification mechanism in the alvinnelid polychaete worm (Juniper et al., 1986). Similarly, the ctenidial mucus on the gill of bivalves is exposed to the water to be filtered (MacGinitie, 1941; Ballantine and Morton, 1956; Fretter and Graham, 1976). Therefore it is likely that mucus traps Hg and acts as a barrier to uptake in this study. Under normal filtering conditions, the mucus layer is continuously renewed, providing new binding sites for the metal, and thus restricting uptake of Hg from the water column by binding to its negatively charged glycoproteins (Conrad et al., 1991). Moreover, elevated post-exposure levels in pseudofaeces indicate an additional role played by mucus in depuration of the metal. Mucus is produced in all tissues of *B. azoricus* as evidenced by the presence of goblet cells in all organs (our unpublished TEM observations; data not shown) and thus may be involved in trapping Hg-bearing organelles that are then eliminated by exocytosis during the depuration. Future in-depth ultrastructural investigations on putative Hg bearing organelles and characterization of *B. azoricus* mucus are needed to confirm this hypothesis.

Considering the individual mass of each organ it is notable

Table 1. *Factorial analysis of variance testing the effect of Hg exposure and of the exposure duration on the sum and frequency of shell gape in the vent bivalve* B. azoricus *exposed to inorganic Hg*

		Sum of shell gape		Frequency of shell gape	
Source of variation	d.f.†	MS‡	F§	MS‡	F§
Time	2	8.9×10^4	0.15	380.7	0.4
Treatment	2	1.7×10^7	28.7***	2.3×10^4	26.4***
Time×Treatment	4	3.3×10^5	0.55	90.0	0.1
Error	18				
Total	27				

Exposure was by daily administration of 20 μg l^{-1} $HgCl_2$ for 3 weeks followed by 3 weeks recovery in Hg-free seawater.
†d.f., degrees of freedom; ‡MS, mean squares; §F, F-ratio; ***P<0.001.

the 75% of total Hg body burden was found in the gills, followed by mantle (13%), digestive gland (7%), byssus (3%) and foot (2%). Byssus thread concentrations, otherwise highest among all organs, declined by the third week of exposure, possibly because of renewal of threads. To prove this hypothesis however, a more in-depth investigation on the physiology and renewal dynamics of this organ is needed. However, Hg levels in byssus were consistently an order of magnitude higher than in soft tissues, which is in agreement with our previous results on several heavy metals, including Hg, being consistently principally bioconcentrated in byssus threads of *B. azoricus* from various vent sites of the MAR (E. Kadar, J. J. Powell, V. Costa and R. S. Santos, unpublished). Byssus is a target for metallic elements such as Zn^{2+}, Cu^{2+} and Fe^{2+} (Gundacker, 1999) since they play a significant role in the structural integrity of the thread and are essential for the normal functioning of this organ (Lucas at al., 2002). Metal chelate cross-links created with histidine were reported in other bivalves (Lucas at al., 2002) that may have accounted for the high Hg concentrations measured in the byssus of Hg-exposed *B. azoricus* in this study. Ultrastructural evidence, both qualitative and quantitative, on the byssus-Hg interaction is in preparation in order to assess its role in detoxification of Hg.

Tissue concentrations remained above control levels in all organs following transfer of mussels to Hg-free seawater for 3 weeks, suggesting that the metal is firmly bound and depuration requires a longer period. Biological half-life of Hg was only reached in gills, while digestive gland and byssus still maintained over 50% of the peak exposure levels by day 21 of recovery. This however, approximates to the biological half-life obtained for littoral bivalves (not exceeding 40 days when administered as inorganic Hg, according to data reviewed by Jorgensen et al., 2000). For instance, biological half life of Hg (acetate) in the American oyster *Crassostrea virginica* exposed to two distinct concentrations of Hg (10 and 100 $\mu g\ l^{-1}$) for 40 days that resulted in tissue concentrations of 12 and 92 $\mu g\ kg^{-1}$ Hg (wet weight), was 16.8 and 9.3 days (Cunningham and Tripp, 1975). In spite of these differences in exposure conditions, there is similarity, to a certain extent, of the Hg half-life of the vent mussel with that of its shore analogues, but there is no exact proof, owing to the lack of exposure experiments focused on this aspect.

The range and form of Hg to which *B. azoricus* is exposed in its natural habitat is virtually unknown because of the wide fluctuations of temperatures, pressures and chemical conditions present in the mixing zone. Owing to the reducing conditions and the sulphide ion load in water surrounding mussel communities at Menez Gwen (Sarradin et al., 1999), formation of the low soluble HgS would prevent much of the mercury from being methylated (Fergusson, 1990). This would indicate an unusual inorganic Hg bioavailability at hydrothermal vents. Our experiment also confirms an enhanced Hg bioavailability to the vent mussel in spite of its widely accepted low absorption rate (7-15%) because of its limited membrane-crossing ability when present as inorganic salts, and it is in agreement with conclusions reached by Andres et al. (2002), who compared the data available in the literature with respect to limited bioavailability of inorganic Hg as compared to MMHg. Additionally, our simplified experimental system without inorganic sulphide supply, permitted calculations of partitioning of the metal within the system. Thus, out of the total 3.36 mg inorganic Hg (i.e. daily administration of 160 µl of 1000 $\mu g\ ml^{-1}$ standard solution) approximately 77.7% was bioaccumulated in tissues, 2.7% was bound to mucus and 19.4% remained in the water column. Post exposure tissue levels indicate slow depuration that is typical for MMHg accumulation (Fergusson, 1990). However, it is unlikely that in our system a significant bacterial methylation took place in the water, even if it was not conducted in sterile seawater. Endosymbiotic methylation can be ruled out since animals were acclimatised for 2 weeks followed by 21-days control period in seawater not supplied with inorganic nutrients that is long enough to lose their natural endosymbionts (E. Kadar, J. J. Powell, V. Costa and R. S. Santos, unpublished). Methylation is also possible in the digestive tract via well-documented mechanisms involving CH_3 donors such as methylcobalamin (a derivative of vitamin B_{12}) (Fergusson, 1990). Nevertheless, until these mechanisms are shown in *B. azoricus* and considering our experimental conditions, it is thought that what was largely bioavailable was in its inorganic form and that bioaccumulation took place without lethal consequences and/or shell closure as observed in other bivalves. Therefore an efficient mechanism may have been responsible for sequestering the metal and preventing its toxicity at these levels. Metallothioneins are small, soluble, heat stable proteins known to be involved in the detoxification of Hg, and other trace elements (Ng and Wang, 2000). Although found in relatively high quantity in *B. azoricus* (Rousse et al., 1998; Fiala-Medioni et al., 2000), their levels did not reflect the metal burden of the tissue especially in the symbiont-bearing organ. In other words no relationship between metals and total metallothionein levels was evident, which may also suggest the prevalence of an alternative detoxification mechanism. Future in-depth ultrastructural investigations on putative Hg-bearing organelles may answer these questions.

The relatively unchanged total Hg concentration in the water column at the end of each consecutive week of exposure may suggest that duration of exposure did not have notable influence on the rate of uptake, which is consistent with the prolonged shell gapes. Up to one third of the total Hg from the water column was retained by 0.45 µm pore-size membranes, indicating the presence of Hg-hydroxipolymers and/or Hg adsorbed onto particles. However, a complete picture on Hg speciation during the experiment is beyond the scope of this study, and it would involve more frequent sampling, and also methyl-Hg analysis. We instead, provide evidence for the bioavailability of inorganic Hg to the vent bivalve that resulted in the increase in duration, with simultaneous decrease in frequency, of its shell gaping. In spite of high Hg levels recorded in tissues, no mortality was recorded during the 63-day experiment, which might indicate an efficient Hg-

detoxification mechanism developed by the vent mussel, and which deserves further investigations.

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