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Postcapture Depuration of Essential Metals in the Deep Sea Hydrothermal Mussel *Bathymodiolus azoricus*

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Metal bioaccumulation in bivalves results from intake (via ingestion and uptake from solution) and excretion (e.g., by the usual excretory routes, granule release, mucus-bound particle rejection, sequestration via byssus renewal, deposition in shells). Accumulated metals are typically detoxified (unless specifically kept in metabolically available form) and then either stored or excreted. A study examining the dynamics of decontamination, at both the organism and organs level, gives a better understanding of the overall metal handling mechanisms involved. Additionally, there is increasing evidence that the uptake and release of metals in some molluscs are influenced by their pre-exposure history, possibly via the induction of physiologic detoxificatory processes (Blackmore and Wang, 2002; Boisson et al., 1998; Ng and Wang, 2004; Rainbow et al., 2004). However, this may not be the general trend for all molluscs (Wang and Rainbow, 2005).

The water surrounding bivalves at deep sea hydrothermal vents is enriched in various metals (Kadar et al., 2005a) with enhanced bioavailability (Fiala-Medioni et al., 2000; Kadar and Costa 2006c; Kadar et al., 2005b, 2006a, 2006b; Ruelas-Izunza et al., 2003). Because of their distinct geochemical setting, these vents provide natural pollution laboratories for the study of adaptations in response to coexposure to metals, which makes vent invertebrates inherently interesting for the investigation of metal uptake and elimination. Moreover, because invertebrates at these vents are exposed to toxicants for durations with a geologic time scale, unlike their shore analogues from contaminated sites in which pollution events are occasional or temporal, hydrothermal species may have developed alternative, effective detoxification mechanisms that deserve investigation. An increasing body of circumstantial evidence supports the putative presence of such alternative metal handling, other than that developed in littoral species in response to metal exposure (Company et al., 2004; Hardivillier et al., 2006; Kadar et al., 2005b; Leignel et al., 2005).

Hydrothermal environments are metal rich due to interactions of the convective seawater with basaltic rocks inside the ocean crust. This metal-enriched thermal fluid mixes with the oxidized sea water (Prol-Ledesma et al., 2002), forming a mixing zone in which physicochemical parameters fluctuate sharply.

Such dynamic environments host dense macroinvertebrate communities, including the mytilid bivalve *Bathymodiolus azoricus* (Von Cosel et al., 1999), endemic at many hydrothermal vents of the Mid-Atlantic Ridge (MAR). Bathymodiolids are unusual in having a mixotrophic nutrition based on (1) endosymbiosis (harboring within their gill cells both methanotroph- and sulphur-oxidizing bacteria) (Fiala-Medioni et al., 1986, 2002; Kadar et al., 2005b), (2) direct ingestion or filtration of particulate organic matter (Le Pennec and Bejaoui, 2001; Page et al., 1991), and (3) ability to absorb dissolved amino acids directly (Fiala-Medioni et al., 1994; Fisher et al., 1988). Such mixotrophy not only allows a broad distribution of this species relative to the effluent, as compared with other vent fauna (Fisher et al., 1989), but also renders metals bioavailable in both particulate and dissolved forms.

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Metal exposure levels at the Menez-Gwen hydrothermal vent were reported as being lowest among MAR vent sites, but still significantly above levels in ordinary seawater (i.e., Cu concentrations ranged from 0.17 to 0.6 μM, Fe from 0.2 to 0.3 μM, and Zn from 0.02 to 1.2 μM) (Kadar et al., 2005a). Despite these relatively low exposure levels, the Menez-Gwen hydrothermal vent was chosen as the sampling site because it is the shallowest among MAR sites (–870 m), and thus mussels can overcome decompression stress associated with their collection. Therefore, the long-term laboratory maintenance (over 1 year) is possible at 1 atm, without specialized pressure equipment (Kadar et al., 2006d; and revised in Kadar and Powell, 2006e). Additionally, the geographic location of the Menez-Gwen hydrothermal vent site permits frequent sampling via deployment of acoustically retrievable cages. This ensures sustainable postcapture experimentation on vent bivalves (Kadar and Powell, 2006e).

Although consideration has been given to trace metal accumulation in various hydrothermal species, including *B. azoricus*, no study has focused on metal depuration known to be influenced by both tissue type and age. We have therefore conducted a laboratory experiment aimed at assessing the essential metal loss from the various tissues (gill, digestive gland, mantle, and byssus threads) of the hydrothermal bivalve *B. azoricus* over a 30-day depuration period. In doing so, determination of the biologic half-life and distinctive features of metal handling in different organs and in mussels from distinct size groups was possible. Specific metal-handling strategies that enable these vent bivalves to survive under the hostile hydrothermal environment are discussed with an emphasis on the metal–organism interactions in this unique habitat.

Materials and Methods

Animals were collected at the Menez-Gwen hydrothermal vent site (31°31'W, 37°50'N) during the SEAHMA I cruise in the summer of 2002 using the telemanipulated arm of the remotely operated vehicle Victor 6000 of the R/V Atalante. The health condition of the mussels was satisfactory, as indicated by their gill appearance, foot waving, and lack of mortality during acclimatization to laboratory conditions in the refrigerated unit of the R/V Atalante. The mussels were kept in natural seawater pumped into the aquaria from an unpolluted bay in Horta (38.5°N 28.7°W), which was changed every 48 h. Because the mussels survived for periods exceeding 1 year in natural seawater (Kadar et al., 2006d), we assumed that filter feeding in *B. azoricus* is a viable alternative to harboring endosymbionts.

A total of 64 mussels representing two distinct size groups (shell lengths, 2–4 and 5–7 cm) was used for the study. The experiment lasted for 30 days during the summer of 2002. At each time interval, 16 individuals (8 mussels from each size group) were dissected as follows: initial, day 7, day 15, and day 30 of depuration. The animals were kept in 15-L volume experimental tanks (high-density polyethylene containers) filled with seawater cooled to an average of 8°C. Water parameters were measured daily using portable sensors, and maintained constant during the experiment (pH of 8 ± 0.4, dissolved oxygen (DO) of 75% ± 5 % of saturation, and temperature of 8°C ± 1°C), which was closed to levels in the mussels' natural habitat at the Menez-Gwen vent. Full water changes were completed every 48 h after collection of pseudofeces using decontaminated Pasteur pipettes. We termed pseudofeces the gelatinous, mucus-bound material that was collected. Care was taken to separate it from true feces, which was dark in color and appeared more consistent in texture. Pseudofeces samples were pooled each week and kept frozen until dehydration and analysis.

Whole tissues (gill, mantle, digestive gland, and byssus threads) from mussels dissected at each experimental sampling time (i.e. initial, day 7, day 15, and day 30 of depuration) were lyophilized, as were pseudofeces samples pooled for weekly periods and for the experimental container. Dried samples were subjected to acid digestion before analysis, as previously described (Jugdaohsingh et al., 1998). Briefly, 0.5 to 1 g of dry tissue (previously lyophilized using a Savant refrigerated vapor trap system overnight) was digested with equal volumes (1 mL/0.1 g dry weight) of Aristar grade concentrated nitric acid (69% v/v) and 30% hydrogen peroxide. Tissue digests were diluted ×3 with high-purity deionized water before analysis by flame atomic absorption spectrophotometry (GBC-Avanta Σ, Dandenong, Australia). Standards were prepared using commercially available 1,000-ppm standard solutions (Merck) added to 0.5 N of HNO_3 and ranged between 0-9 ppm for Fe, 0 to 5 ppm for Cu, and 0 to 1.5 ppm for Zn. The accuracy of the analytical method was monitored by analyzing certified reference material: NRCC- TORT (lobster hepatopancreas), DOLT (dogfish liver), CRM 278R (mussel tissue), and CRM 414 (plankton). The recovery of metals in reference samples ($n = 6$) was within 10% of the certified values in the reference materials, and the sample spikes and blank spikes recoveries were between 90% and 116%.

Factorial analysis of variance (ANOVA) was used to test the statistical significance of the influence of shell length and tissue type on metal depuration. Specific differences between treatments were examined a posteriori using the S-N-K multiple-range test (Steel and Torrie, 1980). The normal distribution of data was confirmed by the One-Sample Kolmogorov–Smirnov Test. Calculations were made using SPSS (SPSS Inc., 1989).

Results and Discussion

The analysis of essential metals in various tissues of *B. azoricus* showed that under natural conditions at the Menez-Gwen hydrothermal vent (initial), Fe is the most strongly accumulated metal (average concentration, 266 ± 34 µg/g of dry weight tissue), followed by Zn (157 ± 9 µg/g) and Cu (79 ± 10 µg/g). The highest metal concentrations were detected in the digestive gland and gills (Fig. 1). Mantle accumulated the lowest concentration of both Fe (average, 60 µg/g) and Cu (average, 30 µg/g), as shown in Fig. 1a and b. In contrast, Zn concentrations were equally distributed among tissues (Fig. 1c). The concentration of all metals in byssus threads was more than 10-fold higher than in soft tissues, and had the highest concentration of Zn (average, 2,500 µg/g) followed by Fe (average, 1,400 µg/g) and Cu (average, 500 µg/g) (Fig. 2).

Our results for the initial essential metal concentrations recorded in soft tissues of *B. azoricus* are in agreement with those previously reported for this species from the Menez-Gwen hydrothermal vent (Kadar et al., 2006b, Rousse et al., 1998), and also have an order of magnitude similar to that reported for the hydrothermal vent clam *Vesicomya gigas* from the Guyamas basin (Ruelas-Inzunza et al., 2003). Elevated essential metal concentrations in hydrothermal species are known to be associated with their physiologic adaptations to their environment. Elevated Zn concentration in vent invertebrates is associated with the presence of a sulphide-transporting protein and with Zn at its active site (Childress et al., 1993; Flores et al., 2005), whereas high tissue Fe is associated with abundant intracellular hemoglobin-transporting oxygen to the site of C fixation, that is, to the endosymbiont bacteria within the gill of the vent clam *Calyptogena magnifica* (Ruelas-Inzunza et al., 2003; Terwilliger et al., 1983). Although the presence of such metal-bearing molecules has not yet been described in *B. azoricus*, its high concentration of essential metals even after 30 days of depuration may indeed suggest a physiologic role beyond the function they play in

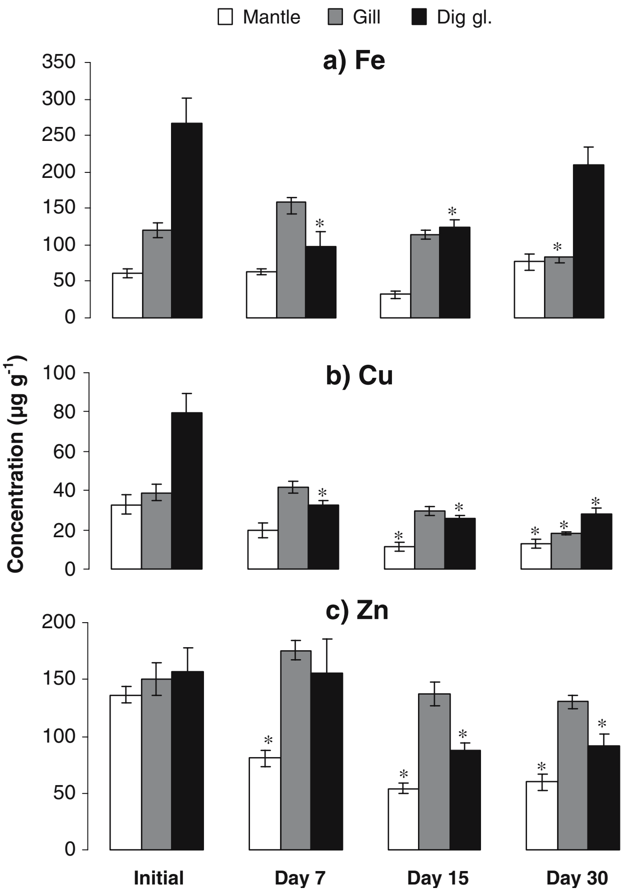

Fig. 1 Concentration of the microessential metals (a) Fe, (b) Cu, and (c) Zn in soft tissues (mantle, gill, and digestive gland) of the hydrothermal mussel *Bathymodiolus azoricus* collected at the Menez-Gwen vent site (initial) and subsequently placed in metal-free seawater for 30 days for depuration. Vertical bars represent means ± standard error of the mean (n = 16), and asterisks represent significant differences as compared with control levels ($p < 0.05$)

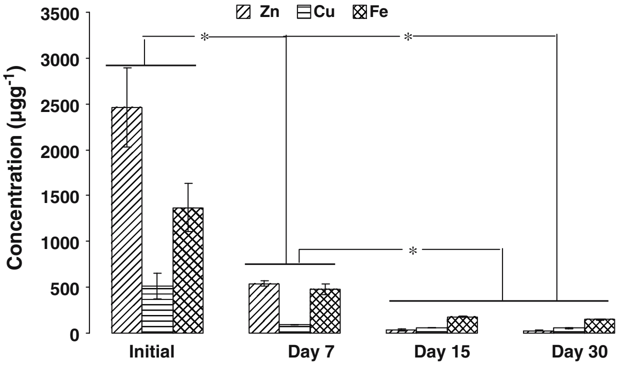

Fig. 2 Concentration of microessential metals (Cu, Fe, and Zn) in byssus threads of the hydrothermal mussel *Bathymodiolus azoricus* from the Menez-Gwen vent site (initial) and during the 30 days of depuration. Vertical bars represent means ± standard error of the mean (n = 5), and asterisks represent significant differences as compared with control levels ($p < 0.05$)

Table 1 Factorial analysis of variance (ANOVA) testing the effect from the duration of depuration, the organ type, and the shell length on the concentration of microessential metals (Fe, Cu, and Zn) in the vent bivalve *Bathymodiolus azoricus* that underwent depuration in metal-free seawater for 30 days

Source of variation	*df* [a]	Fe *MS* [b]	*F* [c]	Cu *MS* [b]	*F* [c]	Zn *MS* [b]	*F* [c]
Organ	2	$1.3\ 10^5$	62.1***	4.3^3	24.1***	$4.1\ 10^4$	19.4***
Size	1	$8.3\ 10^4$	36.9	$2.6\ 10^3$	15.1***	$2.3\ 10^3$	1.2
Depuration time	3	$4.5\ 10^4$	20.2***	$1.1\ 10^4$	59.8***	$1.6\ 10^4$	7.4***
Organ x size	2	$5.4\ 10^3$	2.4	$6.4\ 10^2$	3.57*	24.3	0.01
Organ x depuration	6	$2.3\ 10^4$	10.4***	$2.1\ 10^3$	12.1***	$5.7\ 10^3$	2.6*
Size x depuration	3	$1.3\ 10^4$	6.1**	$1.6\ 10^3$	8.9***	$2.3\ 10^4$	11.1***
Error	136						
Total	160						

[a] df = degrees of of freedom.

[b] MS = mean squares.

[c] F = F-ratio.

Probability levels for significant effects: $p<0.001$(***); $p<0.01$(**); $p<0.05$(*).

nonhydrothermal bivalves, which deserves further investigation.

After the placement of mussels in clean seawater, the concentration of all three metals fell significantly ($p < 0.01$) in all tissues, in a descending order of Cu > Zn > Fe. The concentration decline, however, was both tissue and size group dependent (Table 1). In mantle, the tissue that naturally contained the least of these metals, the concentration of Fe remained close to initial values, whereas Cu and Zn approached depuration half-time (T50, i.e., 50% of the control concentration) by day 15 of depuration (Fig. 1). During the first week, there was no loss from gills either, and T50 was reached only on day 30 of depuration for Cu and Fe, whereas Zn remained elevated (not significantly different from the initial $p > 0.05$ level) (Fig. 1). In the digestive gland, where initial concentrations were highest, T50 for both Fe and Cu was attained before day 7 of depuration, unlike Zn, which remained at the predepuration levels (i.e., as in animals from the wild). Curiously, after further depuration, Fe concentration in the digestive gland increased to 80% of initial values. Concentration of Zn in the digestive gland reached 50% of initial levels by day 15, and remained constant toward the end of the experiment (Fig. 1). Copper levels also remained constant, but below 50% of the initial concentration, which was reached from day 7 of depuration.

Our data obtained over the 30 days of depuration permitted an insight into essential metal dynamics showing specificities when the processes were analyzed at the organ level and in comparison with nonvent species. The bivalve gill, for instance, is widely known to lose bioaccumulated metals rapidly when placed in a metal-free environment (Chong and Wang, 2001; El-Shenawy, 2004; Inza et al., 1998; Ng and Wang, 2004). This does not appear to be the case with *B. azoricus*. The reason may be related to the presence of endosymbiont bacteria within its specialized gill bacteriocytes, which survive up to 15 days in seawater lacking bacterial nutrients, followed by their gradual disappearance within 30 days (Kadar et al., 2005c). Weight deficit in gills resulting from symbiont loss may have masked some metal loss. In other words, concentrations per unit of dry tissue may have remained the same or not much diminished, but actual metal burdens were significantly reduced.

These circumstances become even more complex when symbiotic influences of metal metabolism within the gill are considered as well as mobilization of essential metals under stress caused by nutritional perturbation. Endosymbiotic bacteria of vent invertebrates reportedly contribute to metal sequestration (Kadar et al., 2006a, 2006b), possibly by precipitation within their cell wall (Wang et al., 1997). Thus the fate of bacteria during depuration may have an impact on metal concentration of the host gill. Our postcapture laboratory manipulations showed that endosymbiont loss is associated with an increase in both the number and volume of lysosomes responsible for the intracellular digestion of bacteria (Kadar et al., 2005b).

The implications of the preceding data regarding metal burden would indeed be a delay in depuration during the first weeks of depuration in seawater not supplied with nutrients for endosymbiont bacteria. However, even after a prolonged depuration (i.e., 30 days), only a slow release of metals from gills was recorded. Cu was the only metal to reach depuration half-time by day 30, whereas Fe remained at 80% of initial values, and Zn remained close to the initially measured concentrations in mussels at vents. This may indicate constitutive levels of essential metals in hydrothermal mussels above those in their shore analogues, which was further supported by our recent longer depuration experiment (150 days) (for details see Kadar and Costa, http://www.noc.soton.ac.uk/GDD/DEEPSEAS/symp_pages/list_entries.php?action=view_abstract&id=51).

Alternatively, because metabolically available essential metals may be only a small fraction of the total accumulated form, high concentrations of accumulated/stored Fe and Zn may simply suggest an efficient "normal" detoxified storage system for Fe and Zn (of a different degree of permanence) in *B. azoricus*, as would be expected in a mussel exposed to high metal bioavailabilites. However, until further in-depth postcapture physiologic investigations are performed, these allegations remain highly speculative.

Byssus thread that initially had many times the concentration recorded in soft tissues showed the most dynamic loss, reaching T50 in fewer than 7 days for all metals, with a final concentration of Zn at 1%, Cu at 10%, and Fe at 3% of the concentrations measured initially (Fig. 2). Byssus thread therefore may be considered an important depuration route via sequestration of metals into the threads, followed by their renewal. This may not, however, be considered as active detoxification because our previous investigations showed that metal concentrations in byssus increased proportionally with exposure levels at geochemically distinct vent sites (Kadar et al., 2006b). This is consistent with earlier findings that metal uptake is not regulated in this organ (Szefer et al., 2002; Yap et al., 2003). Moreover, postcapture ecotoxicologic investigations conducted with Hg-exposed *B. azoricus* indicated that byssus is an important route of Hg elimination (Kadar et al., 2005b), also suggesting a nonspecific metal-binding ability. Byssus thread, apparently the main target for metals, exhibited the greater loss with depuration, possibly attributable to the renewal of the threads after detachment caused by frequent water changes.

The metal burden in byssus may not be as significant as expected from concentrations because of its small weight relative to the organism. Consequently, its role in depuration per se is uncertain. However, because of its avid binding capacity for essential metals, byssus–metal interactions deserve a closer scrutiny.

Size-dependent variations of whole-tissue metal concentrations showed that small mussels initially had higher concentrations of Cu and Fe than large mussels, but during depuration tended to lose more of these metals. This relationship, however, was not clear for Zn. Tissue concentrations of both Cu and Zn were similar for the two size groups by the end of 30-days depuration, whereas Fe concentration in small mussels remained consistently higher than that measured in large mussels (Fig. 3). Although findings have shown body size to be a critical factor influencing metal concentration in marine bivalves (Chong and Wang, 2001; Strong and Luoma, 1981), size-dependent depuration data for bivalves are scarce. Few studies are available, suggesting that body weight does have an influence on

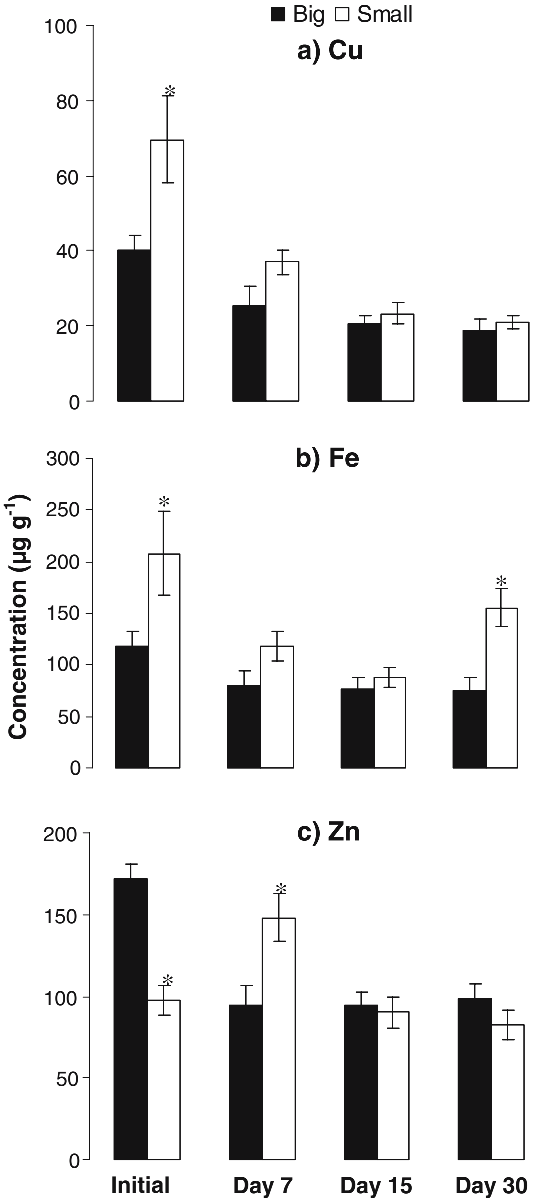

Fig. 3 Depuration of the microessential metals (a) Cu, (b) Fe, and (c) Zn in small (shell length, 3 ± 0.5 cm) versus big (shell length, 8 ± 0.5 cm) *Bathymodiolus azoricus* (total in all tissues) collected at the Menez-Gwen vent site (initial) and subsequently allowed to depurate for 30 days in metal-free seawater. Vertical bars represent means ± standard error of the mean (n = 8), and asterisks represent significant differences between the two size classes ($p < 0.05$)

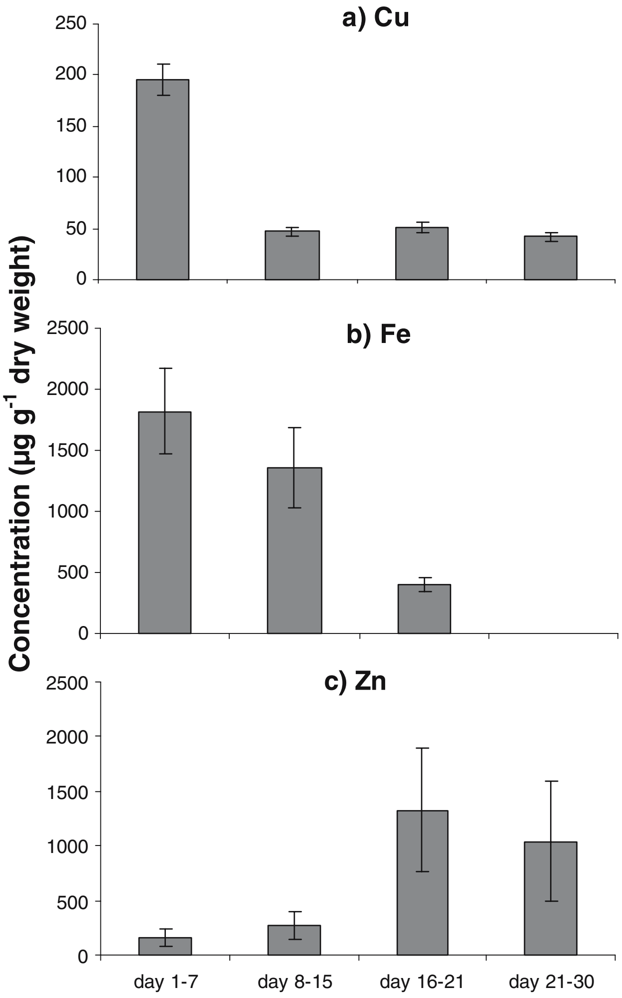

Fig. 4 Concentration of microessential metals (Cu, Fe, and Zn) in pseudofeces pooled for 1-week periods during the 30 days of depuration. Vertical bars represent mean ± standard error of the mean (n = 3 analytical subsamples)

depuration patterns. Using radiolabeled metals to track depuration patterns of Cd, Cr, and Zn, Lee et al. (1998) reported a general trend of metal loss inversely correlated with body weight. These authors suggested that the allometry of the gill surface area determines this relationship. This trend is shown in our study regarding Fe loss, which, however, was not so obvious for Zn, and was significant only for the first week of Cu depuration. Small mussels, possibly because of their higher metabolic activity (Jorgensen, 1954), showed a more dynamic release of metals during depuration.

Concentration of metals in pseudofeces was many times higher in soft tissues, and remained elevated during the whole depuration period, except Fe, which was not detectable in samples collected during the last week of depuration (days 21–30; the detection limit of the instrument was 4×10^{-4} μg/g). Curiously, Zn concentrations in pseudofeces increased with depuration time (Fig. 4). Extremely high concentrations of Fe and Zn in pseudofeces (exceeding tissue levels by an order of magnitude) during the whole period of depuration indicate an important role of mucus in the depuration of metals. This is in addition to its role in preventing uptake by binding positively charged elements to active sites on mucus glycoproteins (Conrad et al., 1991). Similarly, mucus was proposed as a dual internal and external detoxification mechanism in the vent alvinnelid polychaete worm (Juniper et al., 1986). Ctenidial mucus excreted by gill cells in bivalves comes first in contact with the water to be filtered (Ballantine and Morton, 1956; MacGinitie, 1941). It is therefore likely that mucus acts as a barrier to prevent diffusion of metals into the animal's tissue because of the strong affinity to positively charged ions (Whitehead et al., 1996). Elevated metal levels in pseudofeces produced by *B. azoricus* even after 30 days in clean seawater indicate a putative role of mucus in postexposure depuration. It is interesting to speculate on the fate of pseudofaeces and the extent to which those deposited near mussel beds are involved in metal cycling at hydrothermal vents.

The digestive gland seems to act as an acceptor organ as soon as Fe levels in gills. This suggests the possible transport between tissue compartments that resulted in 80% of initial Fe levels by day 30 of depuration. The mechanism involved may imply that metals from the aforementioned lysosomal granules in gills containing digested bacterial remains follow a path that reaches the digestive gland. Alternatively, an increase in hemocytes as an immune response may be responsible for augmented Fe levels in tissues. This is likely not an experimental artifact because an increase in Fe concentration with depuration time also was observed in the mantle. A similar increase in metal concentrations (Cd, Cr, and Zn) with depuration time was observed in soft tissues of nonhydrothermal mussels and clams, and was attributed to organ-to-organ transport (Chong and Wang, 2001).

Our results compared with those obtained from studies on nonvent bivalves with similar preexposure histories and depuration parameters show clear differences not only regarding the magnitude of metal loss, but also concerning its dynamism. For instance, *Ruditapes decussatus* collected from an industrially polluted site in Egypt, and with a similar baseline of Fe (370 μg/g) and Cu (25 μg/g) concentrations, lost more than 99% of its initial burden, reaching 14 and 1.8 μg/g, respectively, after 4 days depuration (El-Shenawy, 2004). The comparative study of Zn depuration in intertidal mussels versus clams (Chong and Wang,

2001) showed a linear decrease in concentration with time for the digestive gland of both species, reaching less than 10% of the initial concentration in mussels and 30% in clams by day 30. This unusual lack of metal depuration in the hydrothermal mussel may be a physiologic adaptation to the typical hydrothermal vent environment, whereas in other macroinvertebrates, Zn is required in sulphide transport to endosymbiont bacteria (Childress et al., 1993; Flores et al., 2005). Likewise, Fe is indispensable in the maintenance of endosymbiosis because of its role in oxygen transport (Ruelas-Inzunza et al., 2003; Terwilliger et al., 1983). These functions may justify an increased constitutive metal requirement in hydrothermal species, as compared with their shore analogues, which deserves further investigation.

In summary, the hydrothermal mussel is physiologically adapted to atypically high metal exposure in both the soluble and particulate phases. By exposing it to metal-free seawater over 30 days, an insight into the essential-metal dynamics was possible, which then could be compared with depuration data available from the literature on nonhydrothermal bivalves. The conclusion can be drawn that (1) depuration of essential metals in *B. azoricus* is slower than the extent reported for nonhydrothermal species, and this may indicate a higher essential metal requirement; (2) there were indications of organ-to-organ metal transport at the onset of depuration; (3) mucus produced by *B. azoricus* plays an important role in the depuration of metals, and this complements its preventive role in metal uptake; (4) it is probable that mucus greatly influences metal cycling at vents, especially considering the number of mucus-producing organisms here (including bacterial biofilms); and (5) small mussels originally had higher concentrations of essential metals and tended to lose Fe and Cu more rapidly than larger mussels.

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