Temporal variation in the antioxidant defence system and lipid peroxidation in the gills and mantle of hydrothermal vent mussel *Bathymodiolus azoricus*

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Received 7 June 2005; received in revised form 16 May 2006; accepted 19 May 2006
Available online 25 July 2006

Abstract

Hydrothermal vent mussels are exposed continually to toxic compounds, including high metal concentrations and other substances like dissolved sulphide, methane and natural radioactivity. Fluctuations in these parameters appear to be common because of the characteristic instability of the hydrothermal environment. Temporal variation in the antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), total glutathione peroxidases (Total GPx), selenium dependent glutathione peroxidases (Se-GPx)), metallothioneins and lipid peroxidation (LPO) in the gills and mantle of the mussel *Bathymodiolus azoricus* from Menez–Gwen hydrothermal vent site was evaluated and related to the accumulated metal concentrations (Ag, Cu, Cd, Fe, Mn and Zn) in the tissues. Maximum antioxidant enzyme activities in the gills were detected in the beginning of summer, followed by a gradual decrease throughout the following months. One year after, the levels of antioxidant enzyme activities were similar to those reported one year before. LPO in this tissue exhibited a similar temporal variation trend. A different pattern of temporal variation in antioxidant enzyme activities was observed in the mantle, with a gradual increase from summer to the end of autumn (November). LPO in the mantle exhibited an almost reverse trend of temporal variation to that of antioxidant enzyme activities in this tissue. Antioxidant defences in the gills of *B. azoricus* were significantly enhanced with increasing concentrations of Ag, Cu and Mn, while negative relationships between antioxidant enzymes and Cd, Cu, Mn and Zn concentrations in the mantle were observed, suggesting different pathways of reactive oxygen species (ROS) production and that these tissues responded differently to the metal accumulation. However, temporal variation in biomarkers of defence and damage were in general similar to coastal bivalve species and can be associated with temporal variations of the physiological status due to reproduction. These variations might also be linked to the highly unstable nature of the hydrothermal environment.

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1. Introduction

The Mytilid mussel *Bathymodiolus azoricus* is a key species in Mid-Atlantic Ridge hydrothermal vents in the Azores region being the most common organism in the Menez-Gwen, Lucky Strike and Rainbow vent fields, covering extensive areas around the active hydrothermal area in high-density clusters at the base and walls of vent chimneys (Desbruyères et al., 2000). The presence of symbiotic sulphide-oxidizing chemosynthetic bacteria in the guts of *B. azoricus* provides the major source of food for these mussels, and it is one of the most important physiological differences between coastal and hydrothermal vent mussels (Kochevar et al., 1992; Nelson et al., 1995). Nevertheless, these mussels also filter the surrounding water, which contains bacterioplankton (Utsumi et al., 1994), holoplanktonic organisms (Berg and Van Dover, 1987; Wiebe et al., 1988; Burd and Thomson, 1994; Kaartvedt et al., 1994; Burd and Thomson, 1995) and planktonic larval stages of vent species (Khripounoff et al., 2000). Hydrothermal vents are deep-sea structures characterized by a relatively hostile environment compared to other ecosystems (Pruski and Dixon, 2003) and enriched in potentially toxic species (sulphide and heavy metals) to which the organisms are exposed (Desbruyères et al., 2000). In Menez-Gwen, the hydrothermal vent fluid is characterized by high metal concentration, including Cd (2 nM), Cu (2 μM), Zn (2 μM) and Ag (4.3 nM), high temperatures (271–284°C), low pH (4.4–4.5), high CO₂ (17–20 mmol Kg⁻¹) and H₂S (<1.5 mM) (Desbruyères et al., 2001; Douville et al., 2002).

A common pathway of toxicity induced by a wide range of environmental toxic compounds is the enhancement of intracellular generation of reactive oxygen species (ROS), “oxygen-derived species” or oxyradicals and comprises both radical and non-radical species (Darley-Uslmar et al., 1995; Reist et al., 1998; Livingstone, 2001). Organisms are able to deal with these radicals by several mechanisms, including the production of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx). When antioxidant systems are insufficient to neutralize the oxyradicals within the cells, damage can occur to the biological membranes resulting in lipid peroxidation (Halliwell and Gutteridge, 1999; Livingstone et al., 2000).

Metals in particular are known to enhance the production of ROS, and consequently increase the oxyradical stress in the tissues, with a direct influence on the antioxidant enzyme levels and lipid peroxidation. Several studies with coastal bivalves, including the mussels, *Mytilus galloprovincialis* (Viarengo et al., 1991), *Mytilus edulis* (Géret et al., 2002a) and *Unio tumidus* (Doyotte et al., 1997), the clams, *Ruditapes decussatus* (Géret et al., 2002b), and the oysters, *Crassostrea virginica* and *Crassostrea gigas* (Ringwood et al., 1998; Géret et al., 2002b), showed the influence of some metals (Cd, Cu, Zn, Ag, Hg) on the antioxidant defence system and/or lipid peroxidation levels. The metal content in hydrothermal vent bivalves has been followed for several years, mainly in the clam *Calyptogena magnifica* (Roesjadi and Creco, 1984; Roesjadi et al., 1985), and the mussel *Bathymodiolus* sp. (Rousse et al., 1998; Smith and Flegal, 1989) and so have metal detoxification mechanisms in vent organisms (Cosson-Mannevy et al., 1988; Cosson and Vivier, 1995; Cosson, 1997; Géret et al., 1998).

The relation between metals and metallothioneins (MT) in marine bivalves has also been extensively studied (Langston et al., 1998). These low molecular weight proteins are considered the major metal detoxification mechanism by their capacity to bind covalently the metal ions in excess within the organisms. The protective role of MT against oxidative damage caused by ROS has been a focus, as these proteins bind and sequester transition metal ions, which contain unpaired electrons and strongly accelerate free radical formation (Cui et al., 2004), or scavenge directly oxyradicals like hydrogen peroxide (Anderson et al., 1999).

Biochemical parameters in coastal species can be influenced by seasonal factors, including temperature, salinity, sunlight exposure, diet and gametogenesis (Cotelle and Férand, 1999; Livingstone et al., 1990; Sheehan and Power, 1999; Sleiderink et al., 1995). Thus, fluctuations in antioxidant enzyme activities and MT may be related to seasonal effects, whether exogenous or endogenous, as well as contaminant exposure, or interactions of both. Several studies point out that temporal variations of antioxidant defences in bivalves are related to the reproductive cycle and food availability (Viarengo et al., 1991; Solé et al., 1995; Power and Sheenan, 1996; Cancio et al., 1999; Regoli et al., 2002). No information is available about temporal changes of antioxidant enzymes in hydrothermal vent species or the possible parameters that might control those changes. The hydrothermal vent environment is highly variable and the instability can occur at temporal scales ranging from minutes to decades.
(Lalou et al., 1984). Moreover, vent communities are relatively isolated from the rest of the oceanic ecosystems, and consequently most of the factors that determine seasonal changes in coastal species may not exist in the deep-sea vents. In fact, these environments are typically considered aseasonal because of their dependence on continuous geochemical energy (Dixon et al., 2002). However, seasonal variations in particle flux have been identified in the Lucky Strike hydrothermal region (Khripounoff et al., 2000), and seasonality of the reproductive cycle in vent mussels has been recently suggested (Tyler and Young, 1999; Le Pennec and Beninger, 2000).

The aim of the present work was to study the natural variability of antioxidant enzyme activity (SOD, CAT, Total-GPx and Se-GPx), MT concentrations and metallothioneins and lipid peroxidation (LPO) in the gills and mantle of *B. azoricus* collected over several months at the Menez-Gwen vent site and also to assess the relationship between metal concentrations in the two tissues and the antioxidant parameters considered.

2. Materials and methods

2.1. Sample collection and preparation

During the ATOS cruise (June 22–July 21, 2001; Sarradin et al., 2001) six cages were deployed with the French ROV Victor 6000 (IFREMER) in the Menez-Gwen hydrothermal vent field (37° 51′N, 32° 31′W, 850 m), located on the Mid-Atlantic Ridge near the Azores Triple Junction (Dixon et al., 2001). The cages were filled with approximately six hundred vent mussels (*B. azoricus*) each. Afterwards the cages were recovered periodically from July to November 2001 using the R/V Arquipelago by an acoustic retrieval system, a fast recovery method (approximately 20 min to surface) (Pruski and Dixon, 2003). The mussels were maintained in temperature-controlled tanks (9 ± 1 °C) until arrival at the laboratory (LabHorta) at the University of Azores. One year after the ATOS cruise, a new mussel sampling was carried out during the SEAHMA cruise (July 29–August 14, 2002) in the same period of the year to complete an annual cycle.

Ten mussels from each cage were dissected for biochemical determinations and the gills and mantle immediately frozen in liquid nitrogen and stored at −80 °C prior to analysis. An additional 10 organisms for chemical analysis were stored at −20 °C until use.

2.2. Biochemical analysis

Symbiotic bacteria were not separated from the tissues; thus the enzymatic activities obtained reflect both host and symbiont contributions. The activity of SOD (EC 1.15.1.1) (McCord and Fridovich, 1969), CAT (EC 1.11.1.6) (Greenwald 1985) and GPx (Lawrence and Burk 1976) were determined in the gills and mantle by spectrophotometric assays at 550, 240 and 340 nm, respectively. The activities of these enzymes (SOD, CAT and GPx) were expressed respectively as U mg⁻¹, mmol min⁻¹ mg⁻¹ and μmol min⁻¹ mg⁻¹ of total protein concentrations.

MT concentrations were determined in the heat-treated cytosol by differential pulse polarography in accordance with the method of Olafson and Sim (1979) modified by Thompson and Cosson (1984) (VENTOX samples) and according to Bebianno and Langston (1989) (SEAHMA samples). The standard addition method was used for calibration with rabbit liver MT (Fluka) in the absence of *B. azoricus* MT standard. Results obtained by the two different methods are comparable. The levels of MT are expressed as mg g⁻¹ wet weight.

Total protein content of gills and mantle tissues was measured by the Lowry method (Lowry et al., 1951) using Bovin Serum Albumin (BSA) as reference standard material. Lipid peroxidation was assessed by determining malondialdehyde (MDA) and 4-hydroxyalkenal (4-HNE) levels at 586 nm using malonaldehyde bis (tetrametoxypropan, SIGMA) as standard following the method described by Erdelmeier et al. (1998). Data are expressed as nmoles of MDA and 4-HNE g⁻¹ total protein concentration.

2.3. Statistical analysis

Statistical analyses were performed using STATISTICA/w v.5.1. Results are presented as mean ± standard deviation (SD). Significant differences between groups were studied using *t*-test and one-way analysis of variance (ANOVA), and only *p* < 0.05 was accepted as significant.

3. Results

3.1. Temporal variation of antioxidant enzymes in *B. azoricus*

Fig. 1 shows the temporal variability of enzyme activity of SOD, CAT and GPx in the gills and
mantle of *B. azoricus*. Although with different patterns, temporal changes were observed for all antioxidant enzyme activities in both tissues of *B. azoricus*.

Cytosolic SOD activity in the gills increased linearly from June to the beginning of September (0.401 U mg\(^{-1}\) protein d\(^{-1}\), \(r = 0.874, p < 0.05\)), when a maximum activity was found (36.4 ± 5.7 U mg\(^{-1}\) protein) \((p < 0.05)\), and significantly decreased in November (14.9 ± 1.32 U mg\(^{-1}\) protein). SOD activity in the mussels collected during the SEAHMA cruise in July 2002 was similar to that found in the previous year (16.1 ± 5.2 U mg\(^{-1}\) protein) (Fig. 1A). With the exception of SOD activity in June (6.9 ± 3.5 U mg\(^{-1}\) protein), when a maximum was observed, the mitochondrial SOD exhibited a temporal variation similar to that of cytosolic SOD, with a maximum in September \((p < 0.05)\) similar to that in June and a minimum in the gills of the mussels collected during the SEAHMA cruise (1.60 ± 0.29 U mg\(^{-1}\) protein) \((p < 0.05)\) which was significantly different from that in the previous year (Fig. 1B).

CAT activity in mussel gills remained constant during June and July (0.029 ± 0.005 mmol min\(^{-1}\) mg\(^{-1}\) protein)
protein) \((p<0.05)\), followed by a rapid decrease in August \((0.013 \pm 0.004 \text{ mmol min}^{-1} \text{ mg}^{-1} \text{ protein})\). From August to November, CAT activity increased linearly \((0.0001 \text{ mmol min}^{-1} \text{ mg}^{-1} \text{ protein day}^{-1}, r = 0.995, p<0.05)\). In the mussels collected in the following July, CAT activity in the gills was similar to that of the previous year \((0.024 \pm 0.002 \text{ mmol min}^{-1} \text{ mg}^{-1} \text{ protein})\) (Fig. 1C).

Both total glutathione peroxidases (total GPx) and selenium dependent glutathione peroxidases (Se-GPx) activities in the gills of \(B. azoricus\) exhibited a similar temporal variability, with a rapid increase from June to July, when activities of these enzymes were maximum \((p<0.05)\), followed by a decrease throughout the summer period. For total GPx this inhibition was exponential \((\text{Total GPx [mmol min}^{-1} \text{ mg}^{-1} \text{ protein]} = 0.02e^{-0.016t \text{ [days]}}, r = 0.872, p<0.05)\). Surprisingly, the activities of both enzymes in the gills of the mussels collected a year later were 3- to 5-fold higher than those found in the previous year (Fig. 1D and E).

The mantle had significantly lower enzymatic activities (mainly cytosolic and mitochondrial SOD and CAT) \((p<0.05)\) compared to the gills. Concerning total and selenium-dependent GPx activity, no significant differences between tissues were observed \((p>0.05)\), with the exceptions that TGPx and Se-GPx were significantly higher in the gills in July of 2001 and 2002 \((p<0.05)\) and higher in the mantle in November 2001 \((p<0.05)\).

The seasonal pattern of the activity of CAT in the mantle was similar to that of SOD, with an increase from July to November although not linear. The maximum activity of CAT in the mantle was in November \((0.01 \pm 0.001 \text{ mmol min}^{-1} \text{ mg}^{-1} \text{ protein})\) \((p<0.05)\), decreasing in the following July to the values observed in the previous year (Fig. 1C).

Contrary to what was observed in the gills, both total and Se dependent GPx activity in the mantle showed a similar pattern with SOD, increasing linearly throughout the seasonal study \((\text{Total GPx [mmol min}^{-1} \text{ mg}^{-1} \text{ protein]} = 0.0001t \text{ [days]} - 4.11, r = 0.893, p<0.05; \text{Se-GPx [mmol min}^{-1} \text{ mg}^{-1} \text{ protein]} = 0.0001t \text{ [days]} - 4.47, r = 0.957, p<0.05)\) and remaining unchanged until July 2002 (Figs. 1D and E).

The temporal variability of MT and LPO levels in the gills and mantle of \(B. azoricus\) is presented in Fig. 2. LPO levels in the gills followed the same pattern as GPxs (Total and Se-dependent) with significantly higher levels in July \((280 \pm 40 \text{ nmol g}^{-1} \text{ protein})\) \((p<0.05)\), when a maximum activity of total and selenium-dependent GPx was also reported. During the other months, LPO remained unchanged \((128 \pm 21 \text{ nmol g}^{-1} \text{ protein})\), and after a year LPO levels were similar (Fig. 2A). MT concentrations in the gills remained unchanged from June to July \((23 \pm 4 \text{ mg g}^{-1} \text{ protein})\) and increased from August to November. The mussels collected in the following year exhibited significantly lower MT concentrations \((12.0 \pm 1.7 \text{ mg g}^{-1} \text{ protein})\) compared to an equal period of the previous year (Fig. 2B).

LPO in the mantle was significantly lower \((p<0.05)\) compared to the gills, although both tissues exhibited similar variation patterns. As observed in the gills, the level of lipid peroxidation in the mantle was significantly higher at the end of July \((117 \pm 10 \text{ mg g}^{-1})\) \((p<0.05)\) and showed a marked temporal variability (Fig. 2A). MT concentrations
in the mantle were approximately 2-fold lower than in the gills. MT levels, unlike those in the gills, increased linearly from July to September 2001 (0.393 mg g\(^{-1}\) protein d\(^{-1}\), \(r = 0.999, p < 0.05\)) and remained unchanged in the organisms collected in the following summer (Fig. 2B).

In the gills, total and Se-GPx activities were correlated with LPO levels (Total GPx = 0.0001 [LPO] - 0.004, \(r = 0.937, p < 0.05\) and Se-GPx = 7 \(\times\) 10\(^{-5}\) [LPO] - 0.004, \(r = 0.994, p < 0.05\) (Fig. 3), suggesting that when GPX in the gills was not able to counteract ROS, LPO occurred.

### 3.2. Relationship between metal concentrations and antioxidant parameters

In order to verify if there is any relationship between antioxidant parameters LPO and MT and metals, both essential (Cu, Fe, Mn and Zn) and non-essential metals (Ag and Cd) were analysed in the gills and mantle of *B. azoricus* during the same period (Fiala-Médioni et al., in preparation). Statistical analysis showed that some of the metals are positively related with antioxidant enzymes in the gills and negatively related in the mantle.

In the gill tissue, cytosolic SOD activity increases exponentially with the increase of Ag concentrations in the soluble fraction (SOD Cyt = 7.89e\(^{0.28[}\text{Soluble Ag}\]), \(r = 0.794, p < 0.05\) (Fig. 4A). Moreover, mitochondrial SOD activity also increases, but in this case linearly with the enhancement of soluble Ag concentrations in the gills tissue (SOD Mit = 2.40 [Soluble Ag] - 4.04, \(r = 0.979, p < 0.05\) (Fig. 4B).

Similarly, for the essential metals, mitochondrial SOD activity also increases linearly with the increase of total Cu (SOD Mit = 0.10 [Total Cu] - 1.31, \(r = 0.800, p < 0.05\) and soluble Cu (SOD Mit = 0.10 [Soluble Cu] - 10.0, \(r = 0.877, p < 0.05\) accumulated in gill tissue, with a similar induction rate (\(p < 0.05\)) (Fig. 4C). Moreover, mitochondrial SOD, a Mn-containing enzyme, is linearly induced with the increase of Mn concentrations, either total or in each of the subcellular fractions (soluble and insoluble), in the gills (SOD Mit = 3.20 [Total Mn] - 8.42, \(r = 0.850, p < 0.05\); SOD Mit = 4.79 [Soluble Mn] - 1.91, \(r = 0.730, p < 0.05\); SOD Mit = 4.14 [Insoluble Mn] - 6.34, \(r = 0.870, p < 0.05\) (Fig. 4D). In this case mitochondrial SOD is induced more rapidly because of the increasing concentrations of soluble Mn, followed by insoluble Mn and total Mn concentrations in the gills (\(p < 0.05\)). Furthermore, both total and Se-GPx activities in the gills also increase linearly with Zn accumulated in the insoluble fraction (Total GPx = 0.001 [Insoluble Zn] - 0.042; \(r = 0.783; p < 0.05\) and Se-GPx = 0.0008 [Insoluble Zn] - 0.038; \(r = 0.773; p < 0.05\) (Fig. 4E) with similar rates (\(p > 0.05\)).

In the mantle, in contrast with the gills, the activities of some of these antioxidant enzymes were significantly inhibited by the accumulation increase of some of the metals (Cd, Cu, Mn and Zn) in this tissue. Thus, CAT is linearly inhibited by the accumulation of total and insoluble Zn concentrations in the mantle (CAT = -0.0001 [Total Zn] + 0.016, \(r = 0.893, p < 0.05\); CAT = -0.0001 [Insoluble Zn] + 0.013, \(r = 0.789, p < 0.05\) with
similar inhibition rate ($p > 0.05$) (Fig. 5A). Similarly, total GPx is linearly inhibited by the increase in Zn concentrations in the insoluble fraction (Total GPx = $-0.0004 \cdot [\text{Insoluble Zn}]+0.03$, $r = 0.823$, $p < 0.05$) (Fig. 5B) as well as Mn in the soluble fraction (Total GPx = $-0.029 \cdot [\text{Soluble Mn}]+0.03$; $r = 0.868$, $p < 0.05$) (Fig. 5C).

Furthermore, the increase of total Cd concentrations accumulated inhibited linearly both CAT and mitochondrial SOD activity in the mantle (CAT = $-0.012 \cdot [\text{Total Cd}]+0.001$, $r = 0.814$, $p < 0.05$; SOD Mit = $-3.32 \cdot [\text{Total Cd}]+2.51$, $r = 0.939$, $p < 0.05$) (Figs. 6A and B), while this inhibition is exponential between Se-GPx and insoluble Cd (Se-GPx = $0.019e^{-3.14[\text{Insoluble Cd}]$, $r = 0.899$, $p < 0.05$) (Fig. 6C). However, changes in metal concentrations in both gills and mantle of $B. azoricus$ had no effect on LPO or MT levels ($p > 0.05$).
4. Discussion

4.1. Methodology

The traditional methods for seasonal studies are compromised at hydrothermal vents by technical constraints of sampling in these environments. Analyses of time-series from deep-sea organisms are rare and have often been accomplished by sampling over many years and integrating data from different years (Gage and Tyler, 1991). Because of the difficulty of sampling in the deep sea in general and at hydrothermal vents in particular, knowledge has to be extrapolated from a limited amount of data or derived from comparison with known established patterns (Tyler and Young, 1999). The acoustically retrievable cages used in this study are a useful and innovative approach that proved to be very promising for periodic sampling of sessile deep-sea organisms and consequently for monitoring biochemical parameters over a long period of time. This was the first time that hydrothermal vent mussels were collected from the same vent site during a period of several months after a scientific mission. Unfortunately, no representative samples from the winter and spring periods were collected because of technical difficulties in cage recovery under adverse weather conditions.

4.2. Antioxidant enzymes

Our knowledge of antioxidant systems in hydrothermal vent organisms is still scarce. Earlier studies provided evidence of the presence of these enzymes (CAT, SOD, and GPx) in two important vent organisms, the tube worm Riftia pachyptila and the clam Calyptogena magnifica (Blum and Fridovich, 1984). Only recently, antioxidant enzymes were determined in the vent mussel B. azoricus from

![Graphs showing relationships between antioxidant enzymes and metals in B. azoricus mantle.](image-url)
different Azores hydrothermal vent sites (Bebianno et al., 2005). The levels of antioxidant enzymes found in *B. azoricus* were of the same order of magnitude as those reported in coastal mussels such as *M. galloprovincialis* (Bebianno et al., 2005). Considering that vent fluids are loaded with potentially toxic chemical species and the organisms exposed to extreme temperatures, pH and pressure, higher levels of oxidative stress biomarkers would be expected. Antioxidant enzymatic activities and LPO and MT concentrations were periodically analysed in two tissues of *B. azoricus*, gills and mantle, and related to the temporal changes in metal content of both tissues.

The results provide strong evidence for significant temporal variations in all the parameters mentioned above and in particular antioxidant enzymes; nevertheless, this variation was different between tissues. In the gills, SOD, CAT and GPx activities were higher in the summer (Fig. 1) which also corresponds to a maximum of LPO levels (Fig. 2A). In fact, levels of malondialdehyde and 4-hydroxyalkenal (MDA and 4-HNE) were almost 3-fold higher in July than in the other months, suggesting that although enhanced, the antioxidant defence system was not able to protect gill tissues from ROS mediated damage. Metal concentrations in the gills during these months were also significantly higher compared to other periods, especially Ag, Fe, Mn and Zn (Fiala-Médioni et al., in preparation).

In the mantle, a different temporal pattern was observed, with a gradual increase of antioxidant enzyme activities from July to November, when a maximum for SOD, CAT and GPx was registered (Fig. 4). In this tissue, as in the gills, maximum MDA and 4-HNE levels occurred in July, but the activities of these antioxidant enzymes were lower. Moreover, the variation of the metal content in this tissue was not significant during this period (Fiala-Médioni et al., in preparation), which suggests that
damage to the lipid membranes is probably related to factors, other than metals, capable of inducing oxidative stress, for example, hydrogen sulphide. Interestingly, vestimentiferan mitochondria are inhibited by moderate sulphide concentrations, as are those of most metazoans (Flores et al., 2005).

Many aspects of the biochemistry of marine coastal bivalves are under marked seasonal control and probably reflect variations in environmental conditions like temperature and changes in food availability and also endogenous factors like reproductive status and fluctuations in the physiological condition of the organisms. In the mussels *M. edulis* (Viarengo et al., 1991; Power and Sheehan, 1996; Sheehan and Power, 1999), *M. galloprovincialis* (Cancio et al., 1999; Solé et al., 1995; Orbea et al., 2002) and *Perna perna* (Filho et al., 2001), antioxidant enzymes exhibit marked seasonal variations. These seasonal variations of antioxidant enzymes in the coastal mussels, despite some small differences, have a pattern similar to that observed in the present study, where the highest antioxidant activities correspond to spring-summer months, and lower levels occur during autumn-winter. One exception to this behaviour was the digestive gland of *M. galloprovincialis*, where CAT and SOD showed maximum activities at the beginning of April, followed by a decrease and reaching a minimum in June (Solé et al., 1995).

The proposed temporal variation of antioxidant enzymes for *B. azoricus* is thus in agreement with the majority of seasonal studies in coastal mussel species, even though the temporal changes found seemed tissue-dependent. The increase of antioxidant defences in coastal mussels was frequently linked to the increase of metabolic activity related to seasonal temperature rise, as well as intense reproductive activity that occurs in summer months (Filho et al., 2001). On the other hand, reduction in antioxidant defences occurs during the winter months and has been correlated with the increase of LPO (Viarengo et al., 1989; Power and Sheehan, 1996). Concerning the reproduction of hydrothermal vent mussels, including *B. azoricus*, previous investigations suggested that these vent organisms were continuously spawning in a supposedly aseasonal deep-sea environment (Tyler and Young, 1999). However, later studies concluded that *B. azoricus* has episodic spawning events occurring in May (Comtet et al., 1999; 2000). More recently, with the possibility of obtaining a more frequent access to samples with the use of retrievable acoustic cages, the temporal nature of the reproductive cycle was confirmed and related to the need for a particulate food supply during the larval dispersal phase (Dixon et al., 2002; Dixon pers. obs.). This information may contribute to an explanation of the temporal variation in antioxidant enzymes in this species. If *B. azoricus* exhibits a specific reproductive period, instead of a continuous reproduction during the entire year, this clearly indicates that, like their coastal counterparts, the physiological status of the organisms also fluctuates seasonally, and consequently the susceptibility to oxidative stress and antioxidant defences display seasonal changes. Nevertheless, this still remains controversial without strong complementary data on reproductive development of *B. azoricus*.

Another source of seasonal variations in hydrothermal vent ecosystems seems to be the regular emissions of mineral and biological materials and their subsequent dispersal (Khripounoff et al., 2000). The emission and dispersal of these materials depend on several factors, namely (1) the eruption of vent fluids that precipitate chemicals (especially metals) as fine particles carried by currents and the hydrothermal plume, (2) direct erosion of the chimneys by biological activities or mechanical erosion by currents and (3) hydrothermal production of “living particles” such as bacterioplankton, holoplanktonic organisms and planktonic larval stages of vent species (Khripounoff et al., 2000). In the MAR Lucky Strike hydrothermal vent, a seasonal pattern of total particulate flux was detected, with a maximum observed in June and May, whereas during the winter period these emissions were 3-fold lower (Khripounoff et al., 2000). This variation is typical of deep-sea areas with pronounced seasonal changes in the overlying production, and it can be assumed that the same occur in other vent fields such as Menez-Gwen. Moreover, the increase of total particulate flux coincided with the maximum abundance of bivalve larvae (Khripounoff et al., 2000) and corresponds to the maximum activity of the antioxidant enzymes in the gills of *B. azoricus* found in this study.

4.3. Metals

It seems that the increase of Ag, Cu, Mn and Zn levels in the gills of *B. azoricus* ultimately enhances the production of hydrogen peroxide although by different mechanisms. Ag is a non-essential metal with no recognized biological functions and
therefore can be extremely toxic for organisms even at low concentrations, particularly when present in the ionic form (Ag⁺) (Grossel et al., 2002). In the gills of B. azoricus, this metal seems to increase the production of superoxide radical (O₂⁻) with subsequent induction of Cu/Zn-SOD (cytosol) and Mn-SOD (mitochondria matrix) to detoxify this radical by converting it to hydrogen peroxide.

On the other hand, Mn is an essential metal and therefore required by the organisms and found in many enzymes, including glutamine synthetase, alkaline phosphatase, and arginase besides Mn-SOD (Zhang et al., 2003). At low concentration, Mn²⁺ can have a protective effect by reducing the highly reactive OH⁻ radical to yield Mn(OH)²⁺ (Chang and Kosman, 1989). However, although an essential metal, Mn can be toxic at high concentrations (Zhang et al., 2003). Several studies showed that this metal is able to enhance ROS production in both Mn²⁺ and Mn³⁺ forms (Ali et al., 1995; Soliman et al., 1995). The levels of this metal in Mid-Atlantic Ridge hydrothermal vent environments (59–2250 µM) are extremely high compared to average seawater (0.0013 µM) (Douville et al., 2002), and therefore the accumulation of Mn by B. azoricus can greatly exceed the amounts required for normal cellular function. In B. azoricus Mn enhances superoxide radical production in the gills, since mitochondrial SOD is linearly induced by this metal either in the soluble or insoluble fractions.

Similarly, although Cu is an essential metal and a large number of enzymes require this metal as a cofactor for structural and catalytic properties (like the cytosolic enzyme Cu/Zn-SOD), it is also a redox-active metal involved in ROS formation by Fenton-like reactions (Aust et al., 1985; Cheeseman and Slater, 1993). The reduction of Cu²⁺ to Cu⁺ that occurs in the presence of superoxide radical (O₂⁻), produced by the Ag accumulation in this tissue, is capable of catalysing the formation of hydroxyl radicals (HO⁻) from hydrogen peroxide (H₂O₂) through the Haber–Weiss reaction (Brenner, 1998; Kadiiska and Mason, 2002).

Zn, like Mn and Cu, is also an essential metal involved in numerous biological functions, an essential constituent of over 300 enzymes including the Cu/Zn-SOD (Olin et al., 1995; Larsen et al., 2000) and it acts as an antioxidant at different cellular levels (Bray and Bettger, 1990). Although considered a non-redox metal (Maret, 2000) and therefore unable to directly produce reactive oxygen species by Fenton-like reactions, at high concentra-

ions Zn seems to increase the production of hydrogen peroxide or organic hydroperoxides in the gills of B. azoricus, with a subsequent induction of GPx activity in order to detoxify the excess of peroxides. Zn ions play a key role in mediating sulphide binding in the vesicomyid clam Calypto-genia elongate. The Zn serum-borne sulphide-binding protein present in this vent species fosters uptake and transport of sulphide from the reducing environment to chemoautotrophic endosymbionts (Flores et al., 2005).

The excess of hydrogen peroxide accumulates inside gill cells at a higher rate than GPx is able to detoxify it. This is supported by the direct linear relationship between GPx (both total and Se-GPx) and LPO levels (Fig. 3). Although elevated, the activities of these enzymes are not completely effective in preventing ROS-mediated damages like lipid peroxidation. In this case, the excess of hydrogen peroxide seems to be transformed into the hydroxyl radical (OH⁻) by Haber–Weiss reactions (Kehrer, 2000). This radical is known to be one of the best LPO initiators by the subtraction of a hydrogen atom from a methylene group of a polyunsaturated fatty acid (Di Giulio et al., 1995).

The presence of symbiotic bacteria in the gill cells of B. azoricus may also have an important role in the accumulation and detoxification of metals (Fiala-Médioni et al., 2000; Hardivillier et al., 2004). Preliminary studies indicated the presence of significant SOD activity in the symbionts present in the gills of B. azoricus (Hoarau, unpublished data) and consequently ROS detoxification in this bivalve is likely to involve both host and symbiotic contributions, although this hypothesis needs further investigation. To avoid the toxic effects of sulphide while supplying it to their internal chemoautotrophic symbionts, the vestimentiferan tube-worm Riftia pachyptila possesses several large extracellular haemoglobins that simultaneously and reversibly bind sulphide and oxygen (Flores et al., 2005). Therefore, the role of chemoautotrophic endosymbionts in B. azoricus may be more complex than previously supposed.

Contrary to what was observed in the gills, the accumulation of some metals had an inhibitory effect on antioxidant enzyme activities in the mantle. The increase of Zn concentrations inhibits the enzymes responsible for detoxifying hydrogen peroxide (CAT and total GPx). It is known that, although both tissues are in direct contact with the surrounding water and therefore exposed to the
same metal concentration, *B. azoricus* accumulates in the mantle approximately half of the Zn concentration in the gills (Bebianno et al., 2005). It seems that mantle tissue, even though accumulating significantly lower Zn concentrations, might be more susceptible to Zn toxicity. In the same way, the increase of soluble Mn concentrations in the mantle also inhibits total GPx, which in turn impairs one of the most important enzymes involved in the detoxification of hydrogen peroxide. Also, the increase of Cd concentrations inhibits CAT, mitochondrial SOD (total Cd) and Se-GPx (insoluble Cd). Although Cd is unable to generate ROS by Fenton-type reactions (Watanabe et al., 2003; Stohs et al., 2001), this metal is known to induce superoxide anion and nitric oxide (Yang et al., 1997; Hassoun and Stohs, 1996) and is considered one of the most toxic non-essential metals to biological systems. Several studies showed that Cd affects the antioxidant defence system and inhibits the activity of SOD, GPx and CAT (Jurczuk et al., 2004; Leon et al., 2002; Muller, 1991; Hussein et al., 1987), confirming the results obtained in the mantle of *B. azoricus*. Although more research is needed, the reduced presence of symbiotic bacteria in the mantle (Hoarau, unpublished data) compared with the gills may inhibit the level of antioxidant protection against metals or other toxic species (e.g. hydrogen sulphide) mediated by ROS.

MT concentrations show a similar pattern of temporal variation in both tissues, with a linear increase from July to November (gills) or September (mantle) (Figs. 2B). Although these proteins have a high affinity for metals and have been largely used as metal exposure biomarkers in coastal mussels *M. galloprovincialis* (Bebianno and Machado, 1997), in *B. azoricus* from Menez-Gwen these proteins are not involved in metal detoxification. This may suggest that the main function of *B. azoricus* MT may be constitutive (Fiala-Medioni et al., 2000; Hardivillier et al., 2004). Moreover, although MT appears to be regulated by oxidative stress (Sato and Bremer, 1993; Maret, 2000; Andrews, 2000; Viarengo, 2000) and free radical scavenging capacity due to their high thiol content (Chubatsu and Meneghini, 1993; Markant and Pallau, 1996; Rossman and Goncharova, 1998), temporal MT fluctuations are not related to antioxidant enzyme activities or LPO. In this case, MT temporal variations may be related to other factors like temperature fluctuations and reproductive status of *B. azoricus* as suggested for coastal bivalves (Baudrimont et al., 1997).

5. Conclusions

Evidence that hydrothermal vents in the Azores region of the Mid-Atlantic Ridge area are not an aseasonal environment as supposed earlier is increasing. Therefore, temporal variations of biochemical parameters need to be followed systematically. Temporal variation of antioxidant defence systems, lipid peroxidation and MT levels are described here for the first time in *B. azoricus* and related to accumulated metal concentrations in the gills and mantle. Different patterns of temporal changes occur in both tissues due to their different physiologic functions. This variability must be taken into account in future studies of oxidative stress and related biomarkers in hydrothermal environments. Metal accumulation within tissues induces antioxidant protection in the gills, while an inhibitory effect was observed in the mantle.

Acknowledgements

The authors would like to thank Pierre M. Sarradin, chief scientist of ATOS cruise and the crew of N/O L’Atalante and Victor 6000 (IFREMER). This work was largely funded by the VENTOX project (EVK3 CT1999-00003). R. Company was supported by an FCT Ph.D. Grant (Fundação para a Ciência e a Tecnologia) of the Ministry of Science and Technology of Portugal (reference SFRH/BD/904/2000).

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