Universidade do Algarve
Faculdade de Ciências do Mar e do Ambiente

Antioxidant defence systems in the deep-sea mussel *Bathymodiolus azoricus* from Mid-Atlantic Ridge hydrothermal vents

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Resumo

A descoberta das fontes hidrotermais em 1977 foi um dos eventos oceanográficos mais importantes do século 20. Estes sistemas marinhos localizados nas cordilheiras médio-oceânicas, são caracterizados pela presença de compostos considerados tóxicos noutros ambientes, como o sulfureto de hidrogénio, metano, dióxido de carbono e em particular metais que provêm dos fluidos hidrotermais e por isso são geralmente considerados um dos ambientes mais tóxicos do planeta. Durante a última década foram identificadas várias fontes hidrotermais na Cordilheira Médio-Atlântica perto dos Açores, incluindo Menez-Gwen, Lucky Strike e Rainbow. Nessas fontes hidrotermais foram descobertos mexilhões *Bathymodiolus azoricus*, descritos pela primeira vez em 1999, identificados como uma das espécies mais abundandes nestas fontes hidrotermais. Estes mexilhões têm a capacidade de acumular tanto metais essenciais como não essenciais. Alguns dos mecanismos de desintoxicação metálica do *B. azoricus* têm sido recentemente alvo de investigações, no entanto os sistemas de defesa antioxidante nunca foram estudados nesta espécie hidrotermal. Sabe-se que os metais têm a capacidade de aumentar a produção de espécies reactivas de oxigénio, tais como o anião superóxido ($O_2^{\bullet-}$), radical hidroxilo (OH•) e peróxido de hidrogénio (H_2O_2), tanto pela participação em reacções do tipo Fenton e Haber Weiss (metais redox activos) como por vias indirectas (metais redox inertes). Estes radicais são posteriormente desintoxicados pela acção de diversas enzimas antioxidantes, incluindo a superóxido dismutase, catalase e glutationa peroxidases que fazem parte do sistema de defesa antioxidante.

Assim, o objectivo desta tese foi estudar os mecanismos de defesa antioxidante no mexilhão *Bathymodiolus azoricus* de fontes hidrotermais ricas em metais da Cordilheira-Médio Atlântica.

Para isso, esta investigação envolveu dois estudos de campo (Capítulos 2 e 3) de forma a determinar tanto as variações espaciais como variações sazonais do sistema de defesa antioxidante em *B. azoricus*. Posteriormente, verificou-se o efeito que vários metais, tanto essenciais (Cu e Zn) como não essenciais (Ag, Cd, Hg) exercem nas defesas antioxidantes, através de experiências de contaminação em laboratório de curto e longo prazo, onde *B. azoricus* foram expostos em condições pressurizadas (sistema IPOCAMP) e a pressão atmosférica, respectivamente (Capítulos 4, 5 e 6).

De forma a estudar as actividades basais e a variação espacial das enzimas antioxidantes (SOD, CAT, GPx) em *B. azoricus*, estes mexilhões foram recolhidos em cinco fontes hidrotermais, Menez-Gwen (ATOS8 e ATOS10), Lucky Strike (Bairro Alto e Eiffel Tower) e Rainbow durante o cruzeiro científico ATOS no Verão de 2001 (Projecto Europeu VENTOX). Os resultados mostraram que as defesas antioxidantes e os danos oxidativos no *B. azoricus* dependem do tecido considerado (brânquias > manto) e surpreendentemente são da mesma ordem de grandeza de outros moluscos não hidrotermais. Também foi demonstrado que entre os ambientes de fontes hidrotermais, Menez-Gwen parece ser menos stressante comparativamente com Lucky Strike e Rainbow, uma vez que a formação de ROS é eficientemente neutralizada pelo sistema de defesa antioxidante. Por outro lado, as enzimas antioxidantes e os danos oxidativos nos mexilhões de Lucky Strike parecem ser induzidos como consequência da presença de elevadas concentrações de Ag, Cd, Cu e Zn nos seus tecidos, enquanto que os mexilhões do Rainbow parecem responder às elevadas concentrações de Fe (Capítulo 2).

O estudo sazonal envolveu a recolha de mexilhões no Menez-Gwen ao longo de vários meses através do uso de gaiolas com detecção remota previamente cheias durante o cruzeiro ATOS. Para completar um ciclo anual foram também recolhidos mexilhões um ano depois, durante o cruzeiro SEAHMA (Verão de 2002). Os resultados evidenciaram que as enzimas antioxidantes nos mexilhões *B. azoricus* têm uma marcada influência sazonal, contrariamente ao que se pensava inicialmente relativamente aos ambientes hidrotermais. Estas variações dependeram da época do ano e dos tecidos considerados. Nas brânquias os máximos de actividade enzimática ocorrem nos meses de Verão, seguidos de um declínio gradual ao longo dos meses. Após um ano, os níveis foram semelhantes aos encontrados no ano precedente. No manto a variação das enzimas antioxidantes teve um padrão de variação diferente, com um aumento gradual desde do Verão até ao final do Outono. As defesas antioxidantes nas brânquias foram significativamente induzidas pelo aumento das concentrações de Ag, Cu and Mn, enquanto que no manto foram inibidas, o que sugere vias distintas na produção de ROS e que estes os tecidos respondem de forma diferente à acumulação metálica. Embora o sistema de defesa antioxidante no *B. azoricus* seja dependente do tecido, parece ser independente do tamanho dos mexilhões (Capítulo 3).

As experiências laboratoriais realizadas de forma a compreender o efeito dos metais no sistema de defesa antioxidante do *B. azoricus* consistiram na exposição de mexilhões recolhidos no Menez-Gwen a vários metais essenciais e não essenciais durante curtos períodos de tempo (variando entre 12 horas e 6 dias) ou períodos de tempo maiores (30 dias).

Nas experiências de curta duração a 85 bars (a mesma pressão hidrostática a que estão sujeitos os mexilhões do Menez-Gwen a 850 metros de profundidade) os exemplares de *B. azoricus* foram expostos separadamente a Cd (100 µg l^{-1}), Cu (25 µg l^{-1}), Zn (1000 µg l^{-1}), Ag (20 µg l^{-1}) e Hg (25 µg l^{-1}) (Capítulos 4, 5 e 6). Os resultados obtidos mostraram que o sistema de defesa antioxidante é afectado de forma diferente por cada um dos metais, pelo tempo de exposição e consoante o tecido considerado. Também é de supor que as bacterias simbiontes possam ter um importante papel antioxidante e que as diferenças encontradas entre tecidos possam estar a reflectir esse aspecto. Algumas das interações observadas entre os metais testados em IPOCAMP (Cd, Cu, Zn and Ag) e as enzimas antioxidantes confirmam as relações obtidas nos estudos de campo para as brânquias e o manto. Surpreendentemente, um dos metais mais tóxicos considerado neste estudo (Hg), provavelmente devido à elevada concentração usada nas experiências, não teve efeito na maior parte das enzimas antioxidantes ou nos níveis de peroxidação lipídica.

As exposições de longo prazo a pressão atmosférica consistiram em 24 dias de exposição, seguidos de 6 dias de depuração. Nestas condições os exemplares de *B. azoricus* foram expostos a Cd (50 µg l^{-1}) e Cu (25 µg l^{-1}). Provavelmente devido à perda dos simbiontes nos tecidos ou ao prolongado tempo de permanência em condições artificiais (em termos de pressão hidrostática, composição química da água entre outras) o sistema de defesa antioxidante dos mexilhões reflectem não o efeito dos metais, mas provavelmente as fracas condições fisiológicas destes organismos (Capítulos 4 e 5).

Como resultado desta investigação é possível concluir que *B. azoricus* é capaz de tolerar as elevadas concentrações metálicas encontradas no meio natural e que o seu sistema de defesa antioxidante parece bem adaptado às condições hidrotermais potencialmente tóxicas.

Résumé

La découverte des sources hydrothermales en 1977 constitue l'un des événements en océanographie les plus importants du 20ème siècle. Ces systèmes marins localisés sur les dorsales médio-océaniques sont caractérisés par la présence de composés considérés toxiques dans d'autres environnements, tel que l'hydrogène sulfuré, le méthane, le dioxyde de carbone et en particulier des métaux provenant des fluides hydrothermaux et sont, par conséquence, généralement considérés comme l'un des environnement les plus toxiques de la planète. Durant la dernière décennie, plusieurs sources hydrothermales ont été identifiées sur la Dorsale Médio-Atlantique près des Açores dont Menez-Gwen, Lucky Strike et Rainbow. Les moules *Bathymodiolus azoricus*, décrites pour la première fois en 1999, représentent l'une des espèces les plus abondantes de ces sources hydrothermales. Ces bivalves ont la capacité d'accumuler aussi bien des métaux essentiels que des métaux non essentiels. Certains mécanismes de détoxification des métaux du *B. azoricus* ont récemment fait l'objet de recherches mais les systèmes de défense antioxydant n'ont cependant jamais été étudiés sur cette espèce hydrothermale. Les métaux ont la capacité d'augmenter la production de dérivés réactives de l'oxygène tel que le radical superoxyde ($O_2^{\bullet-}$), le radical hydroxyle (OH•), le peroxyde d'hydrogène (H_2O_2) aussi bien à travers la participation à des réactions du type Fenton et Haber Weiss (métaux redox actifs) que par voie indirecte (métaux redox inertes). Ces radicaux sont à posteriori désintoxiqués par l'action de diverses enzymes antioxydants comme la superoxyde dismutase, la catalase et les glutathiones peroxydases qui font partie du système de défense antioxydant.

L'objectif de cette thèse est l'étude des mécanismes de défense antioxydant de la moule *Bathymodiolus azoricus* prélevée au niveau de sources hydrothermales riches en métaux de la Dorsale Médio-Atlantique.

Deux études de terrain (Chapitres 2 et 3) ont été menées de manière à déterminer aussi bien les variations spatiales que saisonnières du système de défense antioxydant du *B. azoricus*. A posteriori, les effets de plusieurs métaux essentiels (Cu et Zn) ou non (Ag, Cd, Hg) sur le système de défense antioxidant on été determinés grâce à des expériences de contamination à court et long terme en laboratoire des *B. azoricus* étant exposés respectivement sous conditions pressurisés (système IPOCAMP) et sous pression atmosphérique (Chapitres 4, 5 et 6).

De manière à étudier les activités basales et la variation spatiale des enzymes antioxydants (SOD, CAT, GPx) du *B. azoricus*, ces bivalves ont été prélevés au niveau de cinq sources hydrothermales, Menez-Gwen (ATOS8 et ATOS10), Lucky Strike (Bairro Alto et Eiffel Tower) et Rainbow, lors de l'expédition scientifique ATOS de l'été 2001 (Projet Européen VENTOX). Les résultats démontrent que les défenses antioxydantes et les dommages oxydatifs du *B. azoricus* dépendent du tissu considéré (branchie > manteau) et sont curieusement du même ordre de grandeur que chez d'autres mollusques non hydrothermaux. Parmi les sites hydrothermaux, Menez-Gwen s'est avéré être moins stressant par comparaison des sources Lucky Strike et Rainbow du fait que la formation de ROS est neutralisée de manière efficace par le système de défense antioxydante. Par ailleurs, les enzymes antioxydants et les dommages oxydatifs des moules de Lucky Strike semblent être induites par la présence de concentrations élevées de Ag, Cd, Cu et Zn dans ces tissus, alors que les moules de Rainbow semblent répondre à des concentrations élevées de Fe (Chapitre 2).

L'étude saisonnière a consisté en la récolte de moules sur le site de Menez-Gwen pendant plusieurs mois à l'aide de cages de prélèvement acoustique lors de l'expédition ATOS. Une récolte de moules a été effectuée un an plus tard pendant la campagne SEAHMA (Eté 2002) pour compléter un cycle annuel.

Les résultats mettent en évidence que les enzymes antioxydants des moules *B. azoricus* subissent une influence saisonnière contrairement a ce que l'on pensait au préalable concernant les environnements hydrothermaux. Les variations dépendent de l'époque de l'année et des tissus considérés. Dans les branchies, l'activité maximale enzymatique est détectée pendant les mois d'été suivi d'un déclin graduel au cours des mois. Après un an, les niveaux sont similaires à ceux détectés l'année précédente. La variation d'enzymes antioxydants au niveau du manteau a montré une augmentation graduelle depuis l'été jusqu'à la fin de l'automne. Les défenses antioxydants des branchies ont été induites de façon significative par l'augmentation de concentrations de Ag, Cd, Cu et Zn, alors qui au niveau du manteau elles ont été inhibées, suggérant des voies distinctes dans la production de ROS et également que ces tissus répondent de manière différente a l'accumulation des métaux. Bien que le système de défense antioxydant de *B. azoricus* dépende du tissu, il semble être indépendant de la taille des moules (Chapitre 3).

Les expériences réalisées en laboratoire dans le but de comprendre l'effet des métaux sur le système de défense antioxydant du *B. azoricus* ont consisté en l'exposition de moules récoltées sur Menez-Gwen à plusieurs métaux essentiels et non essentiels pendant de courtes périodes de temps (entre 12 heures et 6 jours) ou des périodes plus étendues (30 jours).

Lors des expériences de courte durée à 85 bars (la même pression qui affecte les moules de Menez-Gwen à 850 mètres de profondeur) les échantillons de *B. azoricus* ont été exposés séparément à Cd (100 µg l^{-1}), Cu (25 µg l^{-1}), Zn (1000 µg l^{-1}), Ag (20 µg l^{-1}) et Hg (25 µg l^{-1}) (Chapitres 4, 5 et 6). Les résultats obtenus démontrent que le système de défense antioxydant est affecté de manière différente par chacun des métaux, suivant le temps d'exposition et le tissu considéré. Les bactéries symbiotiques jouent probablement un rôle antioxydant important et les différences constatées entre les tissus peuvent s'y rapporter. Certaines des interactions observées entre les métaux testés sur IPOCAMP (Cd, Cu, Zn et Ag) et les enzymes antioxydants confirment les rapports obtenus lors des études de terrain concernant les branchies et le manteau. Curieusement, l'un des métaux les plus toxiques considéré dans cette étude (Hg), et ce probablement à cause de la concentration élevée utilisée lors des expériences, n'a eu aucun effet sur la plupart des enzymes antioxydants ou sur les niveaux de la péroxidation lipidique.

Les expositions à long terme sous pression atmosphérique ont été effectuées sur 24 jours d'exposition, suivis de 6 jours de dépuration. Dans ces conditions les échantillons de *B. azoricus* ont été exposés au Cd (50 µg l^{-1}) et au Cu (25 µg l^{-1}). Probablement lié à la perte de bactéries symbiotiques dans les tissus ou à la longue période de temps en conditions artificielles (pression, composition chimique de l'eau, entre autres) le système de défense antioxydant des moules reflète non pas l'effet des métaux mais plus probablement les faibles conditions physiologiques de ces organismes (Chapitres 4 et 5).

En résumé, il est possible de conclure que *B. azoricus* est capable de tolère des concentrations métalliques élevées en milieu naturel et que son système de défense antioxydant semble bien adapté aux conditions hydrothermales potentiellement toxiques.

Abstract

The discovery of hydrothermal vents occurred in 1977 and was one of the most important events in biological oceanography of the 20th century. These deep-sea marine systems located in mid-ocean ridges are characterized by the presence of compounds considered toxic in other environments, such as hydrogen sulphide, methane, carbon dioxide and especially metals that rise from the hot hydrothermal fluids and therefore were recurrently considered one of the most toxic environments on earth. In the Mid-Atlantic Ridge near Azores several hydrothermal vent fields were discovered in the last decade, including Menez-Gwen, Lucky Strike and Rainbow, and the mussels *Bathymodiolus azoricus*, firstly describe in 1999, were found to be one of the most abundant species in these vent fields. These mussels are known to accumulate both essential and non-essential metals. Some of the mechanisms of metal detoxification in *B. azoricus* have been the subject of recent investigations, however the antioxidant defence systems were never studied before in this vent species. Metals are known to enhance the production of reactive oxygen species such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2), either by participation in Fenton-type and Haber Weiss-type reactions (redox-active metals) or by more indirect pathways (redox-inert metals). These oxyradicals are subsequently detoxified by several antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidases, which are part of the antioxidant defence systems.

Therefore, the aim of the present thesis was to study this antioxidant defence mechanisms in the mussel *Bathymodiolus azoricus* from metal rich Mid-Atlantic Ridge hydrothermal vents.

In order to accomplish this, the present research involved two field studies (Chapters 2 and 3) to determine both spatial and seasonal variations of the antioxidant defence systems and other metal detoxification mechanisms in *B. azoricus*. Afterwards, the effects of several metals, both essential (Cu and Zn) and non-essential (Ag, Cd, Hg) on the antioxidant defences were accessed through short term and long term laboratory contaminations where *B. azoricus* were exposed in pressurized (IPOCAMP) and atmospheric pressure conditions respectively (Chapters 4, 5 and 6).

To study the basal and spatial variability of antioxidant enzyme activities (SOD, CAT and GPx) in two tissues (gills and mantle) of *B. azoricus*, these mussels were collected in five hydrothermal vent fields: Menez-Gwen (ATOS8 and ATOS10), Lucky Strike (Bairro Alto and Eiffel Tower) and Rainbow, during the first and second legs of the ATOS cruise in summer 2001 European project VENTOX. The results of this field study showed that antioxidant defences and damage in *B. azoricus* are tissue dependent (gills > mantle) and surprisingly are of the same order of magnitude of other non vent molluscs. Also, Menez-Gwen seems a less stressful environment than Lucky Strike and Rainbow, since the formation of ROS is effectively counteracted by the antioxidant defence system. Antioxidant protection enzymes and damages in the mussels from Lucky Strike are enhanced by the presence of higher amounts of metal levels (Ag, Cd, Cu and Zn) in their tissues, while in mussels from Rainbow may reflect the concentrations of Fe (Chapter 2).

The seasonal study involved the collected of mussels from Menez-Gwen throughout several months using acoustic release cages, previously filled with mussels during the ATOS cruise. Mussels were also sampled during the SEAHMA cruise in summer 2002 to complete an annual cycle. The results of this research showed that *B. azoricus* are under marked seasonal influence, contrarily to what was previously supposed for hydrothermal vent environments. The antioxidant systems fluctuate significantly during the sampling months and these changes were also tissue dependent. Maximum activities in antioxidant enzymes in the gills were detected in the beginning of summer, followed by a gradual decrease throughout the months. One year after, the levels were similar to those reported one year before. A different pattern of seasonal variation in antioxidant enzyme activities was observed in the mantle, with a gradual increase from summer to the end of autumn. 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 ROS production and that these tissues responded differently to the metal accumulation. Although tissue dependent, the antioxidant defence system in *B. azoricus* seems independent of size (Chapter 3).

Laboratory experiments aiming to understand the effects of metals in antioxidant defence mechanisms of *B. azoricus* consisted in expose the mussels collected in Menez-Gwen vent field during short periods of time (ranging from 12 hours to 6 days) or longer periods (30 days) to several essential and non-essential metals.

Short term exposure experiments at 85 bars (the same hydrostatic pressure that affects mussels at 850 meters deep in Menez-Gwen) exposed *B. azoricus* separately to Cd (100 $\mu g\ l^{-1}$), Cu (25 $\mu g\ l^{-1}$), Zn (1000 $\mu g\ l^{-1}$), Ag (20 $\mu g\ l^{-1}$) and Hg (25 $\mu g\ l^{-1}$) (Chapters 4, 5 and 6). Results obtained demonstrate that antioxidant defence systems in this hydrothermal mussel are differently affected by each metal, by the time of exposure and the tissues considered. It is also suggested that symbiotic bacteria may have an important antioxidant role in this species and differences between tissues might reflect this. Some interactions between the metals tested in IPOCAMP (Cd, Cu, Zn and Ag) and antioxidant enzymes confirm the relationships obtained in the field studies for the gills and mantle tissues. Surprisingly, one of the most toxic metals (Hg) has no effect on most antioxidant enzymes or lipid peroxidation levels probably due to the high concentration used in the experiment.

Long time exposure experiments at atmospheric pressure consisted in 24 days of exposure, followed by 6 days of depuration. In these conditions *B. azoricus* were separately exposed to Cd (50 $\mu g\ l^{-1}$) and Cu (25 $\mu g\ l^{-1}$). Probably because of the loss of symbionts in their tissues and prolonged maintenance of the mussels in artificial conditions (in terms of hydrostatic pressure, water chemistry among others) the antioxidant defence systems of *B. azoricus* in these experiments may reflected the poor physiological conditions of the organisms rather than the effects of metals (Chapter 4 and 5).

In result of this research is possible to assume that *B. azoricus* are able to cope with high metal concentrations in their natural environment and its antioxidant defence mechanisms appears well adapted to the potentially toxic hydrothermal conditions.

Index

Chapter 1 General Introduction

Chapter 2

Antioxidant systems and lipid peroxidation in *Bathymodiolus azoricus* from Mid-Atlantic Ridge hydrothermal vent fields

Chapter 3

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

Chapter 4

Effect of cadmium on antioxidant responses and susceptibility to oxidative stress in the hydrothermal vent mussel *Bathymodiolus azoricus*

Chapter 5

Metal detoxification mechanisms in the hydrothermal vent mussel *Bathymodiolus azoricus* exposed to Copper

Chapter 6

Short term effects of zinc, silver and mercury on stress related biomarkers in the gills and mantle of *Bathymodiolus azoricus* from Mid-Atlantic Ridge

Chapter 7 General Discussion

Annex

Figure Index

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Table Index

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Abbreviations and Acronyms

4-HNE	4-hydroxyalkenals
ABAP	2-2'-azo-bis-(2 methyl-propionamidine)-dihydrochloride
ANOVA	Analysis of Variance
AsPx	Ascorbate peroxidase
ATJ	Azores Triple Junction
ATOS	Cruise during VENTOX Project
BSA	Bovine Serum Albumin
CAT	Catalase
cDNA	Complementary DNA
Cu/Zn-SOD	Copper and zinc containing superoxide dismutase
DNA	Deoxyribonucleic acid
DPP	Differential Pulse Polarography
DTD	DT-diaphorase
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
EC	Enzyme Classification number
EDTA	Ethylenediaminetetraacetic acid
EPR	East Pacific Rise
ET-AAS	Electrothermal Atomic Absorption Spectrometry
FAAS	Flame Atomic Absorption Spectrophotometry
Fe-SOD	Iron containing superoxide dismutase
FID	Flame Ionization Detector
GERL	Golgi-Endoplasmic Reticulum-Lysosome complex
GPx	Glutathione peroxidases
GPx1	Glutathione peroxidases of family 1
GPx2	Glutathione peroxidases of family 2
GPx3	Glutathione peroxidases of family 3
GPx4	Glutathione peroxidases of family 4
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase
HDL	High Density Lipoprotein
ICP-AES	Inductively coupled plasma-atomic emission spectrometry
IFREMER	French Research Institute for Exploitation of the Sea
IPOCAMP	Incubateurs Pressurises pour l'Observation en Culture d'Animaux Marins Profonds (Pressurised tank)
kDa	Kilodaltons

KMBA	α-keto-γ-methiolbutiryc acid
LabHorta	Land based laboratory in Horta (University of Azores)
LDL	Low Density Lipoprotein
LPO	Lipid peroxidation
MAR	Mid-Atlantic Ridge
MDA	Malondialdehyde
Mn-SOD	Manganese containing Superoxide dismutase
MPA	Marine Protected Area
MT	Metallothionein
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ORF	Open Reading Frame (section of DNA that could potentially be translated into a polypeptide)
PC1	Principal Component 1
PC2	Principal Component 2
PCA	Principal Components Analysis
PUFA	Polyunsaturated fatty acid
R/V	Research Vessel
ROS	Reactive Oxygen Species
ROV	Remote Operated Vehicle
SD	Standard Deviation
SEAHMA	Seafloor and Sub-seafloor Hydrothermal Modeling in the Azores Sea (Portuguese Project PDCTM/P/MAR/15281/1999)
Se-GPx	Selenium dependent glutathione peroxidase
SIN-1	3-morpholinosydnomine
SMDE	Static Mercury Drop Electrode
SOD	Superoxide dismutase
TGPx	Total glutathione peroxidase
TOSC	Total oxyradical scavenging capacity
Tris	Tris (hydroxymethyl) aminomethane
VENTOX	Deep-Sea Hydrothermal Vents: A Natural Pollution Laboratory (EU Funding Project EVK3-CT1999-00003)
Victor6000	Remote Operated Vehicle used during VENTOX Project

Chapter 1

General Introduction

1. General Introduction

Deep-sea hydrothermal vents comprise one of the most extraordinary and extreme marine environments identified and its discovery was one of the most exciting and important events in biological oceanography of the 20th century (Tyler *et al.*, 2003; Van Dover & Lutz, 2004). Since the discovery of hydrothermal vents, many scientific expeditions were made over the past three decades to collect samples of rocks, fluids, organisms and microorganisms to better understand these environments. The study of hydrothermal vents has been driven by multidisciplinary teams that comprise very distinct scientific areas, such as chemistry, geology, biology, microbiology biotechnology and many others.

Recently, hydrothermal vents were focused in a new ecotoxicological perspective due to the presence of compounds considered toxic in other environments, such as hydrogen sulphide, methane, carbon dioxide and especially metals (Pruski & Dixon, 2003). One of the many surprises about vent sites is that these apparently toxic hydrothermal fluids directly support exceptionally productive biological communities in the deep sea (Little & Vrijenhoek, 2003). In this context, one of its important characteristic is the extremely high metal concentrations (both essential and non-essential for marine organisms) present in both hydrothermal vent fluids (Von Damm 1990; Lowel *et al.*, 1995; Von Damm *et al.*, 1995; Sarradin *et al.*, 1998; Douville *et al.*, 2002) and organisms (particularly in bivalves) (Roesijadi & Crecelius, 1984; Roesijadi *et al.*, 1985; Smith & Flegal, 1989; Cosson, 1997; Rousse *et al.*, 1998; Martins *et al.*, 2001; Ruelas-Inzunza *et al.*, 2003). The concentrations of these metals in both these compartments are extremely high when compared to the average seawater and coastal organisms. Metals are known to be toxic to marine organisms when present in high concentrations. Several studies undergo to understand the specific adaptations that hydrothermal vent organisms have to deal with such potentially toxic environment (Cosson-Mannevy, *et al.*, 1988; Cosson & Vivier, 1995; Géret *et al.*, 1998; Fiala-Médioni *et al.*, 2000).

In many metal contaminated environments, the resistance to metal toxicity involve several detoxification mechanisms, identified in many organisms. These metal detoxification mechanisms comprises the synthesis of metallothionein (MT), a small cytosolic protein able to bind metals (Langston *et al.*, 1998; Dabrio *et al.*, 2002). Another detoxification mechanism is the sequestration of metals in vesicles (lysosomes), or other membrane-bound structures, which keeps the metal from interacting at sensitive sites in the organism (Chassard-Bouchaud *et al.*, 1986, Viarengo & Nott, 1993).

Metals are also able to increase oxidative stress and oxidative damage in organisms by increasing the production of reactive oxygen species (Aust *et al.*, 1985; Hassoun & Stohs 1996; Fridovich, 1998) inside cells and enhancing lipid peroxidation in tissues (Regoli *et al.*, 2000). Therefore, antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidases have an important role in metal detoxification. Although the responses of antioxidant defence systems to metals have been extensively studied in organisms from coastal areas (Viarengo *et al.*, 1990; Regoli & Principato, 1995; Rómeo & Gnassia-Barelli, 1997; Cossu *et al.*, 1997; Matozzo *et al.*, 2001; Géret *et al.*, 2002a,b,c; Almeida *et al.*, 2004; Géret & Bebianno, 2004; Manduzio *et al.*, 2003), the role of these enzymes in most hydrothermal vent species is unknown (Blum & Fridovich 1984).

1.1. Hydrothermal vents discovery

Currently, deep-sea research and especially scientific missions to study hydrothermal vent sites involve a broad number of knowledge in scientific areas. However, only during the second half of the 19th century was discovered that life exists below 600 m depth (Mills, 1983).

The development of marine depths exploration occurred with the advance of communication and the use of telegraphic cables in transoceanic connections. During this time, sea bottoms with more than 5000 meters deep were

discovered as well as the existence of sea mountains in the middle of the ocean (Reyss, 1991).

The exploration of the sea floor at such high depths give rise to several arguments between the scientific community, as the establishment of the "azoic zone" theory was questioned. The "azoic zone" concept was proposed by Edward Forbes and suggested that no life was present in the oceans below 600 meters (Tyler & Gage, 1996; Tyler *et al.*, 2003). In 1861, the French naturalist Henry Milne-Edwards discovered several animals fixed to a deep-sea cable immersed at 1800 meters, including anthozoan and calcareous worm tubes (Mills, 1983). At the same time the Norwegian scientist Michael Sars documented the presence of a large number of taxa in Norwegian fjords at 600 meters (Mills, 1983).

Afterwards, Wyville Thompson persuaded the Royal Society of London in order to get the British Royal Navy to help him organize a scientific mission undertaking the exploration of deep-water. During these pioneering expeditions new forms of live were found from depths down to 4289 meters (Linklater, 1972). Thompson performed a circumnavigating voyage with the H.M.S. *Challenger* from 1872 to 1876. The results of this cruise were later compiled in a 50 volume report (Buchanan *et al.*, 1895) and laid down the foundations for the present knowledge of life at deep-sea floor (Mills, 1983).

The last great expedition was the Danish *Galathea Deep-Sea* mission around the world between 1950-1952 (Bruun *et al.*, 1956). In this expedition one of the latest frontiers of the world was sampled, the Philippine trenches with 10190 meters deep (Mills, 1983).

Between 1960 and 1970 an important change in the approach of deep-sea biological research occurred, with descriptive biology giving way to a more rigorous scientific method (Hessler & Sanders, 1967; Grassle & Sanders, 1973; Grassle, 1977).

At the same time, research advances in deep-sea technology led to the development of manned and unmanned submersibles. The capacity to dive into the sea floor to collect samples and conduct manipulative experiments open the way for possibly the greatest excitement in marine biology in the 20th century: the discovery of hydrothermal vents and their associated fauna (Tyler *et al.*, 2003).

By 1977, geologists had predicted that the heat-loss balance on earth could only occur if heat was lost at mid-ocean ridges by convective hydrothermal processes as well as by conductive processes (Corliss *et al.*, 1979). In 1977, an expedition to the Galapagos Ridge in the East Pacific Ocean documented this convective process as a flux of low temperature vent fluids from basalts (Corliss *et al.*, 1979). During this expedition the first hydrothermal vent was discovered with the manned submersible Alvin and the local fauna described (Corliss & Ballard, 1977; Ballard & Grassle, 1979; Corliss *et al*, 1979). Large colonies of 2-m-long tubeworms *Riftia pachyptila* and dense beds of large bivalves (*Calyptogena magnifica* and *Bathymodiolus thermophilus*) were observed growing in water a few degrees above the ambient temperature of 2 ºC (Van Dover & Lutz, 2004). Since this first contact with hydrothermal vents, similar structures were discovered at mid-ocean spreading centres in the Pacific, Atlantic, Arctic and Indian Oceans (Van Dover, 2000) and it is likely that as scientists explore more of the oceanic ridges more deep-sea hydrothermal vent sites will be discovered.

1.2. Hydrothermal vents characteristics

Hydrothermal vents are deep-sea chimney-like structures located in mid-ocean ridges where the seafloor is spreading. Seafloor spreading, a concept proposed in 1960 by geologist Harry Hess based on his observations of ocean ridges (Coulomb, 1972; Kious & Tilling, 1996), occurs when liquid rock denominated basaltic magma rises from the upper mantle at the spreading centre. In such areas, the seawater, driven by convection, penetrates into the ocean crust, and circulates within the newly formed rock, is heated and rises to the seafloor. The

hot water that ejects from these vents flows at velocities up to five meters per second and reaches temperatures of 350°C (German *et al.*, 1995).

Figure 1.1 resumes a model of the hydrothermal vent process. In this model, hydrothermal process can conceptually be divided into three phases.

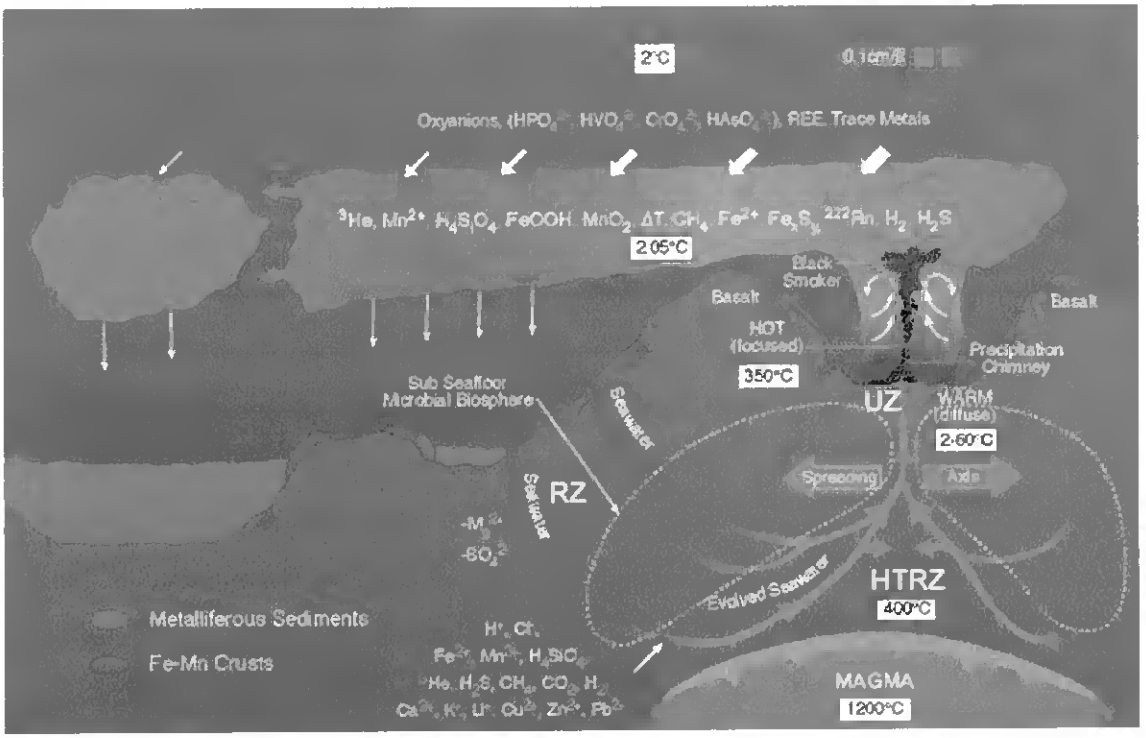

Figure 1.1 – Schematic model of the hydrothermal vent process. RZ - recharge zone; HTRZ - heat-temperature reaction zone and UZ - upflow zone (adapted from Lilley *et al.*, 1995).

First, in the recharge zone (RZ) (Figure 1.1) the cold seawater penetrates into ocean crust composed of highly porous and permeable volcanic rocks and is gradually heated along its flow path (Lilley *et al.*, 1995). As a consequence the reactions between seawater and rocks occur at relatively low temperatures up to about 60°C. Although reactions are relatively slow at these low temperatures, they begin to change the composition of the seawater through two processes (Humphris & McCollom, 1998). First, the seawater partially oxidizes the crust, resulting in the removal of oxygen from the seawater. Minerals containing iron in the rocks are replaced by iron oxides and hydroxides, which also fill the pore spaces in the upper crust (Humphris & McCollom, 1998). Second, the reactions

with seawater break down the original rock minerals, replacing them with alteration minerals such as mica and clay. In the process, potassium and other elements, such as rubidium and cesium, are transferred from seawater into the rocks (Humphris & McCollom, 1998).

As the fluid (already oxygen- and alkali-depleted relative to seawater) continues to penetrate downward toward the heat sources, it becomes heated further, and other reactions occur (Humphris & McCollom, 1998). At temperatures above 150°C, clay minerals and chlorite precipitate out of the fluid, essentially removing all of the magnesium originally present in the fluid. The formation of clay minerals and chlorite also removes hydroxyl ions from the fluid, resulting in an increase in acidity (low pH) (Humphris & McCollom, 1998). This increase in acidity, in combination with the breakdown of the original minerals in the rocks, causes Ca, Na, K, and other elements to be leached from the rock into the fluid (Figure 1.1). Another important reaction results in the formation of the mineral anhydrite (calcium sulphate) (Humphris & McCollom, 1998). This mineral instead of becoming more soluble with increasing temperature as most minerals do, it becomes less soluble. At the pressures found at the bottom of the ocean, this result in anhydrite precipitating from seawater when temperatures rise above about 150°C (Humphris & McCollom, 1998). This process removes about most of the sulphate initially present in seawater and also limits the calcium concentration of the fluid. At temperatures higher than 250°C, the remaining sulphate in the fluid reacts with iron and other metals in the crust to form metal sulphide minerals (Humphris & McCollom, 1998).

The heat-temperature reaction zone (HTRZ) (Figure 1.1) designates the region where high-temperature, water-rock reactions occur. This zone is near the heat source that drives the circulation system. The depth of the reaction zone depends on the depth of the heat source and varies from one mid-ocean ridge to another (Humphris & McCollom, 1998). The reactions in this zone determine the final chemical characteristics of the hydrothermal fluid. Reactions at such high temperatures (up to 350° to 400°C) produce a characteristic suite of alteration minerals (chlorite, sodium-rich feldspar, amphibole, epidote, and quartz), which, in turn, controls the fluid composition (Humphris & McCollom,

1998). Metals, such as Cu, Fe, and Zn, as well as sulphur, are leached from the rock by the acidic fluid. This provides the source of metals for the massive sulphide deposits observed at the seafloor, as well as the hydrogen sulphide (Lilley *et al.*, 1995; Humphris & McCollom, 1998).

Hot hydrothermal fluids are buoyant and rise toward the ocean floor in the upflow zone (UZ) (Figure 1.1). Initially, the upflow is focused along a channel of high permeability, such as a fault surface. As it reaches shallow depths, the flow may continue to be focused and then discharge through a chimney, or may follow through indirect pathways and be discharged as a more diffuse flow (Humphris & McCollom, 1998).

Although the residence time in this zone is probably very short, fluids continue to react with the rock (Lilley *et al.*, 1995). The constant reactions between the rock and the upward-flowing, metal-rich, magnesium-depleted hydrothermal fluid produce an "alteration pipe" of highly altered rocks with an interconnected network of channels filled with sulphides, silica, and chlorites (Humphris & McCollom, 1998). Sulphide minerals crystallize from the contact of hot water directly onto the volcanic rocks at the same place where hot mineral rich water flows from the ocean floor (German *et al.*, 1995). Fluids cool slightly through decompression (adiabatic cooling) and may cool significantly by losing heat to the surrounding rock (conductive cooling) or by mixing with cold seawater-like fluids below the sea floor (German *et al.*, 1995).

Cooling may cause the hydrothermal fluid to become supersaturated and particles precipitate. These particles are predominantly a mixture of sulphides (e.g. pyrrhotite, FeS, sphalerite ZnS, chalcopyrite $CuFeS_2$, etc) and sulphates (anhydrite $CaSO_4$, barite $BaSO_4$). This mineral precipitation forms a hollow, chimney-like structure through which the hot water flows. As the mineral rich hot water rushes out of the structure and mixes with the cold ocean water, many minerals precipitates and causes the vent water to appear black or white colour which are known as "black" and "white smokers" (Tivey, 1995). The high temperature vent fluids are characteristically depleted in Mg^{2+}, SO_4^{2-}, NO^{3-}, PO_4^{3-}, and total S and enriched in silica (SiO_2), metals (e.g. Fe, Mn, As, Cu, etc)

and dissolved gases (e.g. H_2S, CH_4, H_2, CO_2) (Von Damm, 1990; 1992; Lowel *et al.*, 1995; Von Damm *et al.*, 1995; Sarradin *et al.*, 1998) and have low oxygen concentrations (Childress & Fisher, 1992).

The circulation of seawater through mid-ocean crustal materials is responsible not only for dissipating a large amount of heat, but also for important seawater-rock interaction. These interactions constitute a major exchange of ions between the ocean and the igneous material, and provide a mechanism to transport selected chemical constituents into the deep-sea (Corliss *et al.*, 1979; Edmond *et al.*, 1979). Many of these dissolved ions and solutes (NH_4^+, Fe^{2+}, Mn^{2+}, organic compounds) and volcanic gases (H_2S, CH_4, H_2, CO) have potential to support primary and secondary bacterial production.

The chemical variability observed between individual hydrothermal vents or fields, is due to variable mixing between the two-end member fluids. End-member fluids consist in pure hydrothermal fluid that has seen no dilution since contact with the heat source (Tunnicliffe, 1991). Initial geochemical investigations at Galapagos Rift hydrothermal vents, indicated that temperature and fluid chemistry of the water samples were the result of a two end-end member mixing (Edmond *et al.*, 1979). This process is controlled by subsurface plumbing and by the mechanisms of hydrothermal circulation, which can vary both in time and space. More recently, it was found that phase separation occurred during seabed boiling of hydrothermal vent fluids (Massoth *et al.*, 1989), complicating the mixing process, therefore when studying low-salinity fluids from the condensed vapour phase and residual brine these characteristics must be taken in account (Von Damm, 1988).

As mentioned before, active hydrothermal vent fields were identified in several oceans (Van Dover, 2000) and have been intensively studied in recent years. This study was focused in three hydrothermal vent sites in the Mid-Atlantic Ridge near the archipelago of Azores, Menez-Gwen, Lucky Strike and Rainbow described below.

1.3. The Mid-Atlantic Ridge

The Mid-Atlantic Ridge (MAR) was discovered by Bruce Heezen in the 1950s and is an underwater mountain range of the Atlantic Ocean that extends from Iceland to Antarctica (Kious & Tilling, 1996) (Figure 1.2A). This ridge is an oceanic rift that separates the North American Plate from the Eurasian Plate in the North Atlantic, and the South American Plate from the African Plate in the South Atlantic. The MAR comprises several known hydrothermal vents separated in Northern vent fields, which include the Menez-Gwen, Lucky Strike, Rainbow and Saldanha sites located near Azores Triple Junction and the Southern vent fields, that comprises Broken Spur, TAG, Snake Pit and Logatchev sites (Figure 1.2B).

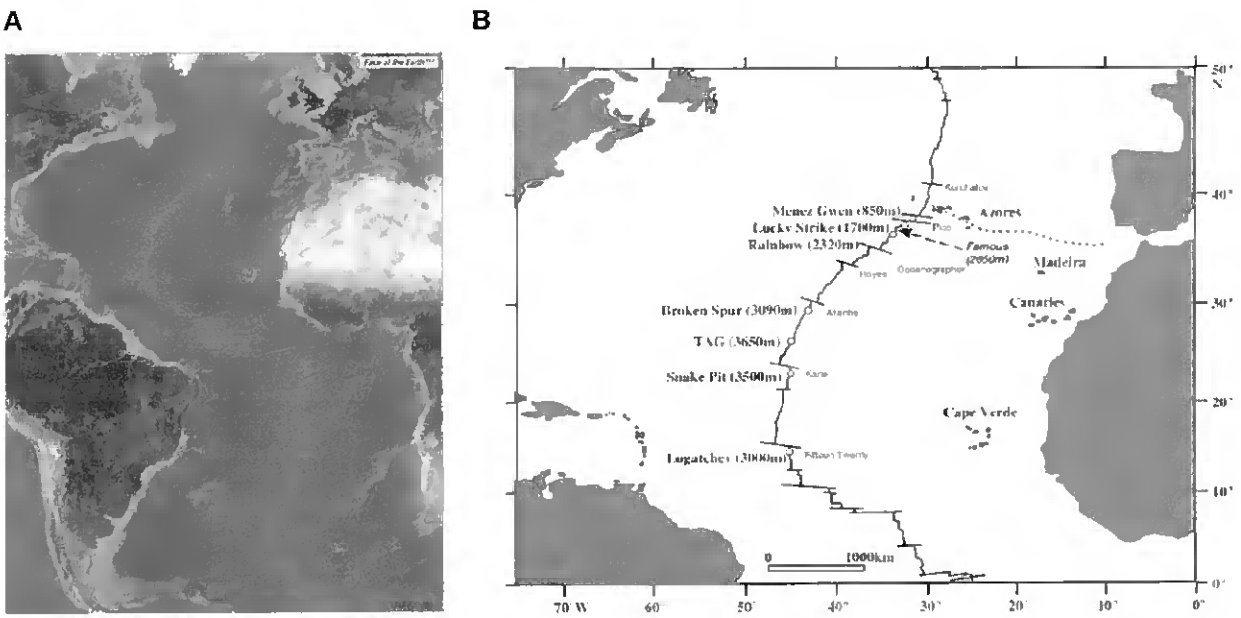

Figure 1.2 – Mid-Atlantic Ridge (MAR) extension (A) and known hydrothermal vent sites (B) (adapted from Desbruyères *et al.*, 2001).

Menez-Gwen (37°51'N, 31°31'W, 850 m), Lucky Strike (37°17'N, 32°16'W, 1700 m) and Rainbow (36°13'N, 33°54'W, 2300m) are among the most visited and sampled sites from MAR hydrothermal vents and their main characteristic are further described (sections 1.3.1 to 1.3.3).

1.3.1. Menez-Gwen (37°51'N, 31°31'W, 850 m)

The Menez-Gwen area was discovered during the French cruise DIVA I in 1994 (Fouquet *et al.*, 1994, 1995). One of the characteristics of the Menez-Gwen segment is the absence of a central rift. The main volcanic feature is a circular volcano at the central part of the segment (Fouquet *et al.*, 1995). This volcano is 700 meters high, with a diameter of 17 km. At its top, there is an axial graben (an elongate crustal block that is relatively depressed between two fault systems) 6 km long, 2 km wide and 300 meters deep (Charlou *et al.*, 2000). The Menez-Gwen site is composed by several active sites located on the southeast and east slopes of this volcano at depths ranging from 840 to 865 m. The volcano is composed entirely of extremely fresh pillow lava with no sediment cover (Fouquet *et al.*, 1995). Chimneys are typically small and essentially composed of white anhydrite, formed by the mixing of seawater and hydrothermal fluid. Around these small chimneys, some mounds with hot water diffusing through all surfaces are found (Fouquet *et al.*, 1995).

Menez-Gwen vents exhibit temperatures between 271° C and 284° C with pH between 4.2 and 4.8 (Table 1.1) (Douville *et al.*, 2002). The hydrothermal fluids in Menez-Gwen have relatively low H_2S concentrations when compared to other hydrothermal fields (< 1.5 mM), while enriched in CH_4 (1.35-2.63 mM) (Table 1.1). Since this hydrothermal site is located in a basaltic environment, methane is produced by out-gassing of carbon from the mantle and is related to the carbon-enriched character of basalt (Charlou *et al.*, 1997). This gas enrichment is consistent with the fact that Menez-Gwen is considered a young site at the beginning of its activity (Douville *et al.*, 1997; Douville *et al.*, 2002). The metal concentration in this hydrothermal vent site, although clearly higher than in average seawater is relatively lower compared to other MAR vent sites, especially Ag (4.3-17 nM), Cd (9-12 nM), Cu (2 µM), Fe (2-18 µM), Mn (59-68 µM) and Zn (2 µM) (Table 1.1). This metal composition is consistent with a short duration of fluid-rock interaction linked to a shallow circulation system induced by the Azores Hot Spot (Douville *et al.*, 1999).

1.3.2. Lucky Strike (37°17'N, 32°16'W, 1700 m)

The Lucky Strike vent field was discovered in 1992, during the joint US-French FAZAR expedition (FAZAR Scientific Team, 1993) and is one of the largest hydrothermal areas known, with 21 active chimneys (Langmuir *et al.*, 1993). The Lucky Strike segment has a wide median valley with a "box-car" shape of parallel valley walls, and a well-developed central volcano (where the hydrothermal site is located). Numerous surveys have been carried out in this segment. Underway bathymetry, gravity and magnetic surveys were carried out during the SIGMA cruise in 1992 (Needham *et al.*, 1992) and SudAçores cruise in 1998 (Cannat *et al.*, 1999). Seafloor reflectivity was mapped with deep-tow instruments during two scientific missions: the HEAT cruise (1994) mapped the axial valley floor over the entire length of the segment and the LUSTRE cruise (1996) produced a highly detailed reflectivity map of the central volcano (German *et al.*, 1996). The hydrothermal site has been repeatedly visited during multidisciplinary diving missions and a large number of rocks, fluids and biological samples collected. The chimneys are distributed around a fossil lava lake in the central caldera of an axial volcano, located in the centre of the Lucky Strike segment. Much of the periphery of the Lucky Strike lava lake appears to be underlain by a hydrothermally altered rock formation that provides a relatively impermeable barrier, referred as "slab". The abundance of slab found suggests that this hydrothermal vent site has been active in the past and has been recently reactivated (Von Damm *et al.*, 1998).

Although deeper than Menez-Gwen vent field, this hydrothermal vent site is relatively shallow (1700 m) and the maximum temperature of hydrothermal fluids (320°C) is just beneath the boiling point (340°C) at that depth. A high variability in fluid composition between vents was observed at Lucky Strike and two sources of fluids were hypothesised (Von Damm *et al.*, 1998). Lucky Strike is usually associated with Menez-Gwen when comparing vent fluid characteristics. Both are basalt-hosted sites, strongly affected by recent volcanic activity and shallow circulation systems. A higher range of temperature (185-324°C) and pH (3.4-5.0) is observed in Lucky Strike fluids compared to other MAR vent sites (Table 1.1) (Douville *et al.*, 2002). This site is relatively

depleted in CH_4 (0.5-0.97 mM), while high concentrations of H_2S (0.6-3.4 mM) are present in Lucky Strike fluids compared to Menez-Gwen and Rainbow (Table 1.1) (Douville *et al.*, 2002). The metal content in Lucky Strike fluids are higher than those found in Menez-Gwen, particularly Ag (4.7-25 nM), Cd (18-79 nM), Cu (2-30 µM), Fe (70-920 µM), Mn (77-540 µM) and Zn (2-40 µM) (Table 1.1) (Douville *et al.*, 2002).

1.3.3. Rainbow (36°13'N, 33°54'W, 2300m)

The Rainbow vent site was discovered in 1997 and is the deepest vent site considered in this dissertation, located at 2300 m depth in the axial discontinuity south of the AMAR (ALVIN Mid-Atlantic Ridge) segment (Fouquet *et al.*, 1997). Rainbow site is set on outcrops of serpentinized peridotites in the tectonized setting of an axial discontinuity. The Rainbow vent field comprises more than 30 groups of active small sulphide chimneys over an area of 15 km^2. There are numerous inactive structures among a large number of rather short-lived active venting sites. It is one of the most active hydrothermal vent fields along the MAR in terms of heat and chemical flux. Chimneys and massive sulphides are enriched in Cu, Zn, Co and Ni compared to other sites in basaltic environments (Fouquet *et al.*, 1998). This enrichment could be due to interactions between the hydrothermal fluids and the basement of serpentinized peridotites.

Rainbow hydrothermal fluids are uniform in composition and influenced by phase separation (Douville *et al.*, 1997; 1999). Fluid temperature in Rainbow is higher (365°C) compared to Menez-Gwen and Lucky Strike, just beneath the boiling point at that depth, extremely acidic (pH = 2.8) and relatively low in H_2S content (1.0 mM) (Table 1.1) (Douville *et al.*, 2002). The metal concentrations observed in Rainbow fluids are the highest observed in the MAR hydrothermal area, particularly for Ag (47 nM), Cd (130 nM), Cu (140 µM), Fe (24000 µM), Mn (2250 µM) and Zn (160 µM), which are several fold higher than those found in Menez-Gwen and Lucky Strike fluids (Table 1.1) (Douville *et al.*, 2002). This metal enrichment is likely to be related to the formation of chlorine-complexes at the high temperature reached by these fluids (Douville *et al.*, 1999).

Table 1.1 – Temperature and concentration of chemical species in end-member fluids for different MAR vent fields, Menez-Gwen, Lucky Strike and Rainbow compared to average seawater (adapted from Douville *et al.*, 2002).

Site	Menez-Gwen 37°51'N, 31°31'W	Lucky Strike 37°17'N, 32°16'W	Rainbow 36°13'N, 33°54'W	Seawater
T (°C)	271 - 284	185 - 324	365	-
pH	4.5	3.4 - 5.0	2.8	7.8
H_2S (mM)	1.5	0.6 - 3.4	1.0	~0
CO_2 (mM)	17 - 20	8.9 - 28	< 16	-
CH_4 (mM)	1.35-2.63	0.5-0.97	2.2-2.5	~0
Ag (nM)	4.3 - 17	4.7 - 25	47	0.023
Cd (nM)	<9 - 12	18 -79	130	0.7
Cl (mM)	380 - 400	413 - 554	750	546
Co (µM)	< 2	< 2	13	< 2
Cu (µM)	< 2	< 2 - 30	140	0.0033
Fe (µM)	< 2 - 18	70 - 920	24000	0.0045
Mn (µM)	59 - 68	77 - 450	2250	0.0013
Ni (µM)	< 2	< 2	3	< 2
Si (mM)	8.2 - 11.2	8.2 - 16	6.9	<0.2
Zn (µM)	< 2	< 2 - 40	160	0.028

Recently, there has been an increasing interest by the scientific community in protect vulnerable deep-sea ecosystems and hydrothermal vent systems in particular (Gjerde & Breide, 2003). Canada was the first country to protect a deep-sea hydrothermal vent system, when in 1998 the Endeavour Hydrothermal Vents, located at 2250 m deep, 250 km southwest of Vancouver Island, were proposed as a Marine Protected Area (MPA) (Andrie, 2001). In 2002 the regional government of the Azores give an important step towards the conservation of these ecosystems, and declared two vent fields in the MAR (Menez-Gwen and Lucky Strike) as MPA (Christiansen, 2003).

After that, Rainbow hydrothermal vent field has been also proposed as a potential Marine Protected Area by WWF under the OSPAR convention (Oslo

and Paris Convention for the Protection of the Marine Environment of the Northeast Atlantic) (Christiansen & Gjerde, 2003). Thus, the three hydrothermal vents considered in this study are the first deep-sea marine protected areas in the Northeast Atlantic under the OSPAR convention.

1.4. Life in hydrothermal vents

Although hydrothermal vent environments have extremely high temperature, low oxygen and are extremely rich in potentially harmful chemical species, mainly hydrogen sulphide (H_2S), methane (CH_4) and both essential and non-essential metals (Mn, Fe, Cu, Zn, Ag and Cd) (Table 1.1) and therefore considered one of the most toxic environments on earth, these sites capture the attention of scientists because of its luxuriant animal communities (Lonsdale, 1977; Corliss *et al.*, 1979).

The discovery of these luxuriant ecosystems immediately raised questions about the energy base that could sustain such a large biomass in the food-limited deep sea (Lonsdale, 1977; Jannasch & Wirsen, 1979). Free-living and symbiotic microorganisms were soon implicated in chemoautotrophic primary production through the oxidation of sulfide-rich fluids emanating from cracks in the basalt seafloor and the use of energy derived from redox reactions in carbon-dioxide fixation (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981).

Therefore, contrarily to most marine ecosystems that depend on sunlight and therefore on photosynthesis as primary source of energy, hydrothermal vent biological communities depend on a very different energy source: chemosynthesis (Van Dover & Lutz, 2004). The abundant reduced chemical species found in hydrothermal vent fluids, particularly H_2S, are used as an energy source by chemosynthetic bacteria for carbon fixation. For the vent organisms, this offers the potential for a large food source more or less independent from the photic zone (Jannasch, 1989).

Most vent organisms rely entirely on this chemosynthetic primary production by actively feeding on free-living bacteria or by forming symbiotic relationships with chemosynthetic bacteria for the bulk of their food supply (Childress & Fisher, 1992; Kennicutt & Burke, 1995; Fisher, 1996). The extraordinarily high level of chemosynthetic-based primary production that is supported by venting is one of the outstanding differences between these regions and the average deep-sea environment.

The needs of the symbiont or free-living bacteria for sulphide and oxygen, and of the animal for additional oxygen, limit vent fauna to areas where the hot vent fluids with sulphide and cold, oxygen-bearing ambient water actively mix (Childress & Fisher, 1992). However, sulphide and oxygen react spontaneously and do not coexist in significant concentrations for significant periods of time (Johnson *et al.*, 1986; Millero, 1986). Animals in those areas therefore experience rapid changes in temperature that coincide with changes in oxygen and sulphide concentration. Animals are found at high densities in hydrothermal vents, but they are of low diversity and have a high degree of endemism (Sibuet & Olu, 1998; Tunnicliffe *et al.*, 1998).

In hydrothermal vents near Azores Triple Junction (Figure 1.2B), especially those described earlier (Menez-Gwen, Lucky Strike and Rainbow) extensive mussels beds of *Bathymodiolus azoricus* dominate the hydrothermal vent fauna (Van Dover, 1995; Colaço *et al.*, 1998; Desbruyeres *et al.*, 2001) as well as three species of shrimps, *Chorocaris chacei*, *Mirocaris fortunata* and *Rimicaris exoculata* (Desbruyères *et al.*, 1994; Colaço *et al.*, 1998).

The *Bathymodiolus* mussels are ideal organisms to consider in this study because of their global distribution in deep-sea hydrothermal vent habitats (see Table 1.2). Since this study was carried out in the hydrothermal vent mussel *B. azoricus*, its main characteristics are presented in more detail in the next section 1.4.1.

1.4.1. Hydrothermal vent mussels (*Bathymodiolus* sp.)

Vent mussels (genus *Bathymodiolus*) are common hydrothermal bivalves and 17 species have been described in different hydrothermal regions (Table 1.2). *B. azoricus* and *B. puteoserpentis* are two important Atlantic species present in the Northern MAR (Menez-Gwen, Lucky Strike and Rainbow) and Southern MAR (Broken Spur, TAG, Snake Pit and Logatchev) hydrothermal sites respectively (Britayev *et al.*, 2003). Other Atlantic species include the *B. heckerae* (Blake Ridge - North Carolina, Florida and Louisiana slopes) (Van Dover *et al.*, 2003), *B. childressi*, *B. brooksi* (Gulf of Mexico) (Gustafson *et al.*, 1998), *B. boomerang* (Barbados) (Von Cosel & Olu, 1998) and *B. mauritanicus* (Mauritania) (Von Cosel, 2002). In the Pacific Ocean, several species of *Bathymodiolus* exist, such as *B. brevior*, *B. elongatus* (North Fiji Basin), *B. thermophilus* (East Pacific Rise) (Berg & Van Dover, 1987), *B. japonicus*, *B. platifrons*, *B. septemdierum*, *B. aduloides* (Japan) (Hashimoto & Okutani 1994; Miyazaki *et al.*, 2004) and two new *Bathymodiolus* species (NZ-1 and NZ-2) recently found near New Zealand (Smith *et al.*, 2004).

Table 1.2 – Identified species of vent mussels *Bathymodiolus* genus.

Species	Location	References
	Atlantic Ocean	
B. azoricus	Northern MAR	Von Cosel *et al.*, 1999
B. puteoserpentis	Southern MAR	Von Cosel *et al.*, 1994
B. heckerae	Blake Ridge	Van Dover *et al.*, 2003
B. childressi	Gulf of Mexico	Gustafson *et al.*, 1998
B. brooksi	Gulf of Mexico	Gustafson *et al.*, 1998
B. boomerang	Barbados	Von Cosel & Olu, 1998
B. mauritanicus	Mauritania	Von Cosel, 2002
	Pacific Ocean	
B. brevior	North Fiji Basin and Lau Basin	Von Cosel *et al.*, 1994
B. elongatus	North Fiji Basin	Von Cosel *et al.*, 1994
B. thermophilus	East Pacific Rise (Galapagos)	Kenk & Wilson, 1985
B. japonicus	Western Pacific Ocean (Japan)	Hashimoto & Okutani, 1994
B. platifrons	Western Pacific Ocean (Japan)	Hashimoto & Okutani, 1994
B. septemdierum	Western Pacific Ocean (Japan)	Hashimoto & Okutani, 1994
B. aduloides	Western Pacific Ocean (Japan)	Hashimoto & Okutani, 1994
B. new species NZ-1	New Zealand	Smith *et al.*, 2004
B. new species NZ-2	New Zealand	Smith *et al.*, 2004
	Indian Ocean	
B. marisindicus	Central and East Indian Ocean	Hashimoto, 2001

One of the most important characteristics of hydrothermal vents mussels of all *Bathymodiolus* genus compared to coastal mussels is the presence of intracellular symbiotic bacteria mainly in the gills. In some of these organisms, intracellular symbionts are sulphur bacteria only (Nelson *et al.*, 1995), which autotrophically synthesize organic matter directly in the gill tissue using the energy of reduced sulphur compounds emanated from hydrothermal fluids. Other *Bathymodiolus* species contain both sulphur and methanotrophic symbiotic bacteria in their gill tissue. Because of such double symbiosis, these animals are able to utilize both the energy of reduced sulphur compounds and methane consumed by methanotrophic endosymbionts (Cavanaugh *et al.*, 1992; Nelson *et al.*, 1995; Southward *et al.*, 2001).

1.4.1.1. *Bathymodiolus azoricus*

The vent mussel *Bathymodiolus azoricus* was firstly describe in 1999 by Von Cosel *et al.* whose systematic classification is in Table 1.3.

Table 1.3 – Systematic classification of *B. azoricus* (adapted from Von Cosel *et al.*, 1999).

Phylum	Mollusca
Class	Bivalvia
Subclass	Pteriomorphia
Order	Mytiloida
Family	Mytilidae
Genus	Bathymodiolus
Specie	*Bathymodiolus azoricus*

As mentioned previously, dense communities of these mussels populations occur in Northern MAR vent fields, including Menez-Gwen, Lucky Strike and in less extent in Rainbow and this mussel is considered the dominant species in the Azores Triple Junction area as can be seen in Figure 1.3 (Van Dover, 1995; Colaço *et al.*, 1998; Desbruyères *et al.*, 2001).

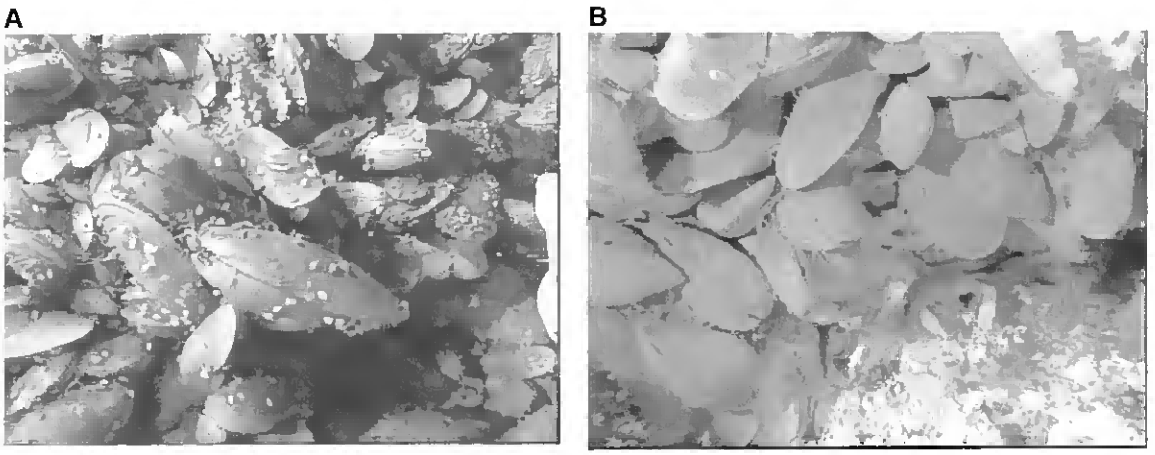

Figure 1.3 – Mussel clusters in MAR hydrothermal vent site Menez-Gwen (A) and Lucky Strike (B) (Fotos from VENTOX Project – © ATOS/IFREMER).

Considering the intracellular symbiotic bacteria in *B. azoricus*, a dual symbioses exist and this mussel derive a substantial portion of its food from free living chemoautotrophic sulphur-oxidizing and methanotrophic symbiotic bacteria that live in mussel gills and use vents substances to produce organic compounds and energy (Fiala-Médioni & Felbeck, 1990; Raulfs *et al.*, 2004).

Although the presence of chemoautotrophic endosymbionts in the gills of *B. azoricus*, this mussel still shows a filtering behaviour, not only for feeding from particulate organic matter (Le Pennec & Hily, 1984; Le Pennec & Prieur, 1984; Le Pennec *et al.*, 1990), but also for providing its symbionts with methane or hydrogen sulphide and for respiration (Desbruyères *et al.*, 2001). It is possible that this filter behaviour may also increase the degree of metal exposure by enhancing the contact between both gills and mantle tissues with the surrounding metal rich water.

In Figure 1.4 is presented one specimen of *B. azoricus* with open valves, showing the gills and mantle, the two tissues selected for this study. These tissues are in direct contact with the surrounding metal rich water of hydrothermal vent environments and consequently can be considered target organs for metal effects. Similarly, in coastal bivalve species, the gills and the mantle has been reported by several authors as main sites for metal accumulation because of its large surface area (Blasco & Puppo, 1999).

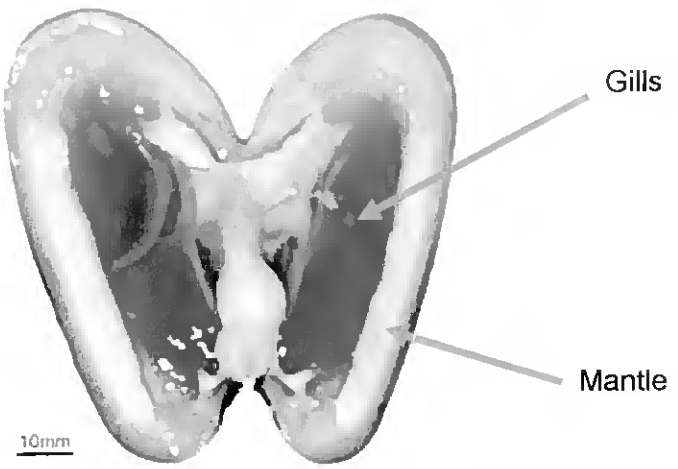

Figure 1.4 – Specimen of *B. azoricus* with open valves. The gills and mantle tissues are indicated.

In addition, the mucus that covers the gills has been indicated as responsible for the capture of metals in dissolved and particulate forms present in water flowing through the cavity of the mantle (Cunningham, 1979). Gills function as both a site for metal uptake, and as an important reservoir for metal storage (Langston *et al.*, 1998).

1.4.1.1.1. Metals in *Bathymodiolus azoricus*

Trace metals of concern in aquatic toxicology include both essential (e.g. Cu, Mn, Zn) and nonessential (e.g. Cd, Ag, Hg) elements. Essential metals are known to be required in small concentrations for metabolism and perform a wide variety of functions including the activation of metalloenzymes, stress proteins, oxygen transport and redox activities, while non-essential ones have no identified biological functions (Soto & Marigoméz, 1995).

Exposure to either essential or nonessential metals can produce toxic effects when the concentrations exceed the capacity of physiological detoxification mechanisms (Rainbow, 1993). These toxic effects generally result from the non-specific binding of reactive metal cations to biologically important macromolecules, causing modification of molecular function (Di Giulio *et al.*, 1995). Thus, metals can bind to sulphydryl, hydroxyl, carboxyl groups, amino

residues of proteins, peptides and aminoacids and phosphate groups of nucleotides and nucleic acids among others (Soto & Marigómez, 1995). However, metals have a preference for sulphur donor groups (compared with oxygen) and therefore tend to form more stable complexes with the sulphydryl residues of amino acids and polypetides (Rainbow, 1993; Soto & Marigómez, 1995).

As a result, the regulation and detoxification of metals within the cells are essential to maintaining optimal cellular function. At the cellular level, metal metabolism involves the sequestration of metals in membrane-bound vesicles such as lysosomes (Sarasquete *et al.*, 1992; Cajaraville *et al.*, 1995). Metals significantly increase lysosomal size and number of these organelles (Cajaraville *et al.*, 1989; Marigómez *et al.*, 1989; Regoli *et al.*, 1998), cause reduction in the stability of lysosomal membranes and increase in lysosomal enzyme activities (Cajaraville *et al.*, 2000). Other metal detoxification mechanisms include the sequestration of metal cations in insoluble granules (Viarengo & Nott, 1993) and the binding of metals to inducible metal-binding ligands such as metallothionein (MT) (Viarengo, 1989; Roesijadi, 1992). These binding sites regulate the availability of both essential and nonessential metals within the cell. For example, they can provide high-affinity sinks for nonessential metals to reduce their interactions with macromolecules. They can also sequester essential metals to modulate their availability for metal-requiring apoproteins, while minimizing their non-specific binding (Chassard-Bouchaud *et al.*, 1986; Viarengo & Nott, 1993).

Recently, several studies were conducted to understand what mechanisms hydrothermal vent organisms developed to survive in such metal rich environments. Concentrations of both essential and non-essential metals in vent bivalves are in general significantly higher, compared to coastal molluscs, such as the mussels *Mytilus galloprovincialis*, *Mytilus edulis* and *Mytilus chilensis* and the clams *Ruditapes decussatus*, *Rudipates philippinarum* and *Scapharca inaequivalvis* (see Table 1.4). The high metal concentrations accumulated in vent mussels tissues can be explained by a long term exposure to hydrothermal fluids (Rousse *et al.*, 1998; Fiala-Médioni *et al.*, 2000).

Table 1.4 – Metal concentrations (μg g^{-1} d.w.) in mollusc tissues from hydrothermal vent sites and coastal environments

Species	Sites	Tissues	Ag	Cd	Cu	Fe	Mn	Zn	References
	Hydrothermal vents								
Bathymodiolus azoricus	Menez-Gwen (ATOS 8)	Gills	4.6±1.1	7.9±2.2	57.3±11.6	186±44	6.3±0.8	161±11	Fiala-Médioni *et al.*, in prep
		Mantle	0.7±0.2	0.6±0.2	11±1.9	10.4±2	3±0.7	42±9	
	Menez-Gwen (ATOS 10)	Gills	1.9±0.5	3.2±0.8	88.9±16.4	206±42	4.8±0.6	173±24	
		Mantle	0.5±0.2	0.3±0.1	39.7±11.7	50.8±13	2±0.3	71±15	
	Menez-Gwen	Gills	3.5±0.4	4.2±1.4	136±15	144±23	4±0.4	136±34	Rousse *et al.*, 1998
		Digestive gland	1.3±0.3	10.6±2.3	65±10	176±40	4.6±0.4	81±10	
		Mantle	0.5±0.3	0.4±0.1	13±3	41±4	3.6±0.7	49±7	
	Lucky Strike (Bairro Alto)	Gills	5.2±1.2	47.2±10	79.7±19.4	303±63	7.6±1.9	1976±570	Fiala-Médioni *et al.*, in prep
		Mantle	0.8±0.2	2.9±1.0	14.2±3.5	235±61	5.8±1.5	124±37	
	Lucky Strike (Eiffel Tower)	Gills	1.7±0.4	17.6±2.1	87.7±15.4	288±53	7.1±1.6	589±135	
		Mantle	0.5±0.1	1.6±0.3	16.2±3.3	100±26	5.5±1.0	65±16	
	Lucky Strike	Gills	1.1±0.3	5.8±2	129±71	347±87	4.0±0.8	653±281	Rousse *et al.*, 1998
		Digestive gland	0.7±0.5	2.8±1.6	52±28	1161±270	4.2±0.3	288±40	
		Mantle	0.1±0.1	0.2±0.08	21±10	180±72	5.5±1.0	79±9	
		Whole organism		1.12±0.82	45.7±30.5			48±21	Geret *et al.*, 1998
	Rainbow	Gills	2.3±0.6	2±0.5	65.5±13.7	2685±663	9.5±2.3	87±13	Fiala-Médioni *et al.*, in prep
		Mantle	0.1±0.02	0.02±0.003	2.3±0.6	186±81	7.2±0.6	28±7	
Bathymodiolus thermophilus	East Pacific Rise	Gills	51.8±49.6	ND	41.3±34.2	310±38.1	111±131	217±279	Smith & Flegal, 1989
		Digestive gland	21.4±8.49	ND	30±25.9	948±281	25.8±17.2	60.4±40.4	
Calyptogena magnifica	East Pacific Rise	Gills	46.5±28	46±12	219±102	1931±626	<7	1560±540	Roesijadi & Crecelius, 1984
		Mantle	50.7±7	1.2±0.4	150±7.2	302±274	13.3±2.4	302±274	
Vesicoma gigas	Guaymas Basin	Gills		115.2±196	8.26±5.8	403.2±242	18±17	884.8±1042	Ruelas-Inzunza *et al.*, 2003
		Mantle		12.3±5.5	29.7±11.1	277.5±27	10.6±1.0	419.1±79.0	
		Gonads		10.1±2.3	22.0±17.1	195.9±26	7.31±3	192.3±32.0	

Table 1.4 – Metal concentrations (μg g^{-1} d.w.) in mollusc tissues from hydrothermal vent sites and coastal environments *(Continued)*

Species	Sites	Tissues	Ag	Cd	Cu	Fe	Mn	Zn	References
	Coastal Areas								
Mytilus galloprovincialis	Korea	Whole organism		0.63±0.37	8.76±1.71	150±53.6	63.5±55.9	124±20.9	Szefer *et al.*, 2004
	Greece	Digestive gland		0.9±0.2	7.1±0.7		6.0±0.5	10.0±0.2	Kalpaxis *et al.*, 2004
	Spain	Whole organism		0.63±0.09	5.57±1.75			283±116	Besada *et al.*, 2002
	Spain	Whole organism		0.20 to 0.77	6.8 to 29.9	174 to 715	4.3 to 15.8	85 to 447	Beiras *et al.*, 2003
	Portugal	Whole organism		2.3±0.1	5.0±0.4	200±147	5.7±3.6	206±101	Beblanno & Machado, 1997
	Italy	Whole organism		0.055±0.007	0.27±0.07			13.8±2.4	Licata *et al.*, 2004
	Italy	Gills		0.6±0.6	14.5±4.3	26.8±16.9	1.7±0.9	115.5±38.2	Irato *et al.*, 2003
	Italy	Digestive gland		1.6±0.4	19.2±3.9	101.7±69.6	2.9±1.1	127.6±20.0	
	Romania	Whole organism		1.08±0.16	8.05±0.26	106±8.0	15.8±1.7	145±19	Roméo *et al.*, 2005
	Morocco	Whole organism		3.21	17	432	11.04	249	Kaimoussi *et al.*, 2001
Mytilus edulis	China	Whole organism	0.02±0.01	2.3±0.3	1.83±0.54	120.6±35.2		76.9±11.1	Fung *et al.*, 2004
	England	Whole organism	5.2	1.8	23.9	478	13.9	118	Giusti *et al.*, 1999
	Chile	Whole organism		1.4 to 3.0	3.0 to 12.2	557.6 to 953.8	12.5 to 19.6	61.4 to 130.9	Manly *et al.*, 1996
Mytilus chilensis	Chile	Whole organism			2.74±1.19	33.84±17.04	2.64±1.05	13.3±4.72	España *et al.*, 1998
Ruditapes decussatus	Spain	Whole organism		0.25±0.06	3.4±0.36	130±25	2.1±0.12	22±1.7	Usero *et al.*, 1997
Ruditapes philippinarum	Spain	Whole organism		0.28±0.02	2.3±0.17	116±24	3.6±0.08	18±1.7	
Tapes philippinarum	Italy	Whole organism		1.1±0.6	10.9±2.3	251.0±86.9	10.0±2.6	101.2±39.0	Irato *et al.*, 2003
	Italy	Gills		0.7±0.6	41.6±11.6	268.2±84.9	8.1±2.2	100.8±21.0	
Scapharca inaequivalvis	Italy	Whole organism		8.9±8.0	6.1±3.0	514.0±103.1	31.5±15.1	229.8±144.5	
	Italy	Gills		0.8±0.2	19.8±3.6	178.1±84.0	2.4±1.5	61.5±8.6	

Moreover, metal concentrations were significantly higher in the gills compared to the mantle in hydrothermal vent mussels *B. azoricus* (Rousse *et al.*, 1998; Fiala-Médioni *et al.*, in prep.) and vent clams *Calyptogena magnifica* (Roesijadi & Crecelius, 1984) and *Vesicoma gigas* (Ruelas-Inzunza *et al.*, 2003) (Table 1.4).

Metal concentrations in *B. azoricus* collected in different MAR hydrothermal vent sites also showed to be site dependent (Table 1.4) (Rousse *et al.*, 1998; Fiala-Médioni *et al.*, 2000) and suggested that the metal content in these mussels are related to some extent with the metal levels present in the hydrothermal vent fluids (see Table 1.1). Due to its wide distribution (see Table 1.2) the *Bathymodiolus* genus was recently proposed to be used as sentinel organisms to evaluate metal concentrations at hydrothermal sites (Hardivillier *et al.*, 2004).

Metallothioneins occur mainly in the cytosol and have also been detected in the nucleus and lysosomes following laboratory exposure or exposure in the field to essential and non-essential metal ions. MTs have high cysteine content (30%), low molecular weight, heat-stability and a strong affinity for binding 6 to 12 atoms of class B metals such as Ag, Cd, Cu, Hg and Zn (Bebianno & Serafim, 1998; Langston *et al.*, 1998; Cajaraville *et al.*, 2000; Dabrio *et al.*, 2002). MT is also known to have a protective role against oxidative damage caused by reactive oxygen species (ROS) by both bind and sequester transitions metals or scavenging oxyradicals like hydrogen peroxide (Anderson *et al.*, 1999; Cavaletto *et al.*, 2002; Cui *et al.*, 2004) and hydroxyl radicals (Hayes & McLellan, 1999; Viarengo *et al.*, 1999; 2000).

Because it is well established that MT acts as a detoxification mechanisms in mussels from metal contaminated coastal environments, these proteins were the first to be studied and quantified in several tissues of *B. azoricus*. The results suggested that the concentrations of these proteins are of the same order of magnitude as those in coastal mussels (Table 1.5). This finding was highly unexpected and therefore there is a need to establish the role of these

proteins in the metal metabolism of hydrothermal mussels (Fiala-Médioni *et al.*, 2000).

Table 1.5 – Metallothionein concentrations in mussels from MAR hydrothermal vent sites and coastal environments.

Species	Sites	Tissues	MT	References
	Hydrothermal vents			
Bathymodiolus azoricus	Lucky Strike	Gills	0.53 [a]	Géret *et al.*, 1998
		Digestive gland	0.97 [a]	
		Remainder	0.19 [a]	
	Menez-Gwen	Gills	3.82±0.79 [b]	Rousse *et al.*, 1998
		Mantle	0.84±0.14 [b]	
	Lucky Strike	Gills	3.42±0.34 [b]	
		Mantle	1.49±0.21 [b]	
	Coastal Areas			
Mytilus galloprovincialis				Bebianno & [illegible]do, 1997
	South coast (Portugal)	Whole organism	3.9 to 13.0 [b]	Serafim *et al.* [illegible]
	Ria Formosa (Portugal)	Gills	2.2 to 3.8 [b]	Ferreira *et al.* [illegible]
	Guadiana (Portugal)	Whole organism	6.8 [b]	Raspor *et al.* [illegible]
	Lim Channel (Croatia)	Gills	0.45 to 0.65 [a]	
		Digestive gland	2.85 to 4.23 [a]	
		Digestive gland	2.1 [a]	Pavicic *et al.* [illegible]
Mytilus edulis	Bay of Bourgneuf (France)	Gills	0.24±0.003 [a]	Géret & Cossa [illegible]
		Mantle	0.38±0.002 [a]	
		Digestive gland	1.6±0.046 [a]	
	Gironde estuary (France)	Gills	0.3 [a]	Amiard *et al.* [illegible]
		Digestive gland	1.5 to 5.5 [a]	
	Gironde estuary (France)	Whole organism	0.5 to 0.6 [a]	Amiard-Triquet *et al.*, 1998a
	Arctic	Gills	1.6 to 2.6 [a]	Amiard-Triquet *et al.*, 1998b
		Digestive gland	8.8 to 19.6 [a]	

[a] mg g^{-1} wet weight
[b] mg g^{-1} dry weight

Recently, complementary DNA (cDNA) sequences of two molecular weigh variants of dimeric and monomeric MT forms (MT-10 and MT-20) were obtained for the Atlantic and Pacific hydrothermal mussels (*Bathymodiolus azoricus* and *Bathymodiolus thermophilus*) and few differences in the aminoacid sequence were observed (Hardivillier *et al.*, 2004). The protein sequence of

Bathymodiolus MT-10 presented 71 aminoacids, 21 of which were cysteines residues, while MT-20 presented 69 aminoacids (with 23 cysteines residues). The comparison between metallothionein cDNA sequences of the *Bathymodiolus* and the *Mytilus* genera also shows strong homologies among metallothioneins of hydrothermal and coastal mussels (more than 80%), with MT-10 of *B. azoricus* close to MT-10III and MT-10IV isoforms of *M. edulis*, while MT-20 of *B. azoricus* close to MT-20II of M. edulis. Interestingly, cDNA sequence of MT-20 of *B. azoricus* and *B. thermophilus* were identical (Hardivillier *et al.*, 2004). Moreover, although MT concentrations found in *B. azoricus* in this study was considered high, the metal content bound to MT was low, supporting that these proteins may not play such an important part in metal detoxication in these mussels as previously supposed (Hardivillier *et al.*, 2004).

Metals can also enhance the production of reactive oxygen species (ROS) and consequently increase oxidative stress in the organisms (Stohs & Bagchi, 1995; Hassoun & Stohs, 1996; Stohs *et al.*, 2001; Harris & Shi, 2003). Transition metals catalyze the formation of ROS, by donating or accepting single electrons. ROS, including hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{-\bullet}$) interact to form the hydroxyl radical ($OH\bullet$) by Fenton-type and Haber Weiss-type reactions (see section 1.5.1.) (Harris & Shi, 2003; Leonard *et al.*, 2004).

Since hydrothermal vent organisms, including the mussel *B. azoricus* are continuously exposed to metal rich hydrothermal fluids (see Table 1.1) and other components known to increase oxyradicals production in marine organisms, and if MT do not seem to act as a major detoxification mechanism was considered important to understand if organisms from these environments developed other detoxification mechanisms such as defence systems against reactive oxygen species.

1.5. Oxidative Stress

Oxidative stress is generally defined as a disruption of the balance between the levels of oxidants (reactive oxygen species) and reductants (antioxidants) in the organisms (Granot & Kohen, 2004). ROS include a variety of both radical and non-radicals molecules that can be produced under natural conditions but are frequently enhanced by the presence of toxic compounds (Matés, 2000) (see section 1.5.1). To avoid ROS-induced injury to tissues, a complex antioxidant system, consisting of both enzymatic and non-enzymatic defenses, has evolved. Traditionally, antioxidants have been defined as substances that prevent the formation of ROS or other oxidants, scavenge them, or repair the damage that they cause (Sies, 1991; Halliwell, 1995) (see section 1.5.2).

1.5.1. Reactive Oxygen Species (ROS)

Molecular oxygen (O_2) is essential to all aerobic organisms, including for *B. azoricus* living in a low oxygen environment. In fact, oxygen in vent habitats varies inversely with temperature and consequently organisms in areas of actively mixing hydrothermal fluid and ambient water may experience rapid oxygen fluctuations (Childress & Fisher, 1992). Animals with chemoautotrophic sulphide-oxidizing symbionts, such as the case of *B. azoricus*, require oxygen for aerobic respiration, as well as both oxygen and sulphide for chemoautotrophic carbon fixation. This dual requirement can prove a challenge for metazoans that are dependent upon chemoautotrophic symbionts, because they will require substantial amounts of both oxygen and sulphide, two chemicals that do not normally co-occur (Millero, 1986).

The main role of molecular oxygen in organisms is that of terminal electron acceptor in mitochondrial respiration, where it is ultimately reduced to water during the process of oxidative phosphorylation, the main source of energy in aerobes (Bandyopadhyay *et al.*, 1999). However, the reduction of O_2 to water requires four electrons, and this reduction proceeds sequentially through the one-, two- and three-electron products, according to the reactions mentioned

below (Gutteridge *et al.*, 1981, Halliwell, 1982, Halliwell & Gutteridge, 1984; 1986):

$$O_2 + e^- \longrightarrow O_2^{-\bullet} \quad (1)$$

$$O_2^{-\bullet} + e^- \xrightarrow{2\,H^+} H_2O_2 \quad (2)$$

$$H_2O_2 + e^- \xrightarrow{H^+} OH\bullet + H_2O \quad (3)$$

$$OH\bullet + e^- \xrightarrow{H^+} H_2O \quad (4)$$

$$O_2 + 4e^- \xrightarrow{4H} 2H_2O \quad (5)$$

The products of the reactions (1) to (3) are the superoxide radical anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•). These reactive oxygen species (ROS), particularly OH•, are very reactive and potentially deleterious to biological systems (Chen & Schopfer, 1999). Both $O_2^{-\bullet}$ and OH• are oxygen-based free radicals (oxyradicals). Although not a free radical, hydrogen peroxide is also reactive via Haber-Weiss reaction with $O_2^{-\bullet}$, and serves as an important precursor to radical hydroxyl (OH•) (Kehrer, 2000) (reaction 6):

$$O_2^{-\bullet} + H_2O_2 \longrightarrow OH\bullet + OH^- + O_2 \quad (6)$$

(Haber-Weiss reaction)

While thermodynamically favourable, this reaction (6) is kinetically slow. However, catalysis by transition metals such as iron and copper facilitates OH• production (Di Giulio *et al.*, 1995). In biological systems, metal (e.g. chelated iron) catalyzed reactions are considered an important source of OH• (reactions 7 and 8) (Kehrer, 2000). In this reaction, the radical $O_2^{-\bullet}$ serves as the reductant for the transition-metal oxidation-reduction catalyst [e.g. chelated iron in (7)]. The reduced metal then reacts with H_2O_2 to yield OH• in a reaction referred to as the Fenton reaction (8) as follows:

$$O_2^{-\bullet} + Fe^{3+}\text{-chelate} \longrightarrow O_2 + Fe^{2+}\text{-chelate} \quad (7)$$

$$Fe^{2+}\text{-chelate} + H_2O_2 \longrightarrow OH\bullet + OH^- + Fe^{3+}\text{-chelate} \quad (8)$$

(Fenton reaction)

Other reactive oxygen species include alkoxyl radicals (RO•), peroxyl radicals (ROO•), hydroperoxyl radicals ($HO_2^{\bullet}$) and non-radicals forms such as singlet oxygen ($^1O_2$), ozone (O_3) and peroxynitrite (HOONO) (Table 1.6) (Di Giulio *et al.*, 1995; Darley-Usmar *et al.*, 1995).

Table 1.6 – Reactive oxygen species (ROS) (Darley-Usmar *et al.*, 1995)

Radicals	Non-radicals
Superoxide: $O_2^{-\bullet}$	Hydrogen peroxide: H_2O_2
Hydroxyl: OH•	Singlet oxygen: $^1O_2$
Peroxyl: ROO•	Ozone: O_3
Alcoxyl: RO•	Peroxynitrite: HOONO
Hydroperoxyl: $HO_2^{\bullet}$	

The mitochondria are thought to consume over 90% of the cellular oxygen and are considered the major site of cellular ROS production (Lenaz, 1998; Staniek & Nohl, 1999; Han *et al.*, 2001), although several other sources of endogenous cellular oxyradicals have been identified (Staniek & Nohl, 2000).

These include the electron transport chain in microsomes (Winston & Cederbaum, 1983; Dicker & Cederbaum, 1991; Winston *et al.*, 1996), and chloroplasts, the respiratory burst associated with active phagocytosis by leukocytes (Anderson & Zeeman, 1995) and the activity of a number of enzymes such as xanthine oxidase, tryptophan dioxygenase, diamine oxidase and prostaglandin synthase (Fridovich, 1989). Although ROS are generally considered in light of their deleterious effects, the examples of prostaglandin synthase and phagocytosis indicate they occasionally play important beneficial roles (Di Giulio *et al.*, 1995).

Nevertheless, ROS are frequently focused in relation to compounds that enhance their production and the resulting damaging effects. Classes of compounds identified for their ability to enhance the flux of oxyradicals include quinones and diols, bipyridyls, aromatic nitro compounds, aromatic hydroxylamines, aromatic azodyes and transition metals (Di Giulio *et al.*, 1995; Stohs *et al.*, 2001)

In Figure 1.5 the redox cycle is briefly described along with the processes capable of producing reactive oxygen species, together with the antioxidant defences (superoxide dismutase, catalase and glutathione peroxidases – that will be further detailed in section 1.5.2) and some of the known toxic consequences, including lipid peroxidation (described in section 1.5.3).

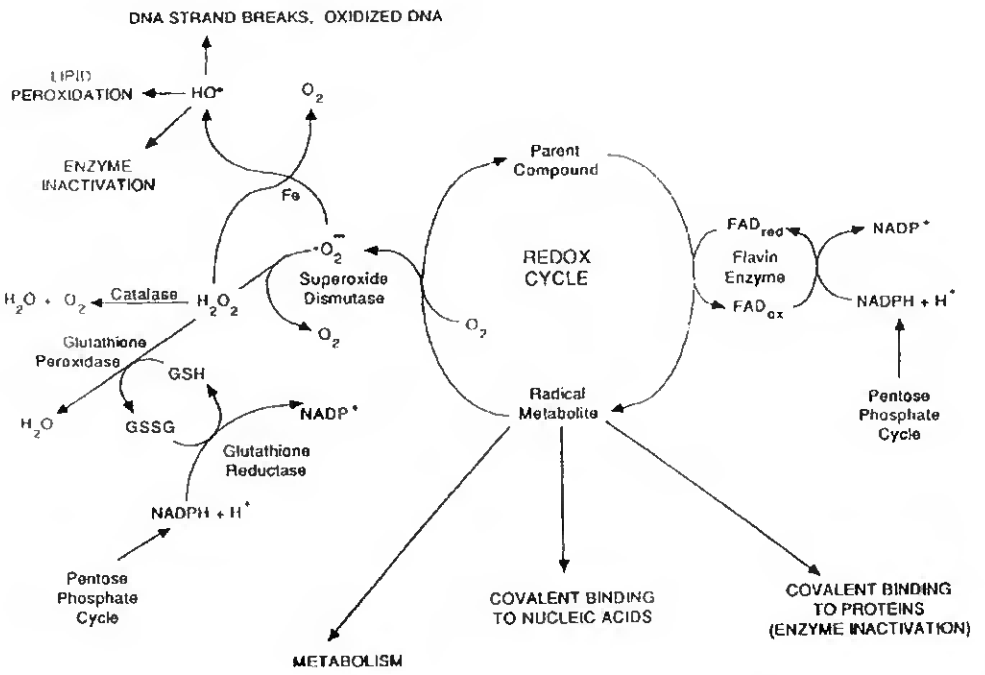

Figure 1.5 – Redox cycle summarizing ROS production, antioxidant defences and toxicological consequences (adapted from Di Giulio *et al.*, 1995).

In this cycle, the xenobiotic compound is first reduced to his corresponding free radical, with reducing equivalents typically provided by NADH or NADPH. This reaction is generally catalyzed by NAD(P)H reductases such as cytochrome P450 reductase, xanthine oxidase, ferrodoxin reductase, and NADH-ubiquinone

oxyreductase. To complete the cycle, the unshared electron of the free radical is transferred to O_2, giving rise to $O_2^{-\bullet}$ and the parent compound. The later can undergo continued futile cycling. Therefore, redox cycling is characterized by concomitant oxidations of NAD(P)H and catalytic yields of ROS. Both events can underlie the toxicities of redox-active compounds (Di Giulio *et al.*, 1995). To counteract these effects, the organisms developed mechanisms to scavenge the ROS produced within their tissues, known as antioxidant defence mechanisms (Bandyopadhyay *et al.*, 1999).

1.5.2. Antioxidants Defence Mechanisms

Antioxidant defence systems are developed by aerobic and aerotolerant anaerobic organisms in order to deal with the potential deleterious effects of ROS (Harris, 1992). These defences provide a fundamental adaptation to both natural and anthropogenic stressors that influence the flux of ROS in these organisms.

Strictly anaerobic organisms do not require oxygen in order to survive. Instead, they use anaerobic respiration to obtain energy and in fact oxygen is considered toxic to these organisms. Therefore it was former thought that these organisms lack antioxidant protection against oxygen toxicity (Fridovich, 1975). However, other studies found that not only aerobic and facultative anaerobic bacteria but also strict anaerobes (*Desulfovibrio* and *Clostridium*) possess SOD activity (Hewitt & Morris, 1975; Gregory *et al.*, 1978). More recently the presence of both SOD and CAT were found in several species of strictly anaerobic species including methanogenic bacteria (e.g. *Methanobrevibacter cuticularis* and *Methanosarcina barkeri*) and sulphate-reducing bacteria (e.g. *Desulfotomaculum nigrificans* and *Desulfotomaculum kuznetsovii*) (Brioukhanov *et al.*, 2002).

Antioxidants can be classified as water-soluble reductants such as glutathione, uric acid and ascorbate (vitamin C) and also lipid-soluble radical scavengers such as α-tocopherol (vitamin E), β-carotene (vitamin A) and various

xanthophylls. Moreover, there are also a variety of enzymes able to detoxify reactive oxygen species within the cells (Remacle *et al.*, 1992).

The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx), which are further explained in this section and studied in this dissertation. Other important antioxidant enzymes include ascorbate peroxidase (AsPx), glutathione reductase (GR) and DT-diaphorase (DTD). Reduced glutathione (GSH) has a major role in detoxification process of contaminants. GSH can scavenge radicals directly and provide reducing equivalents for the reduction of peroxides by GPx. Similarly, ascorbate in chloroplasts can scavenge oxyradicals such as OH•, as well as serve as a cofactor for AsPx (Halliwell & Gutteridge, 1989). GSH and ascorbate are paradoxical in that they possess pro-oxidants as well as antioxidant activity. Their auto-oxidations, particularly in the presence of transitions metals, give rise to oxyradicals (Cohen *et al.*, 1981).

1.5.2.1. Superoxide Dismutase

Superoxide dismutase (SOD) (EC 1.15.1.1) is a group of metalloenzymes that disproportionates the radical $O_2^{-\bullet}$ in H_2O_2 in the reaction below (9) (Fridovich, 1986):

$$2O_2^{-\bullet} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad (9)$$

Superoxide dismutase is considered to play a very important antioxidant role. It occurs in all aerobic, aerotolerant anaerobes and most of the strictly anaerobes organisms examined and catalyzes the dismutation of $O_2^{-\bullet}$ at rates approximating diffusing limits, making it among the most active enzymes described (Di Giulio *et al.*, 1995; Brioukhanov *et al.*, 2002). Specific isozymes are typically found in cytosol, mitochondria and chloroplasts. Numerous studies have indicated induction of SOD in many organisms by factors associated with

increased oxyradicals production, such as elevated O_2 and exposure to redox-active contaminants (Di Giulio *et al.*, 1995).

Four classes of SOD have been identified based on the metal cofactor, which could be either a dinuclear Cu/Zn or mononuclear Fe, Mn or Ni (Whittaker & Whittaker, 1998).

Copper and zinc containing superoxide dismutase (Cu/Zn-SOD) is a dimeric protein whose molecular weight averages 32 kDa (Paoletti & Mocali, 1990) and have been found in the cytosol of eukaryotes, in chloroplasts, and in the periplasm of some prokaryotes (Cannio *et al.*, 2000). The properties of these enzymes have been conserved throughout the evolution. In fact, the classes found in fungi, plants, birds, and mammals, are distinguishable from each other due to a few differences in amino-acid composition and electron-paramagnetic resonance spectra (Beem *et al.*, 1974). There is general agreement that Cu^{2+} is alternatively reduced and oxidized by direct electron transfer during the dismutation of superoxide to hydrogen peroxide and molecular oxygen (Hodgson & Fridovich, 1973).

The iron-containing enzyme (Fe-SOD) is a dimer with a molecular weight of 41 kDa (Salin, 1987) and manganese-containing enzyme (Mn-SOD) is a tetrameric protein with an average molecular weight of 86 kDa (Salin, 1987). Both Fe- and Mn-SOD are somewhat similar and of ancient origin having evolved in prokaryotes, while the clearly different copper-zinc containing enzyme (Cu/Zn-SOD) of eukaryotes represents a more recent evolutionary origin (Ahmad, 1995).

Prokaryotes possess both Mn-SOD and Fe-SOD, and some species contain a heterodimer composed of both Mn-SOD and Fe-SOD (Cannio *et al.*, 2000). Mn-SOD has been well characterized from fungus, yeasts, and plants possess all three types of SOD (Ahmad, 1995). In vertebrates, Cu/Zn-SOD is the predominant form present in the cytosol (about 80% of total SOD), while the remainder is Mn-SOD present in the mitochondrial matrix. A similar pattern of SOD occurs in invertebrates (Ahmad, 1995).

A new type of cytosolic SOD containing nickel as a cofactor has recently been discovered in several *Streptomyces* species (Youn *et al.*, 1996a,b; Chun *et al.*, 1997). Some of these strains also contain Fe-SOD. These enzymes are composed of four identical subunits of 13.4 kDa. Transcriptional analyses revealed that Ni is also involved in activation of the gene encoding Ni-SOD and in the repression of the gene encoding Fe-SOD (Kim *et al.*, 1998). The aminoacid composition, N-terminal sequences, and immunological properties demonstrated that they are distinct from the Mn-, Fe- or Cu/Zn-SOD and thus represent a new class of SOD (Cannio *et al.*, 2000). However, regardless of the metal cofactor, all forms of SOD share the same mechanism and speed in dismutating $O_2^{-\bullet}$ (Ahmad, 1995).

The SOD types can also be distinguished by their differential response to H_2O_2 and KCN (Bowler *et al.*, 1989). Mn-SOD is not affected by either H_2O_2 or KCN, Fe-SOD is sensitive to H_2O_2 but not KCN, while Cu/Zn-SOD is inhibited by both H_2O_2 and KCN. Another feature of these enzymes is their inducibility. Under excessive oxidative stress such as by prooxidants, the eukaryotic Cu/Zn-SOD is usually rapidly induced (Bowler *et al.*, 1989). Prooxidants usually do not induce the Mn-SOD or Fe-SOD, however under intrinsic conditions, which may lead to increased mitochondrial or chloroplasts metabolism, Mn-SOD is induced (Masuda *et al.*, 1988).

Thus, endogenous metabolic signals rather than exogenous factors are more important in inducing the activity of Mn-SOD, and both factors may induce Fe-SOD. SOD appears to be the most important antioxidant enzyme since its point of attack is $O_2^{-\bullet}$, which is the initiator of the oxygen radical cascade that triggers the lipid peroxidation chain reactions (Ahmad, 1995).

1.5.2.2. Catalase

Catalase (CAT) (EC 1.11.1.6) functions subsequently to SOD in radical detoxification from the cellular environment. After $O_2^{-\bullet}$ scavenging by SOD, H_2O_2 is produced which is then degraded to H_2O and O_2 by the action of this enzyme (reaction 10) (Di Giulio *et al.*, 1995):

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \qquad (10)$$

Catalase is a tetrameric haemin enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa. Therefore, it contains four ferriprotoporphyrin groups per molecule, and its molecular mass is about 240 kDa (Matés, 2000). Catalase is one of the most efficient enzymes known which cannot be saturated by H_2O_2 at any concentration (Lledías *et al.*, 1998). Catalase is an unusual enzyme since does not follow the classical Michaelis-Menton kinetics and the activity increases linearly with the available H_2O_2 over a wide range of its concentration (Ahmad, 1995).

Although CAT decomposes H_2O_2 very efficiently, the enzyme is turned on at rather high H_2O_2 concentrations (Halliwell, 1982). Thus, CAT has a relatively minor role in catabolism of H_2O_2 at low rates of H_2O_2 generation, but its role increases and become indispensable when H_2O_2 production is enhanced from oxidative stress (Ahmad, 1995).

Catalase exhibits dual activities, i.e., "catalytic" in which two molecules of H_2O_2 are dismutated, one molecule acting as an oxidant, while the other as a reductant (H donor) in two consecutive steps (Ahmad, 1995). The other reaction is called "peroxidative" in which the reductant is the H donor. In both reactions, an active enzyme-H_2O_2 intermediate (complex I) is formed first (Ahmad, 1995). In the typical catalytic activity of CAT, the reaction between complex I and another molecule of H_2O_2 proceeds very rapidly, while the peroxidative reaction proceeds slowly (Ahmad, 1995).

There is only slight dependence on either temperature or pH for this fast-acting enzyme, and most assays are conducted at pH 7.0 and 20-25 °C. Only at unphysiological levels of substrate (5-50 mM H_2O_2) CAT is inactivated (autoinactivation) (Ou & Wolff, 1996).

The primary role of CAT is to decompose H_2O_2, although some activity occurs with methyl and ethyl hydroperoxides (Ahmad, 1995). CAT is present in most eukaryotic cells and the enzyme is primarily located in the matrix of peroxisomes (Khessiba *et al.*, 2005). The peroxisomal location of CAT is considered strategic since the peroxisomes contain many of the cellular enzymes that generate H_2O_2, like glycolate oxidase and flavoprotein dehydrogenases by a direct two-electron reduction of O_2, and without the intermediacy of $O_2^{-\cdot}$ radical (Khessiba *et al.*, 2005).

1.5.2.3. Glutathione Peroxidases

Glutathione peroxidases (GPx) (EC 1.11.1.9) are ubiquitously expressed proteins, which catalyze the reduction of organic hydroperoxides to alcohols and water (reaction 11) or hydrogen peroxide reduction to water (reaction 12) by reduced glutathione (GSH) forming glutathione disulphide (GSSG) and thus represents an important defence against oxidative damage (Bandyopadhyay *et al.*, 1999; Matés, 2000):

$$ROOH + 2GSH \xrightarrow{GPx} ROH + H_2O + GSSG \quad (11)$$

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG \quad (12)$$

Four different types of GPx are known, GPx1 to GPx4 (Chu *et al.*, 2004). As the first discovered GPx family, the classical cytosolic/mitochondrial GPx1 is a selenium-dependent enzyme (Burk & Hill, 1999). GPx2 is an intracellular enzyme expressed only at the epithelium of the gastrointestinal tract (Burk & Hill, 1999).

GPx1 and GPx2 are closely related cytosolic enzymes in terms of structure and specificity for H_2O_2 and fatty acid hydroperoxides as substrates, although GPx1 is also found in mitochondria (Utsunomiya *et al.*, 1991; Chu *et al.*, 1993; Esworthy *et al.*, 1997).

GPx3 is an extracellular glycosylated enzyme that can use the thioredoxin and glutaredoxin systems in addition to GSH as electron donors to reduce a broader range of hydroperoxides and is mainly expressed by the kidney from where it is released into the blood circulation (Bjornstedt *et al.*, 1994; Tham *et al.*, 1998). GPx3 can reduce H_2O_2, fatty acid hydroperoxides, and phospholipid hydroperoxides, but not cholesterol hydroperoxides; although GPx3 has slower catalytic rates than other GPxs (Esworthy *et al.*, 1991; Yamamoto *et al.*, 1994).

GPx4 is expressed in most tissues and is present in cytosolic, mitochondrial and nuclear forms (Maiorino *et al.*, 2003). GPx4 reduces phospholipid, cholesterol, and thymine hydroperoxides and may function synergistically with vitamin E in the prevention of lipid peroxidation (Bao *et al.*, 1997; Maiorino *et al.*, 2003).

1.5.2.4. Total Oxyradical Scavenging Capacity (TOSC)

The detoxification of reactive oxygen species comprise the action of antioxidant enzymes, such as SOD, CAT and GPx, as mentioned previously. Therefore, variations in these antioxidants are believed to reflect the biological effects of compounds capable to produce ROS. However, the analysis of these single antioxidants may not reveal the overall efficiency of antioxidant system, which can be assessed by the determination of total oxyradical scavenging capacity (TOSC) (Winston *et al.*, 1998). The TOSC quantifies the capability of the whole antioxidant system to neutralize oxyradicals, allowing to discriminate between different forms of ROS (Winston *et al.*, 1998; Regoli & Winston, 1998; 1999), and provides a value which reflects susceptibility to oxidative stress with a greater predictive value, since the overall impairment in neutralizing ROS reactivity will anticipate alterations at other levels (Regoli, 2000; Regoli *et al.*, 2002). The TOSC assay has been extended to various oxyradicals (peroxyl radicals, hydroxyl radicals and peroxynitrite), all antioxidant species with significant damaging potential to biological targets (Regoli *et al.*, 2000; 2002).

The combined analysis of single antioxidant enzymes with TOSC generally represents a more holistic assessment of the capacity of the organisms to absorb different forms of reactive oxygen species (Regoli *et al.*, 2002).

1.5.3. Oxidative Damage

Free radicals, including ROS can react with a large variety of biomolecules and are often non-specific with respect to biochemical targets. This is particularly true for the highly reactive radicals such as OH•. Imbalance or loss of cellular redox homeostasis results in oxidative stress, causing severe alterations and damages to all major classes of biological macromolecules: DNA, proteins and membrane lipids (Halliwell, 1994; Kehrer, 2000). These alterations are believed to underlie specific tissue injuries associated with redox-active contaminants and, more broadly, may be associated with aspects of chemical carcinogenesis and aging (Borg *et al.*, 1978; Freeman & Crapo, 1982; Sies, 1986). There are several examples of primary biochemical and physiological events associated with ROS, such as DNA damage and lipid peroxidation (LPO) (Davies, 1995).

Lipids have a critical structural and functional role in membranes. Any disruption of this role can lead to cell death. The double bonds found in polyunsaturated fatty acids are ready targets for free radical attack (Kehrer, 2000). Lipid peroxidation is the oxidation of polyunsaturated fatty acids (PUFAs) and is considered an important consequence of oxidative stress. Lipid peroxidation occurs by a chain reaction and demonstrates the capacity of a single radical species to propagate a number of deleterious biochemical reactions (Figure 1.6). This process is initiated by the abstraction of a hydrogen atom from a methylene group (—CH_2—) of a PUFA (or "LH"). Oxyradicals, particularly OH•, can readily perform this abstraction, yielding the lipid radical L• (Di Giulio *et al.*, 1995).

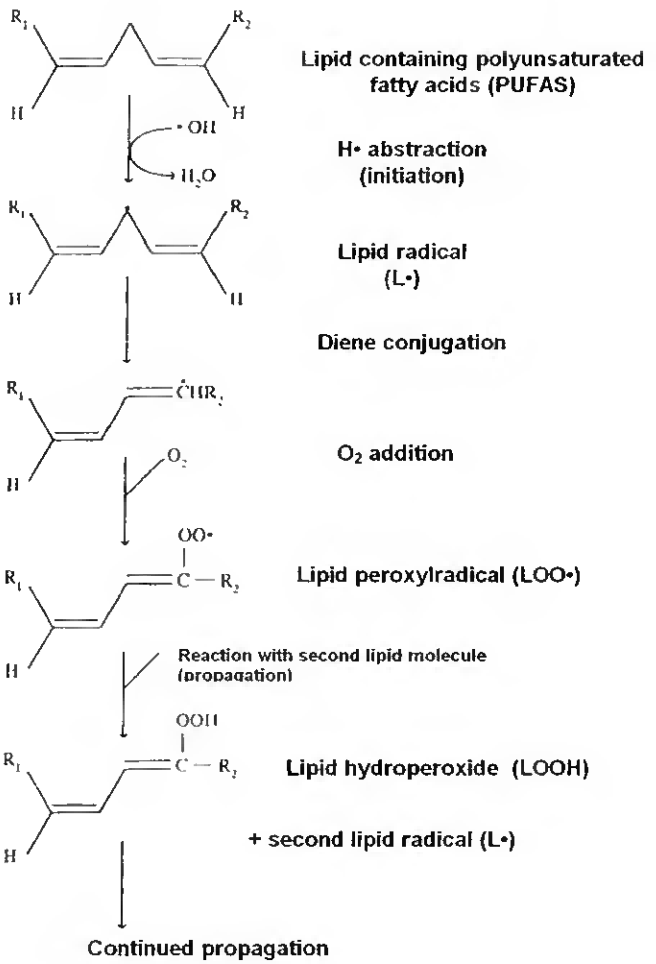

Figure 1.6 – Lipid peroxidation (LPO) mediated by hydroxyl radical (OH•) (adapted from Di Giulio *et al.*, 1995).

This carbon-based radical (L•) tends to be stabilized by molecular rearrangement to a conjugated diene radical. The L• radical readily reacts with O_2 to produce the peroxyl radical LOO•. This radical can easily abstract a hydrogen from another LH, yielding a lipid hydroperoxide (LOOH) and a new L•, which can then continue the chain reaction, propagating additional LOOH and L•. LOOH is relatively stable in isolation but can react with transitional metal complexes (including cytochrome P450) to yield alkoxyl radicals (RO•) (Di Giulio *et al.*, 1995). As these reactions progress, ionic channels may be affected, membrane transport proteins or enzymes can be inactivated, or the lipid bilayer may become more permeable thereby disrupting ion homeostasis (Kehrer, 2000).

1.5.4. Antioxidant parameters in bivalves

The activities of antioxidant enzymes, TOSC and lipid peroxidation have been determined in several bivalve species, in order to understand the deleterious effects of reactive oxygen species in the organisms and to study the antioxidant defence system. In Table 1.7 data concerning antioxidant parameters in several tissues in both hydrothermal vent sites and coastal areas are presented.

In coastal organisms, particular mussels (*M. galloprovincialis*, *M. edulis*, *Unio tumidus*), clams (*Rudipates decussatus*, *Tapes semidecussata* , *Mya truncata*, *Mercenaria mercenaria*) and oysters (*Ostrea edulis*, *Crassostrea gigas*) antioxidant parameters are highly variable and depend on the species, tissues, location, time of the year and reflect the degree of environmental contamination (Table 1.7).

The only information available in hydrothermal organisms report the activity of SOD, CAT and GPx in the giant clam *Calyptogena magnifica* and the tube worm *Riftia pachyptila* collected in the East Pacific Rise (Blum & Fridovich, 1984). There is an evident lack of information concerning the protection mechanisms that hydrothermal vent organisms possess to counteract the effects of ROS, especially for Mid-Atlantic Ridge hydrothermal vent species.

In hydrothermal bivalves, particularly mussels that are wide spread in hydrothermal vent sites around the world, this is the first study to assess the antioxidant defence systems.

Table 1.7 – Specific activities of antioxidant enzymes, TOSC and LPO concentrations in invertebrates from hydrothermal vent sites and coastal environments.

Species	Sites	Tissues	SOD (U mg^{-1} prot)	CAT (nmol min^{-1} mg^{-1} prot)	GPx (nmol min-1 mg-1 prot)		TOSC	LPO (nmol g^{-1} prot)	References
					Total GPx	Se-GPx	Peroxyl		
	Hydrothermal vents								
Calyptogena magnifica	East Pacific Rise	Gills	3.3	ND	0.0009 [d]				Blum & Fridovich, 1984
		Muscle	5.3	ND	0.0017 [d]				Blum & Fridovich, 1984
Riftia pachyptila	East Pacific Rise	Muscle	3.7	ND	0.006 [d]				Blum & Fridovich, 1984
		Trophosome	1.2	ND	0.0025 [d]				Blum & Fridovich, 1984
	Coastal areas								
Mytilus galloprovincialis	Spain	Digestive gland	6-13	2.5±4.0[b]		2.4-3.7			Solé *et al.*, 1995
	Gulf of Mexico (USA)	Digestive gland					630±73		Regoli *et al.*, 1998
Mytilus edulis	Italy	Digestive gland	12±1.5	189±21	7.7±0.4	2.4±0.1			Livingstone *et al.*, 1992
	Plymouth (UK)	Digestive gland	615±52 [a]	5.9±1.1 [b]	604±146 [e]	479±203 [e]			Gamble *et al.*, 1995
	Italy	Digestive gland	569±218 [a]	1.09±0.1 [b]	465±125 [e]	113±40 [e]			Viarengo *et al.*, 1991
		Digestive gland	584±225 [a]	0.95±0.1 [b]	545±138 [e]	115±79 [c]			Viarengo *et al.*, 1991
		Digestive gland	593±86	1.4±0.08 [b]	661±150 [e]	121±34 [c]			Viarengo *et al.*, 1991
	Tyrrhenian Sea (Italy)	Digestive gland					470±48		Regoli *et al.*, 1998
Unio tumidus	Moselle River (France)	Gills	83.0±16.0	20.0±2.0	95.0±1.0	53.0±2.0			Cossu *et al.*, 1997
		Digestive gland	72.0±5.0	120.0±10.0	112.0±10.0	81.0±15.0		2.7±1.0 [f]	Cossu *et al.*, 1997
Rudipates decussatus	Ria Formosa (Portugal)	Gills	39.5±0.2	579±59	9.9±1.6	8.5±1.5		3.1±1.8 [f]	Géret *et al.*, 2004
		Gills	41.2±7.7	614±119				374±45	Géret & Bebianno, 2004
		Digestive gland		229±30				381±38	Géret & Bebianno, 2004
Tapes semidecussata	Spain	Digestive gland	14.6±0.8	4500±146 [c]	2.9±0.4 [f]			840±58	Solé *et al.*, 1994
Mya truncata	Arctic fjord (Norway)	Digestive gland					4010±1339		Camus *et al.*, 2003
Astarte borealis	Kiel Bight (Germany)	Gills	11.1±4.1	134.6±44[b]					Abele-Oeschger & Oeschger, 1995
Mercenaria mercenaria	USA	Gills	10.8	26.0	0.0017				Blum & Fridovich, 1984
		Muscle	11	3.5	0.002				Blum & Fridovich, 1984
Ostrea edulis	Spain	Digestive gland	9.6±0.3	5660±318 [c]	3.5±0.1 f				Solé *et al.*, 1994
Crassostrea gigas	Spain	Digestive gland	10.7±0.3	4110±171 [c]	5.9±0.3 f				Solé *et al.*, 1994
Crassostrea sp.	Spain	Digestive gland	22.83±2.37	2.66±0.38	9.13±0.61 f				Orbea *et al.*, 2002
Adamussium colbecki	Antartic	Digestive gland	12.9±1.7	444-715	8.3-23.7	5.7-11.9			Regoli *et al.*, 1997
Pecten maximus	Plymouth (UK)	Digestive gland	324±6 [a]	36.1±0.6 [b]	450±151c	260±135 [c]			Gamble *et al.*, 1995

[a] U g^{-1} wet weight
[b] mmol min^{-1} g^{-1} wet weight
[c] µmol min^{-1} g^{-1} wet weight
[d] units mg^{-1} protein
[e] nmol min^{-1} g^{-1} wet weight
[f] MDA ng mg^{-1} protein

1.6. Aim and structure of the thesis

The aim of the present thesis was to study the metal detoxification mechanisms in a recently identified hydrothermal vent mussel, *Bathymodiolus azoricus*, to a metal rich environment, especially in regard to antioxidant enzymatic defences, never studied before in vent mussels.

In order to accomplish this main objective, the thesis was structured into several chapters as follows.

The **Chapter 1** introduces the subject of hydrothermal vents, its main characteristics as well as a brief description of three MAR hydrothermal vents included in this study (Menez-Gwen, Lucky Strike and Rainbow). The mussel species selected (*Bathymodiolus azoricus*) is also characterized. This general introduction also describes metal accumulation and detoxification strategies and processes of reactive oxygen species production, their main consequences in biological systems and the importance of antioxidant defence systems in organisms, mainly the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx) and total antioxidant scavenging capacity (TOSC).

In order to understand the antioxidant defence system in *B. azoricus* in response to metals, it was first necessary to determine the basal activities of antioxidant enzymes (SOD, CAT and GPx) in tissues (gills and mantle) of organisms from five hydrothermal vent sites: Menez-Gwen (ATOS8 and ATOS10), Lucky Strike (Bairro Alto and Eiffel Tower) and Rainbow. Furthermore, TOSC and MT concentrations in the same tissues of *B. azoricus* were also investigated. This field study is presented in **Chapter 2**.

After the characterization of basal SOD, CAT and GPx activities in the mussels, it was necessary to understand if age and season interfere with antioxidant enzymatic mechanisms in this vent species. Thus, in **Chapter 3** another field study was made, where vent mussels of two size classes were collected in Menez-Gwen and variability of antioxidant enzymatic mechanisms accessed. Moreover, mussels were collected periodically from the same site, using cages

with retrievable acoustic release, during several months to conclude about the seasonal variability of antioxidant enzymes in tissues of *B. azoricus*.

After these two field studies, several experimental essays were conducted, where the mussel *B. azoricus* was exposed to various metals (both essential and non-essential ones) which are present in high concentrations in MAR hydrothermal vents. These exposure experiments, aiming to understand the effects of metal accumulation in antioxidant defence mechanisms of *B. azoricus*, were done in a pressurized tank (IPOCAMP - *Incubateurs Pressurises pour l'Observation en Culture d'Animaux Marins Profonds*) at 85 bars or at atmospheric pressure.

Thus, in **Chapter 4**, the effects of cadmium (Cd) in *B. azoricus* antioxidant enzymes were assessed. Mussels were exposed to 100 μg l^{-1} Cd during 1, 2 and 6 days (short term experiment) and to 50 μg l^{-1} Cd during 26 days, followed by 6 days of depuration (long term experiment). During the short term experiment the organisms were pressurized in a tank at 85 bars, the same pressure registered in Menez-Gwen vent field, while during the long term experiment the mussels were kept at atmospheric pressure.

Similarly, the effects of copper (Cu) were studied during two assays that are described in **Chapter 5**. The vent mussels were exposed to 25 μg l^{-1} Cu during a short term pressurized (85 bars) exposure experiment (12 and 24 hours) and a long term experiment (24 days exposure; 6 days depuration) at atmospheric pressure.

In **Chapter 6** the results of three separate short term exposure experiments in pressurize tanks (85 bars) are presented. In this study the effects of one essential (Zinc (Zn)) and two toxic metals (silver (Ag) and mercury (Hg)) in the antioxidant defence systems of *B. azoricus* were assessed.

Finally, a general discussion is presented in **Chapter 7** gathering the information obtained in the previous sections, both during field and experimental works, and discussing the possible parameters that interfere with the antioxidant defence mechanism in the hydrothermal vent mussel *B. azoricus*.

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Chapter 2

Antioxidant systems and lipid peroxidation in *Bathymodiolus azoricus* from Mid-Atlantic Ridge hydrothermal vent fields

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2.1. Abstract

The enzymatic defences involved in the protection of the vent organisms from oxygen radical damage were determined in the gills and mantle of *Bathymodiolus azoricus* collected from three contrasting Mid-Atlantic Ridge (MAR) vent fields (Menez-Gwen, Lucky Strike and Rainbow). The activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx) (total and Se-dependent), total oxyradical scavenging capacity (TOSC), metallothioneins (MT) and lipid peroxidation (LPO) were determined in *Bathymodiolus* tissues and the impact of metal concentrations on these antioxidant systems and lipid peroxidation assessed. Antioxidant enzyme systems able to counteract the presence of reactive oxygen species (ROS) in the gills and mantle of *B. azoricus* revealed tissue and site variability. SOD, CAT, TOSC, MTs and LPO levels were higher in *Bathymodiolus* gills while glutathione peroxidases (total and Se-dependent) were in the mantle and with the exception of CAT of the same order of magnitude of other vent and non-vent molluscs. Although present in high levels, MTs do not play a metal detoxification or an antioxidant strategic role in this species. TOSC levels from Menez-Gwen indicate that the vent environment at this site is less stressful and the formation of ROS in mussel is effectively counteracted by the antioxidant defence system. TOSC depletion from the other two vent sites indicates an enhancement in ROS production caused by either the presence of important metal concentrations or a more toxic environment in general. Cytosolic SOD, GPx and LPO are more significant at Lucky Strike (Bairro Alto) where levels of essential (Cu and Zn) and toxic metals (Cd and Ag) were highest in the organisms. CAT activity and LPO were predominant at Rainbow vent site where the excess of iron in mussel tissues and in vent fluid (the highest of all three vent sites) altered the normal cell physiology and cause LPO. Therefore, three distinct pathways for antioxidant enzyme systems and LPO based on environmental metal characteristics of MAR vent fields are proposed for *Bathymodiolus* gills. For Menez-Gwen, TOSC towards peroxyl and hydroxyl radicals and peroxynitrite are predominant, while at Lucky Strike cytosolic SOD activity and GPx are the main antioxidant mechanisms. Finally at Rainbow, catalase and lipid peroxidation are dominant.

2.2. Introduction

Since their discovery in 1977 (Corliss *et al.*, 1979), invertebrate hydrothermal vent populations, particularly Mytillidae, raised special attention due to their capacity to live in one of the most extreme environments known on the face of the earth. This environment is characterised by high temperature and pressure, low pH, enriched in toxic sulphide species (0.9 mM near de vents (Blum & Fridovich, 1984)), radionuclides and naturally high bioavailable metal concentrations that would be toxic or even lethal to non vent marine species (Desbruyères *et al.*, 2001). Apart from being enriched in toxic metal and sulphide species, this environment has periodic access to reduced chemical species (H_2S, CH_4) and seawater oxidized compounds (Géret *et al.*, 1998). To live in this extreme environment, bivalve species such as the Mytilid *Bathymodiolus azoricus* derive a substantial portion of its food from chemoautotrophic sulphur-oxidizing or metanotrophic symbiotic bacteria that live in mussel gills and use vents substances to produce organic compounds and energy (Fiala-Médioni & Felbeck, 1990; Raulfs *et al.*, 2004).

Metals like Fe, Zn, Cu and Mn are essential for these bivalve species but can become toxic if present in excess. Other metals like Cd, Ag, Ba and Sr are toxic metals and do not seem to be related to the normal metabolism of these species (Rousse *et al.*, 1998). Bioaccumulation of metals (Fe, Zn, Mn, Cd, Ag, Ba and Sr) measured in tissues of *Bathymodiolus sp.,* from Mid Atlantic Ridge (MAR), indicate that these species accumulate high metal concentrations, particularly in the gills (main interface between the organism and the hydrothermal source and where symbiotic activity is more important) and digestive gland and accumulation is hydrothermal site dependent and related to the chemical composition of hydrothermal fluid. Metal levels in the mantle are lower when compared with the other two tissues (Rousse *et al.*, 1998).

Bathymodiolus azoricus is one of the dominant species of the biological communities in MAR and typically lives at the diffuse vent area (Desbruyères *et al.*, 2001). The survival of these vent molluscs, is related to its capacity to accumulate metal ions in non toxic forms by immobilization and precipitation (in

insoluble forms) as granules located in lysosomes (Chassard-Bouchaud *et al.*, 1986), excretion or detoxification by binding to specific ligands such as heat-stable low molecular weight cytosolic proteins, known as metallothioneins (MTs) (Rousse *et al.*, 1998). In *Bathymodiolus sp.*, levels of MTs are higher in the gills compared with the mantle. These MT levels could be related with high metals concentrations known to induce MT synthesis (Zn, Cu, Cd and Ag) or to the involvement of thiotrophic and methanotrophic symbiotic bacteria in metal detoxification processes in the gills. However, the role of symbiotic bacteria in metal detoxification processes in this species is still not well understood (Rousse *et al.*, 1998). Furthermore, MTs do not seem to have a dominant role in metal detoxification processes in these species (Rousse *et al.*, 1998).

The accumulation of metals in these mussel species inhibits DNA repair enzymes (Hartwig, 1998) and enhances the production of highly toxic oxyradical species (Fridovich, 1998). Hydrogen sulphide known to be a potent inhibitor of antioxidant enzymes reacts spontaneously with oxygen to generate many toxic oxygen and sulphides which in turn are capable of inflicting DNA damage (Pruski & Dixon, 2003). These radical oxygen species (ROS) include superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH•), peroxyl radicals (ROO•), alkoxyl radicals (RO•) and peroxynitrite (HOONO) (Darley-Usmar *et al.*, 1995). Aerobic organisms possess a baseline status of antioxidant systems, involved in a variety of detoxification reactions, to assure the maintenance of a balance between production and removal of endogenous ROS and other pro-oxidants. This pro-oxidant/antioxidant balance and detoxification of potentially damaging ROS is crucial for cellular homeostasis (Winston & Di Giulio, 1991; Lemaire & Livingstone, 1993; Livingstone, 2001). Cells have evolved an interdependent antioxidant defence system, composed of protective proteins for removing ROS, antioxidant enzymes, molecules that sequester metal ions and enzymes repairing damaged cellular components.

Prominent among these antioxidants are the enzymes superoxide dismutase (SOD, EC 1.15.1.1 - converts $O_2^{-\bullet}$ to H_2O_2), catalase (CAT; EC 1.11.1.6 - converts H_2O_2 to water) present in peroxisomes and glutathione peroxidase

(GPx; EC 1.11.1.9 - detoxifies H_2O_2) and organic hydroperoxides produced by lipid peroxidation present in mitochondria and in the cytosol (Di Giulio *et al.*, 1995; Halliwell & Gutteridge, 1999). In addition, total oxyradical scavenging capacity (TOSC) quantifies the capability of the whole antioxidant system to neutralize oxyradicals, allowing to discriminate between different forms of ROS (ROO•, HO•, HOONO). All these oxidant species have significant damaging potential to biological targets, but different reactivity and formation pathways (Regoli & Winston, 1999; Winston *et al.*, 1998) and therefore TOSC reflects the susceptibility to oxidative stress with a greater predictive value, since the overall impairment in neutralizing ROS reactivity will anticipate alterations at other levels (Regoli, 2000).

Antioxidant enzymes can be induced by various environmental pro-oxidant conditions (i.e. increased ROS generation), e.g. exposure to various types of compounds, temperature (Buchner *et al.*, 1996; Abele *et al.*, 1998) and hypoxia/hyperoxia (Abele-Oeschger *et al.*, 1994; Abele-Oeschger & Oeschger, 1995). Metal ions can also stimulate lipid peroxidation by oxidizing poly-unsaturated fatty acids and other damage. The altered membranes are incorporated into the lysosomes, where they accumulate as insoluble lipofuscin granules (Viarengo & Nott, 1993; Cavaletto *et al.*, 2002). Moreover, MT although not having a determinant role in metal detoxification in these species (Fiala-Médioni *et al.*, 2000), could protect cells from oxidative stress not only by acting as an oxyradical scavenger, but also through metal binding/release dynamic mechanisms (Langston *et al.*, 1998; Viarengo *et al.*, 2000).

Vent mussels are aerobic organisms and the potential for the formation of toxic oxygen radical species during the metabolic processes is unknown. Moreover, the enzymatic activity against oxygen toxicity in the hydrothermal vent animals is sparse. The only information reported deals with enzymatic activity (SOD, CAT and GPx) in tissues of the tubeworm *Riftia pachyptila* and the clam *Calyptogena magnifica* collected at 2500 m depth in the East Pacific Rise vent fields (Blum & Fridovich, 1984). In the present paper the enzymatic defences involved to protect the vent organisms from oxygen radical damage were determined in gills and mantle of *B. azoricus* collected from three contrasting

MAR vent fields (Menez-Gwen, Lucky Strike and Rainbow). The activities of antioxidant enzymes, namely SOD, CAT, GPx (total and Se-dependent), TOSC, MTs and lipid peroxidation (MDA and 4-HNE) in *Bathymodiolus* tissues were determined. Furthermore, the impact of metal concentrations from vent fields on the activity of these antioxidant enzymes, TOSC, lipid peroxidation (LPO) and MTs was assessed.

2.3. Materials and Methods

Deep sea hydrothermal mussels *B. azoricus* were collected from three hydrothermal vent sites located in the Azores Triple Junction (ATJ) in the MAR (Figure 2.1): Menez-Gwen (37º 51'N and 31º 31.2'W, 850 m), in two vent places identified as "ATOS 8" (4.76 ± 0.15 cm shell length) and "ATOS 10" (4.99 ± 0.37 cm shell length); Lucky Strike (37º 17'N and 32º 16'W, 1700 m) in two vent places identified as "Bairro Alto" (7.67 ± 0.66 cm shell length) and "Eiffel Tower" (6.50 ± 0.38 cm shell length) and Rainbow (36º 13'N and 33º 54.1'W, 2300 m) (6.76 ± 0.57 cm shell length), using the remote operated vehicle (ROV) "Victor6000" during the EU-funded ATOS cruise (June 22 - July 21, 2001; Sarradin *et al.*, 2001).

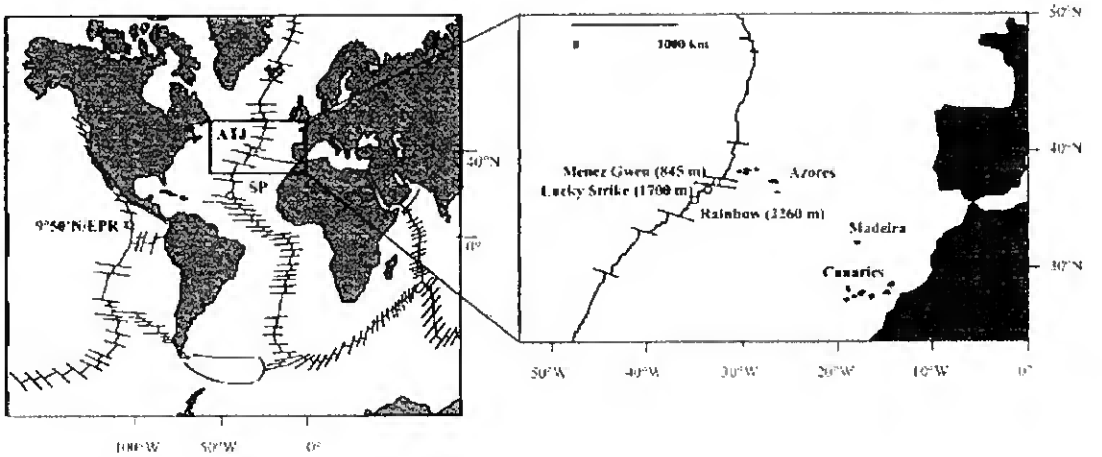


Figure 2.1 – Location of vent fields with detail of the Azores Triple Junction area (Menez-Gwen, Lucky Strike, and Rainbow hydrothermal vent fields) (adapted from Comtet *et al.*, 2000).

Mussels were brought to the surface in an insulated container, at approximately 4 °C, or in an open basket attached to the front of the ROV, measured and dissected on board the French research vessel L'Atalante within a maximum of 3 hours after collection and tissues (gills and mantle) immediately frozen in liquid nitrogen and stored at -80°C until further analysis of total proteins concentrations, enzyme activities (SOD, CAT, GPx (total and Se-dependent)), TOSC, LPO and MTs described below.

Tissue preparation

To determine the levels of enzymatic activity, mussel tissues (gills and mantle) were homogenized in Tris-HCl buffer (20mM), pH 7.6, containing 1mM de EDTA, 0.5M of saccharose, 0.15M of KCl and 1mM of DTT (dithiothreitol) in an ice bath for 2 minutes. The homogenates were centrifuged at 500 *g* for 15 minutes at 4°C to precipitate large particles. The supernatants were centrifuged at 12000 *g* for 45 minutes at 4°C to precipitate the mitochondrial fraction, and purified on Sephadex G-25 gel column to remove the low molecular weight proteins. SOD activity was measured in the mitochondrial and cytosolic fractions. CAT, Selenium-dependent and total GPx activities were measured in the cytosolic fractions.

Total protein concentrations were performed on the supernatants (30000*g* during 30 min at 4°C) of the gills and mantle by the Lowry method (Lowry *et al.*, 1951) using BSA (bovine serum albumin) as a reference standard. Protein concentrations are expressed as mg g^{-1} wet weight tissue. For TOSC, protein concentrations were measured according to Bradford (1976).

Enzymes activities

Superoxide dismutase (SOD) (EC 1.15.1.1) - SOD activity was determined using the method described by McCord and Fridovich (1969), by measuring the absorption of the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at a wavelength of 550 nm. One unit of SOD is the amount of the enzyme that inhibits by 50% the reduction of cytochrome c.

The SOD activity in *B. azoricus* tissues (gills and mantle) is expressed as U mg^{-1} of total protein concentrations.

Catalase (EC 1.11.1.6) - CAT activity was measured following the method described by Greenwald (1985) by the decrease in absorbance at 240 nm due to H_2O_2 consumption. The CAT activity is the difference in the absorbance, at that wavelength, per unit of time (ϵ = -0.04 mM^{-1} cm^{-1}). The CAT activity in the gills and mantle of *B. azoricus* is expressed as mmoles min^{-1} mg^{-1} of total protein concentrations.

Glutathione peroxidases (EC 1.11.1.9) - GPx activities (total and Se-dependent) were measured at 340 nm following NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (Lawrence and Burk, 1976). Cumene hydroperoxide and H_2O_2 were used as substrates for the determination of total and Se-dependent peroxidase activity. The difference in the absorbance per unit of time (the rate of blank reaction was subtracted from the total rate) was taken as the measure of GPx activity (ϵ = -6.22 mM^{-1} cm^{-1}). The GPx activities in the gills and mantle of *B. azoricus* are expressed as µmoles min^{-1} mg^{-1} of total protein concentrations.

Total oxyradical scavenging capacity (TOSC) - The analysis was based on the method described by Winston *et al.* (1998), but buffers used were adjusted for marine bivalves (Regoli *et al.*, 2000). Tissues were homogenized with a Potter-Elvehjem glass/Teflon homogeniser in four or three volumes for gills or mantle, respectively, of 100 mM KH_2PO_4 buffer, 2.5% NaCl, pH 7.5. The homogenate was centrifuged at 100 000 x g for 1 h, and cytosolic fractions were aliquoted and stored at –80°C. Peroxyl radicals are generated by the thermal homolysis of 2-2'-azo-bis-(2 methyl-propionamidine)-dihydrochloride (ABAP) at 35°C. The iron-ascorbate Fenton reaction was used for hydroxyl radicals, while peroxynitrite was generated from 3-morpholinosydnomine (SIN-1), a molecule that releases concomitantly nitric oxide and superoxide anion, which rapidly combine to form HOONO. Final assay conditions were: (a) 0.2 mM α-keto-γ-methiolbutiryc acid (KMBA), 20 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4 for peroxyl radicals; (b) 1.8 µM Fe^{3+}, 3.6 µM EDTA, 0.2 mM KMBA, 180 µM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4 for

hydroxyl radicals; and (c) 0.2 mM KMBA and 80 µM SIN-1 in 100 mM potassium phosphate buffer, pH 7.4 with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) for peroxynitrite. Peroxyl, hydroxyl radicals and peroxynitrite can oxidize the substrate KMBA to ethylene gas, which is measured with gas chromatography. With these assay conditions, the various oxyradicals induce a comparable yield of ethylene in the control reaction, thus the relative efficiency of cellular antioxidants is compared by their ability to counteract a quantitatively similar prooxidant challenge (in terms of KMBA oxidation). Reactions were carried out in 10 ml rubber septa sealed vials in a final volume of 1 ml. Ethylene production was measured by gas-chromatographic analysis of 200 µl taken from the head space of the reaction vials. Ethylene formation was monitored for 96 min with a Hewlett Packard (HP 5890 series II) gas chromatograph equipped with a supelco SPB-1 capillary column (30 m × 0.32 mm × 0.25 µm) and a flame ionization detector (FID). The oven, injection and FID temperatures were 35, 160 and 220°C, respectively; helium was the carrier gas (1 ml/min flow rate) and a split ratio 20:1 was used. The data acquisition system was run by the software Millenium32® (Waters). Each analysis required the measurement of control (no antioxidant in the reaction vial) and sample reactions (biological fluid in the vial). In the presence of antioxidant, ethylene production from KMBA was reduced quantitatively and higher antioxidant concentrations resulted in longer periods in which ethylene formation was inhibited relative to controls. By plotting the absolute value of the difference between the ethylene peak area obtained at each time point for the sample and control reaction it is possible to visualize whether the oxyradical scavenging capacity of the solution is changed. The area under the kinetic curve was calculated mathematically from the integral of the equation that best defines the experimental points for both the control and sample reactions Because the area obtained with the sample is related to that of the control, the obtained TOSC values are not affected by small variations in instrument sensitivity, reagents or other assay conditions. The specific TOSC value was calculated by dividing the experimental TOSC by the concentration of protein used for the assay. Data are expressed as TOSC unit mg^{-1} protein. TOSC analyses were carried out by Dr. Lionel Camus from the University Centre on Svalbard (Norway).

Lipid Peroxidation - the method used was designed to evaluate malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon the decomposition by polyunsaturated fatty acid peroxides (Erdelmeier *et al.*, 1998). This procedure is based on the reaction of two moles of N-methyl-2-phenylindole, a chromogenic reagent, with one mole of either MDA or 4-HNE at 45°C for 60 minutes to yield a stable chromophore that has a maximal absorbance at 586 nm. Therefore, 10 µl of 0.5 M butylated hydroxytoluene, 650 µl of a mixture of 6 ml of methanol with 18 ml of 10.3 mM N-methyl-2-phenylindole and 150 µl of 15.4 M methanesulfonic acid were added to 200 µl of the first cytosolic fraction of the homogenate of the gills or mantle of *B. azoricus* and incubated at 45°C for 60 minutes. MDA + 4-HNE concentrations were estimated by measuring the maximal absorbance of the formed chromophore at 586 nm using malondialdehyde bis (tetrametoxypropan, SIGMA) as a standard. Lipid peroxidation is expressed as nmoles of MDA and 4-HNE g^{-1} of total protein concentrations.

Metallothioneins

To determine MTs concentrations, the tissues were homogenized at 4°C using an electric potter and a Teflon pestle in a Tris buffer (100 mM), pH 8.1, containing 10 mM of β-mercaptoethanol. The soluble and insoluble fractions were separated by centrifugation (30000 *g*, 30 min, 4°C). Aliquots of the supernatants were heated (15 min, 95°C) and allowed to cool on ice. Heat-denatured proteins were separated from heat-stable proteins by centrifugation of the heated supernatants (10000 *g*, 15 min). Supernatants containing the heat-stable proteins, including MTs, were stored at -20°C until use. In the heat-denatured cytosol, the amount of MTs was determined by differential pulse polarography using a PAR 394 analyser and a EG&G PAR 303A static mercury drop electrode (SMDE) in accordance with the method of Olafson & Sim (1979) modified by Thompson & Cosson (1984). The electrochemical detection of MTs takes place in an amnoniacal electrolyte containing cobalt that catalyses the reduction of the cysteine thiol groups (Brdicka, 1933). The standard addition method was used for calibration with rabbit liver MT (Fluka) in the absence of *B. azoricus* MT standard. The levels of MTs are expressed as mg g^{-1} wet weight.

These analyses were performed by Dr. Richard Cosson from ISOMer Marine Biology Laboratory in the University of Nantes (France).

Statistical analysis

Values were expressed as means ± standard deviation (SD). The data were previously tested for normality and homogeneity and analyzed by analysis of variance (ANOVA) to determined significant statistical differences between hydrothermal sites regarding antioxidant enzymatic activity (SOD, CAT and GPx), TOSC levels, MT and LPO concentrations. The level of significance was set at 0.05. Principal components analysis (PCA) was used in the ordination method to compare antioxidant enzyme activities, MDA, MT and metal concentrations within the tissues in each site. Statistical analysis was carried out with Statistica 5.1 and SIMCA-P 10 for windows.

2.4. Results

Superoxide Dismutase

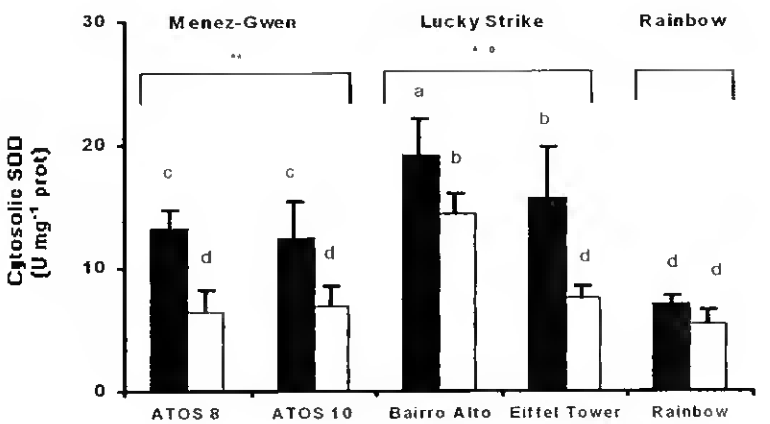
The SOD activity (cytosolic and mitochondria) in the gills and mantle of *B. azoricus* from the hydrothermal vent sites is presented in figures 2.2A and B.

SOD is predominantly present in the cytosolic fraction (>50%) of both tissues in all vent sites. With the exception of Rainbow where no significant differences in SOD activity (cytosolic and mitochondria) between tissues were observed, the SOD activity (cytosolic and mitochondria) was in general significantly higher in the gills (around 2-fold) when compared with mantle ($p>0.05$) (Figure 2.2 A and B).

The cytosolic SOD activity in *Bathymodiolus* gills significantly decreased from Lucky Strike vent site, followed by those of Menez-Gwen and Rainbow ($p<0.05$). In the mantle, a similar cytosolic pattern was observed but no significant differences occurred between mussels from Menez-Gwen and Rainbow vent fields (Figure 2.2A).

In mitochondrial fraction, the higher SOD activity was in the gills of *B. azoricus* from Menez-Gwen, followed by those from Lucky Strike and Rainbow ($p<0.05$). As in the cytosolic fraction, the mitochondrial SOD activity in the mussel mantle was significantly higher at Lucky Strike and not different among the other two sites ($p<0.05$) (Figure 2.2 B).

A



B

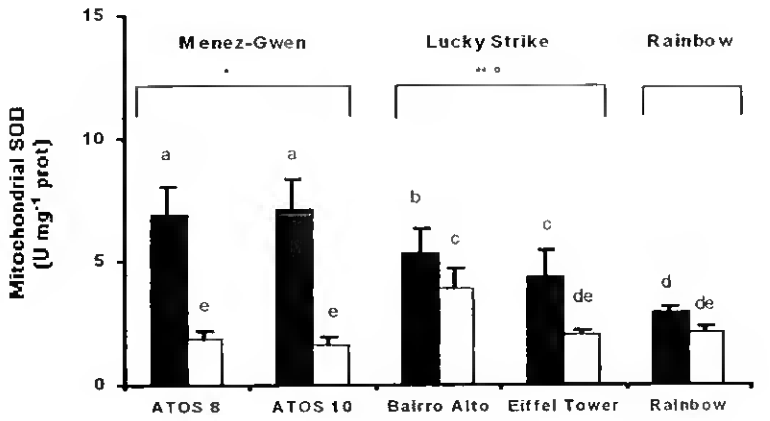


Figure 2.2 – Cytosolic (A) and mitochondrial (B) SOD activity (mean ± SD) (U mg^{-1} protein) in the gills (■) and mantle (□) of *B. azoricus* from several hydrothermal vent sites ($n = 4$). Values followed by the same letter are not statistically different ($p>0.05$). Symbols represent statistical differences between gills (*) and mantle (°) of three main vent sites.

Only *B. azoricus* at Lucky Strike exhibited significantly different SOD activity (cytosolic and mitochondrial) intra sites for both tissues with an SOD activity significantly higher at Bairro Alto than at Eiffel Tower ($p<0.05$) (Figure 2.2A&B).

Catalase

The CAT activity in the gills and mantle of *B. azoricus* from the ATJ vent sites is in figure 2.3. Like for SOD, the CAT activity was significantly higher in the gills (3-fold at Lucky Strike and Menez-Gwen and 7-fold at Rainbow) compared with that of the mantle in all vent sites ($p<0.05$) (Figure 2.3A and B). The enzymatic activity of CAT in the gills was the opposite to that of SOD and significantly different between all three vent sites (Rainbow>Menez-Gwen>Lucky Strike) ($p<0.05$). Contrary, in the mantle, no significant differences in CAT activity were observed between vent sites ($p>0.05$).

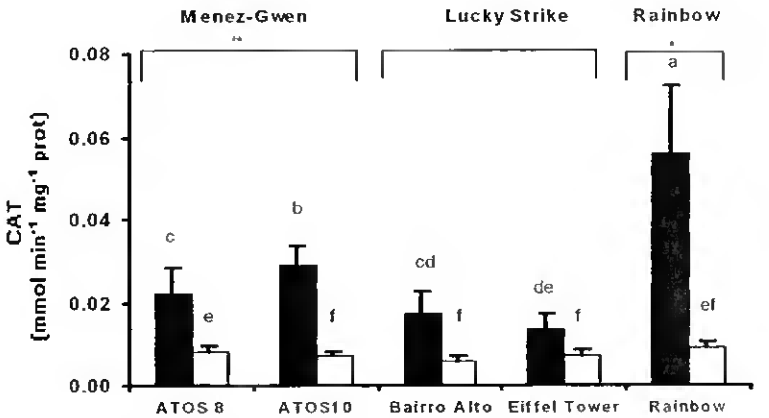


Figure 2.3 – CAT activity (mean ± SD) (mmol min^{-1} mg^{-1} protein) in the gills (■) and mantle (□) of *B. azoricus* from several hydrothermal vent sites ($n = 4$). Values followed by the same letter are not statistically different ($p>0.05$). Symbols represent statistical differences between gills (*) and mantle (º) of three main vent sites.

Glutathione Peroxidases

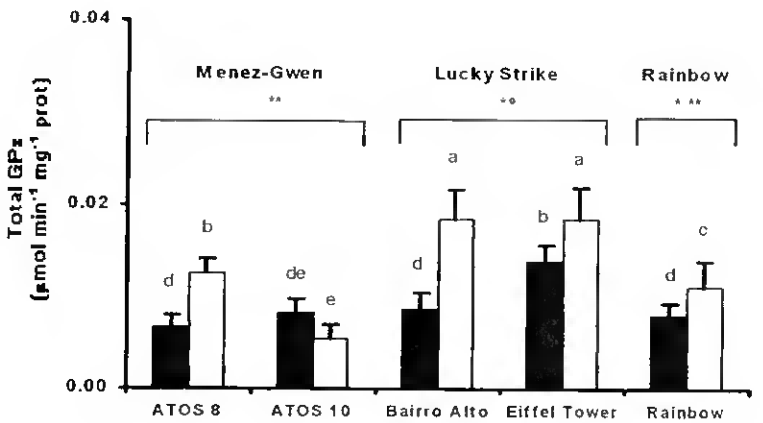
TGPx and Se-GPx activities in the cytosolic fraction of the gills and mantle of *B. azoricus* from hydrothermal vent sites are presented in figures 2.4A and B. The activity of GPx (total or Se-dependent) contrarily to what was observed for SOD and CAT, exhibited higher activity in the mantle than in gills of *B. azoricus*.

Total GPx in *Bathymodiolus* gills (Figure 2.4A) showed the same pattern as SOD with significantly higher TGPx activity in Lucky Strike than in Menez-Gwen ($p<0.05$). However, no significant differences in the activity of this enzyme exist in the gills of the mussels between Lucky Strike and Rainbow and between Rainbow and Menez-Gwen ($p>0.05$) (Figure 2.4A). The TGPx activity in the

mantle also followed the same pattern of SOD and was significantly higher in mussels from Lucky Strike compared with that at Menez-Gwen and Rainbow (p<0.05) (Figure 2.4A).

Within the same sites, significant differences in TGPx only occur in the gills of the mussels from Lucky Strike, and were opposite to that of SOD, with higher TGPx activity at Eiffel Tower when compared with that at Bairro Alto (p<0.05) (Figure 2.4A). In the mantle, significantly higher values of TGPx activity were only observed in mussels from Lucky Strike (Figure 2.4B).

A



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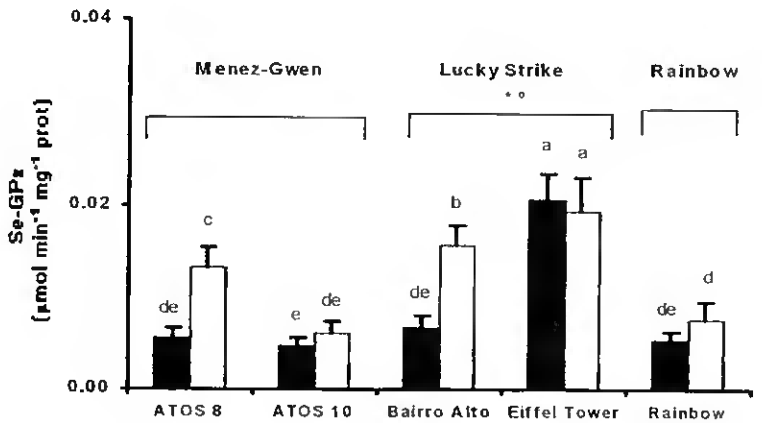

Figure 2.4 – Total (A) and selenium-dependent (B) GPx activity (mean ± SD) (µmol min^{-1} mg^{-1} protein) in the cytosolic fraction of the gills (■) and mantle (□) of *B. azoricus* from several hydrothermal vent sites (n = 4). Values followed by the same letter are not statistically different (p>0.05). Symbols represent statistical differences between gills (*) and mantle (°) of three main vent sites.

Like for TGPx, the Se-GPx activity is higher in the gills and mantle of the mussels from Lucky Strike compared with those of Menez-Gwen and Rainbow ($p<0.05$) (Figure 2.4B). As for TGPx, no significant differences in Se-GPx were observed in the mussels gills intra sites, with the exception of those at Lucky Strike where Se-GPx activity in the mussels from Eiffel Tower was significantly higher than at Bairro Alto ($p<0.05$). In the mantle, the Se-GPX activity was significantly higher at Eiffel Tower and decreased according to the sequence Bairro Alto > ATOS 8 > ATOS 10 = Rainbow ($p<0.05$), with the exception of mussels from Rainbow and ATOS 10 where Se-GPX activity was similar (Figure 2.4B).

TOSC

TOSC were only measured in gills and mantle of mussels from Menez-Gwen (ATOS 10), Lucky Strike (Bairro Alto) and Rainbow and peroxyl, hydroxyl radicals and peroxynitrite are presented in figure 2.5 A-C.

In general, gills exhibited significantly higher levels of total oxyradical scavenging capacity ($p<0.05$) compared with the mantle in all sites. TOSC values towards hydroxyl radicals in *Bathymodiolus* from all sites were significantly lower when compared with TOSC towards peroxyl radicals and peroxynitrite ($p<0.05$) (Figure 2.5B).

The gills and mantle of mussels collected from Menez-Gwen had significant higher TOSC values toward peroxyl, hydroxyl radicals and peroxynitrite ($p<0.05$) indicating that the mussels from this vent field are the most efficient scavengers of peroxyl and hydroxyl radicals and peroxynitrite and therefore have greater capacity to counteract the toxicity of oxyradicals. In the mantle, levels of TOSC were similar between Lucky Strike and Rainbow while in the gills this only occurred in TOSC toward peroxynitrite (Figure 2.5C).

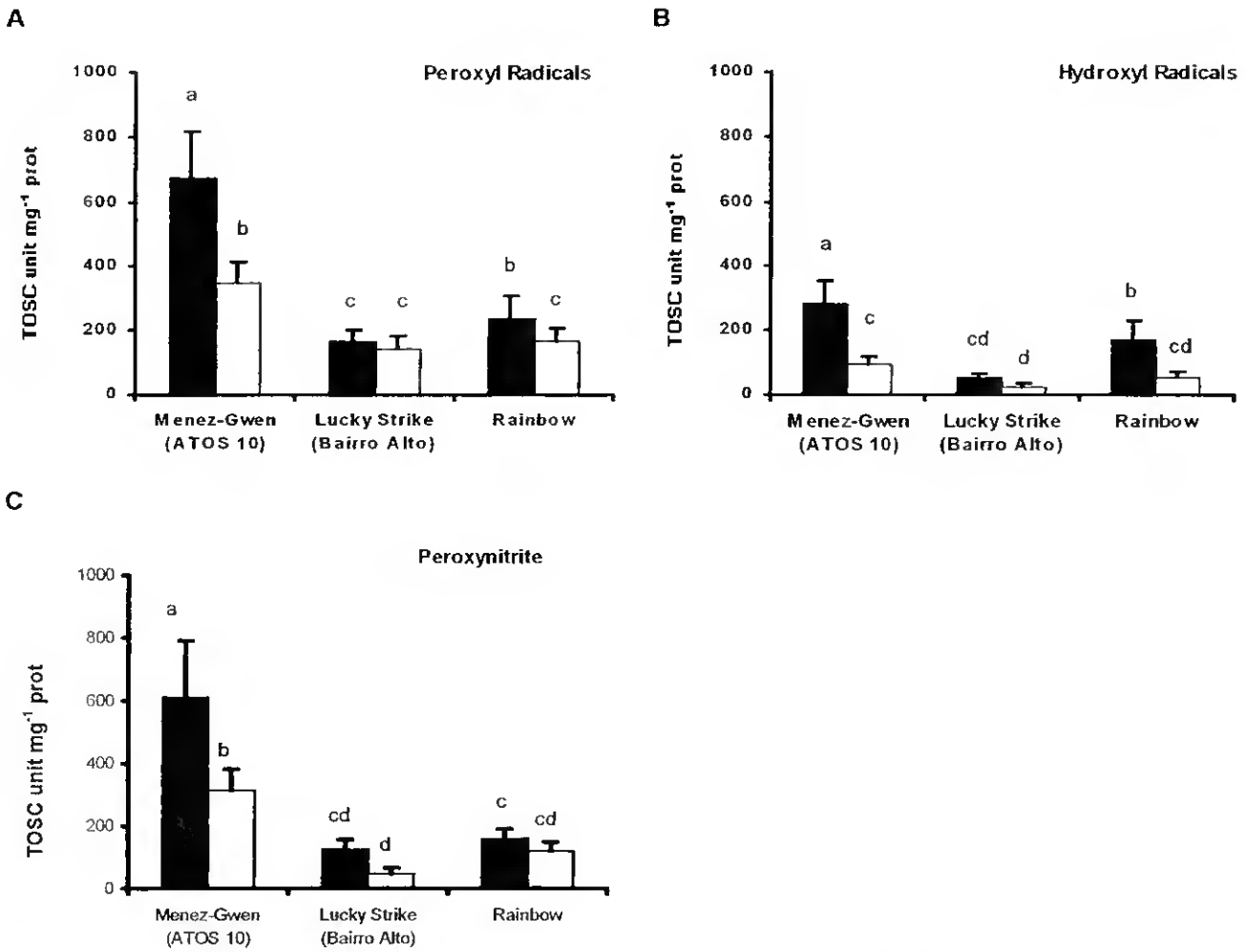

Figure 2.5 – Total Oxyradical Scavenging Capacity (TOSC) values (mean ± SD) towards peroxyl (A) and hydroxyl (B) radicals and peroxynitrite (C) in the gills (■) and mantle (□) of *B. azoricus* from Menez-Gwen, Lucky Strike and Rainbow (n = 5). Values followed by the same letter are not statistically different (p>0.05).

Lipid Peroxidation

The lipid peroxidation (expressed as MDA and 4-HNE compounds) in the cytosolic fraction of the gills and mantle of *B. azoricus* from MAR hydrothermal vent sites are presented in figure 2.6.

The LPO levels were significantly higher in the gills of *B. azoricus* from all vent sites (p<0.05), with the exception of those from Eiffel Tower where lipid peroxidation in the mantle was significantly higher than in the gills (p>0.05) (Figure 2.6). From the three main vent sites, lipid peroxidation in the gills decreased according to the sequence Rainbow > Lucky Strike > Menez-Gwen (p<0.05).

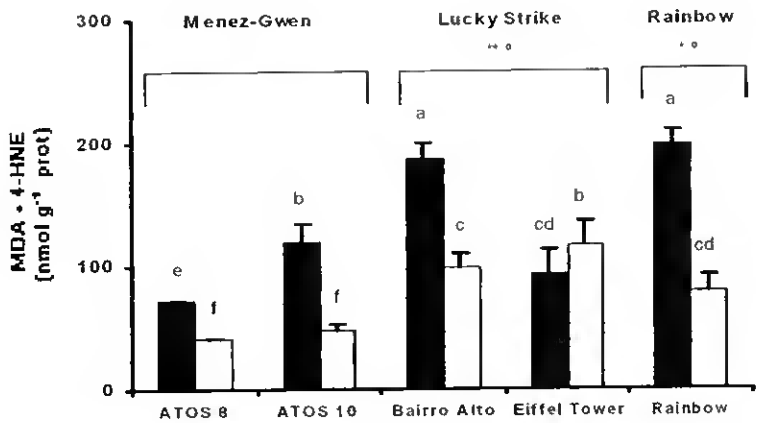

Figure 2.6 – MDA + 4-HNE concentrations (mean ± SD) (nmol g^{-1} protein) in the cytosolic fraction of gills (■) and mantle (□) of *B. azoricus* from several hydrothermal vent sites (n = 4). Values followed by the same letter are not statistically different (p>0.05). Symbols represent statistical differences between gills (*) and mantle (°) of three main vent sites.

However, in the mantle lipid peroxidation was similar in the mussels from Lucky Strike and Rainbow and significantly higher than from Menez-Gwen (p<0.05) (Figure 2.6). Between the same sites, lipid peroxidation in the gills was significantly different intra-sites, with higher levels at ATOS 10 in Menez-Gwen and at Bairro Alto in Lucky Strike (p<0.05). In the mantle, significant differences in lipid peroxidation were only observed in mussels from Lucky Strike with higher concentrations at Eiffel Tower that at Bairro Alto (p<0.05) (Figure 2.6).

MT concentrations

MT concentrations in *B. azoricus* gills and mantle are presented in figure 2.7. MT concentrations were significantly higher in the gills (3-fold), compared to the mantle in all vent fields (p<0.05). Even though no significant differences in MT content in the gills of mussels from the three hydrothermal vent sites were detected, within Menez-Gwen, mussels from ATOS 10 exhibited significantly higher MT concentrations in the gills compared to those at ATOS 8. MT concentrations in the mantle, however, were similar in all sites (p>0.05) confirming that these proteins were not a major detoxification pathway in this species.

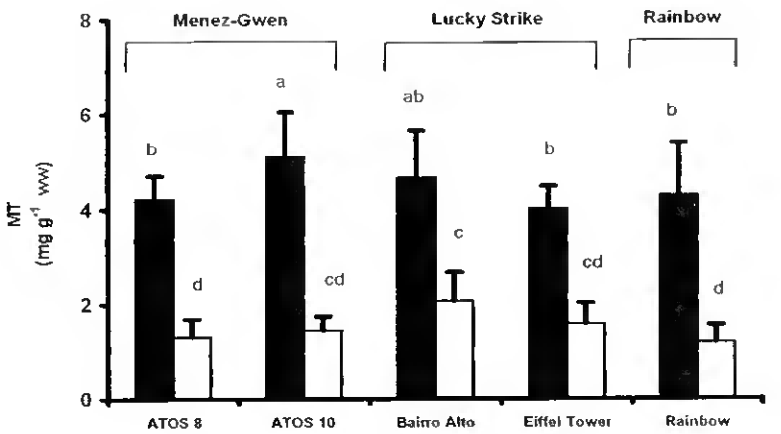

Figure 2.7 – MT concentrations (mean ± SD) (mg g^{-1} w.w.) in the cytosolic fraction of gills (■) and mantle (□) of *B. azoricus* from several hydrothermal vent sites (n = 10). Values followed by the same letter are not statistically different (p>0.05).

Metal (Ag, Cd, Cu, Fe, Hg, Mn and Zn) concentrations in *Bathymodiolus* gills and mantle from the same vent field sites (adapted from Fiala-Médioni *et al.*, submitted) are presented in Table 2.1.

Considering the data of enzyme activities, TOSC, LPO, MTs and metals in gills or mantle of mussels from all the different hydrothermal vent sites together, significant relationships between SOD mitochondrial activity and Mn (r = 0.960, p<0.05), CAT and Fe (r = 0.926, p<0.05), GPx (total and Se-dependent) with Zn (0.974 and 0.986, respectively (p<0.05)) and Cd (r =0.941 and 0.971, respectively (p<0.05)) exist in the gills of *B. azoricus*. The SOD enzymatic activity depends directly on the presence of the anion superoxide to produce hydrogen peroxide, which is a substrate for catalase in the enzymatic reaction.

Since the increase in SOD activity is not followed by an increase of catalase activity (as in the Rainbow site) the main pathway of hydrogen peroxide degradation is probably through glutathione peroxidases with a subsequent formation of water. Moreover, catalase activity in vent mussel gills apparently seems to be inhibited when compared with the other enzymatic systems (SOD, glutathione peroxidases).

Table 2.1 – Metal concentrations in *Bathymodiolus azoricus* collected from three different MAR vent field sites. Similar letters are not statistical different (p>0.05).

	Menez-Gwen				Lucky Strike				Rainbow	
	ATOS 8		ATOS 10		Bairro Alto		Eiffel Tower			
Gills										
Fe	186 ± 44	b	206 ± 43	b	303 ± 63	b	288 ± 53	b	2685 ± 664	a
Mn	6.3 ± 0.8	b	4.7 ± 1.6	c	7.6 ± 1.9	b	7.2 ± 1.6	b	9.5 ± 2.3	a
Cu	57 ± 11	c	89 ± 16	a	79 ± 19	ab	88 ± 15	a	65 ± 14	b c
Zn	161 ± 11	c	173 ± 24	c	1976 ± 571	a	589 ± 138	b	87 ± 13	c
Cd	7.9 ± 2.2	c	3.2 ± 0.8	d	47 ± 10	a	18 ± 2	b	2.0 ± 0.5	d
Ag	4.6 ± 1.1	a	1.9 ± 0.5	b	5.2 ± 1.2	a	1.8 ± 0.4	b	2.3 ± 0.6	b
Mantle										
Fe	10 ± 2	c	51 ± 13	bc	235 ± 61	a	100 ± 26	b	186 ± 81	a
Mn	3.0 ± 0.7	c	1.9 ± 0.3	c	5.8 ± 1.5	b	5.5 ± 1.0	b	7.2 ± 0.5	a
Cu	11 ± 2	b	40 ± 12	a	14 ± 3	b	16 ± 3	b	2.3 ± 0.6	c
Zn	42 ± 9	c	71 ± 14	b	124 ±36	a	65 ± 16	b	28 ± 7	c
Cd	0.6 ± 0.2	c	0.3 ± 0.1	c	2.9 ± 0.1	a	1.6 ± 0.3	b	0.02 ± 0.003	c
Ag	0.7 ± 0.2	ab	0.5 ± 0.2	b	0.8 ± 0.2	a	0.5 ± 0.1	b	0.2 ± 0.03	c

In addition, because no significant relationship occurred between SOD or glutathione peroxidases with LPO in the gills it seems to indicate that these systems are responding to the environmental characteristics of the vent sites, and consequently capable of preventing an increase of LPO in the gill cells of *B. azoricus*. In fact, only catalase activity has a positive linear relationship with MDA concentrations (r = 0.937, p<0.05), suggesting that excess of hydrogen peroxide could increase the hydroxyl radical production and as a consequence enhance lipid peroxidation. Moreover, there is also a linear relationship between LPO and Fe (LPO= 0.03[Fe] + 109.9, r = 0.653, p>0.05), suggesting that the excess of Fe was directly related with LPO.

Principal component analysis (PCA) performed on untransformed antioxidant enzymatic activities, TOSC, LPO, MT and metal concentrations in the gills and mantle of mussels from all vent sites are presented in Figures 2.8-10. PCAs in the gills shows that PC1 and PC2 explain 70% of total variance of the data (38% for the first principal component and 32% for the second principal component) (Figure 2.8).

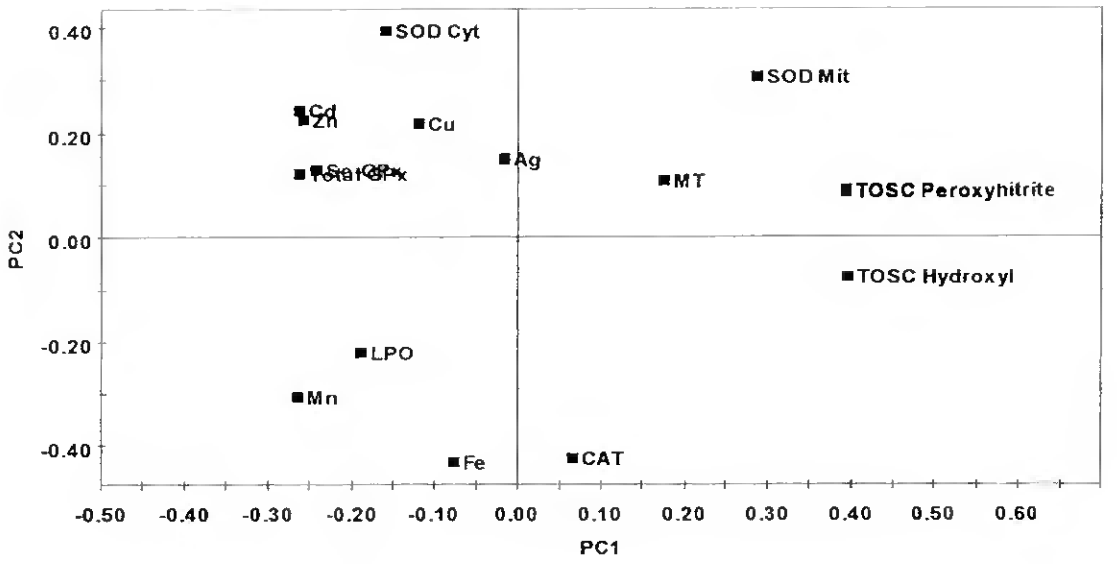

Figure 2.8 – PCA of the antioxidant enzymes activity in the gills of *B. azoricus* showing the loadings of the variables on PC1 and PC2.

Cytosolic and mitochondrial SOD are close to GPx activities, MT, Cd, Ag and Zn while CAT is in the opposite. LPO is higher when SOD and GPx are inhibited, and CAT has a minor role as antioxidant to prevent membrane damage in the gills. PCA on the data scores shows all sites close to origin, suggesting a high similarity in the gills of the mussels between vent sites (Figure 2.9). Although the environment of hydrothermal vent sites is considerably different, in terms of depth, temperature, pH and metal concentrations, these environmental differences do not seem to influence the general function of antioxidant enzymes.

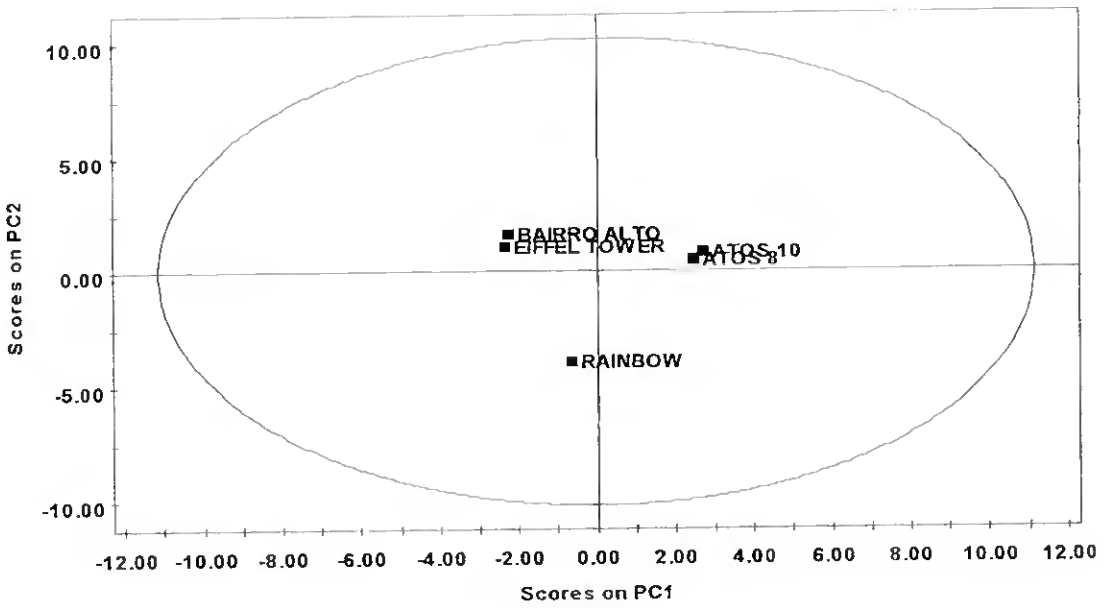

Figure 2.9 – PCA of the antioxidant enzymes activity in the gills of *B. azoricus* showing the data scores labelled as sites.

In the mantle no significant relationship exists between the antioxidant enzymatic activity, MT and metals. However, the cytosolic SOD activities are significantly positively correlated with mitochondrial SOD (SOD_{mit} = 0.24SOD_{cit} + 0.30, r = 0.794, p>0.05), and TGPx with Se-dependent GPx (SeGPx = 0.97 TGPx + 0.51, r =0.932, p<0.05). This suggests that in the mantle of *B. azoricus* the most important pathways of oxyradical detoxification are SOD and GPx (total and Se-dependent), as described for the gills. Also in the mantle, catalase appears to be inhibited.

The MDA+4-HNE concentrations, on the contrary, are significantly correlated with TGPx (r =0.734, p<0.05) and Se-dependent GPx (r =0.698, p<0.05) and also with Fe (LPO = 0.29 [Fe] + 43.5, r =0.844, p<0.05) and the linear relationships between LPO and GPx (total and Se-dependent) (LPO = 4438 TGPx + 27.0, r =0.965, p<0.05) and LPO = 4360 SeGPx + 33.3, (r =0.942, p<0.05), respectively) were similar, which suggests that the responses of the enzymatic system are not sufficient to counter act the formation of reactive

oxygen species and therefore an increase in lipid peroxidation occurs in the mantle.

In the mantle, PCA shows that PC1 and PC2 explain more than 80% of total variance of the data (56% for the first principal component and 27% for the second principal component) (Figure 2.10).

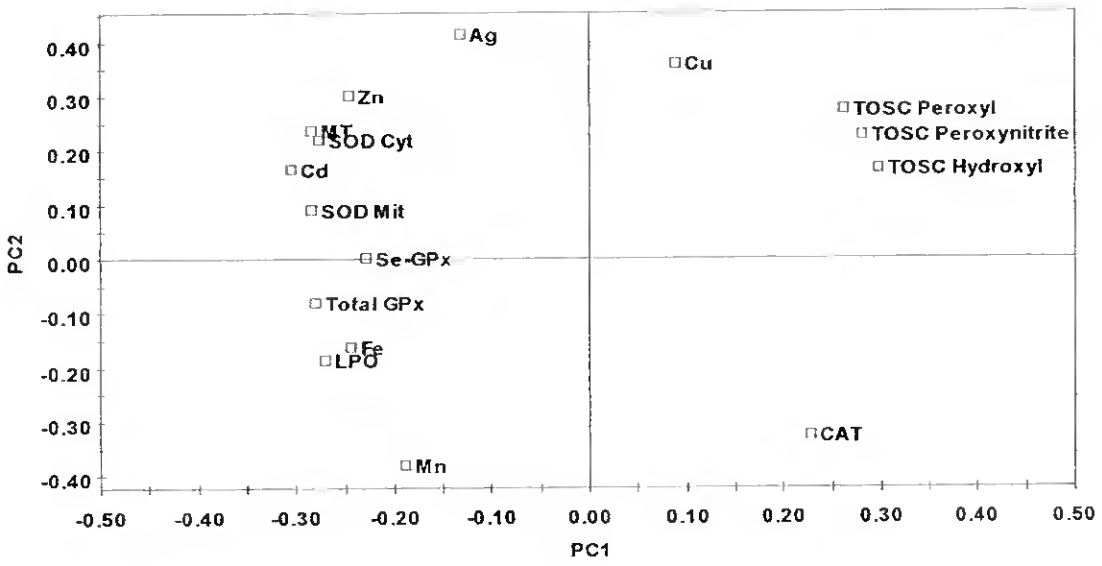

Figure 2.10 – PCA of the antioxidant enzymes activity in the mantle of *B. azoricus* showing the loadings of the variables on PC1 and PC2.

In this case LPO and all antioxidant enzymes are grouped together except CAT and SOD (cytosolic). LPO was considerable lower in the mantle and PCA suggests that antioxidant enzymes are unable to prevent cellular damage in this tissue. As observed for the gills, PCA on the data scores shows that all sites are close to the origin, suggesting a high similarity between mantle tissues from vent sites (Figure 2.11).

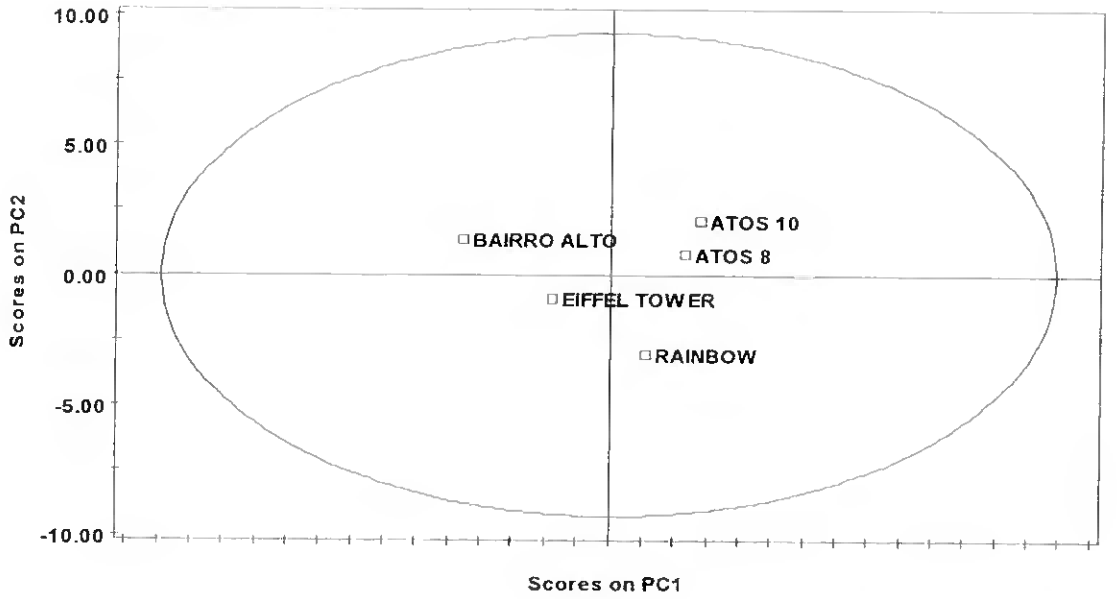

Figure 2.11 – PCA of the antioxidant enzymes activity in the mantle of *B. azoricus* showing the data scores labelled as sites.

2.5. Discussion

The present paper is the first attempt to quantify antioxidant systems, TOSC and LPO in *Bathymodiolus azoricus* from hydrothermal vent fields and to try to explain the role of these antioxidant systems in the resistance and tolerance of vent mussels to a metal-rich reduced environment.

Accumulation of metals by *Bathymodiolus* is tissue and site specific and depends on regulation, accumulation and excretion strategies of this species (Table 2.1). Metal concentrations are lower in the mantle when compared with the gills. These lower metal levels in the mantle indicate that although, like the gills, this tissue is in direct contact with the vent environment, its main function is the accumulation of reserves and secretion for shell formation. The most abundant metals in the gills are in the decreasing order: Fe, Zn, Cu, Cd and Ag while Mn is equally distributed between both tissues (Table 2.1). Moreover, metal accumulation in vent mussels is site dependent and therefore, directly related with the composition and speciation of metals in the vent fluid (Pruski &

Dixon, 2003; Ventox, 2003). Similarly some metal concentrations in *Bathymodiolus* tissues are related with vent field depth. Therefore, levels of Fe and Mn in *B. azoricus* tissues are directly related to vent depth in both tissues (Table 2.1) while is the opposite for Cu in the mantle. Fe and Mn are the most important metals in mussel tissues from Rainbow while Zn, Cd and Ag were higher at Lucky Strike irrespective of the tissue. Similar results were previously detected for the same species and tissues collected from Menez-Gwen and Lucky Strike (Géret *et al.*, 1998; Rousse *et al.*, 1998). However, Ag and Mn accumulated in *B. azoricus* gill tissue were lower, while Mn and Cd higher than those accumulated in tissues from other vent species, namely *Bathymodiolus thermophilus* (Smith & Flegal, 1989) and *Calyptogena magnifica* (Roesijadi & Crecelius, 1984) collected from hydrothermal vent fields from East Pacific Rise.

Vent mussels accumulate significantly high metal concentrations (Ag, Cd, Cu, Mn, Fe and Zn) in the gills and mantle when compared with similar tissues of coastal mussels. A comparison between metal levels in the vent mussel species and those from coastal zones, such as *M. edulis* (Fung *et al.*, 2004) and *M. galloprovincialis* (Bebianno & Machado, 1997; Besada *et al.*, 2002; Kalpaxis *et al.*, 2004; Szefer *et al.*, 2004), reveals that the symbiotic species accumulate higher metal (Fe, Zn, Cu and Cd) levels than those that live in the boundaries of photic zone. This may be related to the metal concentrations in these hydrothermal vent fields that are in general higher (Géret *et al.*, 1998; Pruski & Dixon, 2003) than in heavily polluted coastal environments (Pruski & Dixon, 2003) and to the metal bioavailability in such extreme environments (Ventox, 2003).

Metal partitioning between soluble and insoluble fractions in vent mussels varies, like for total metals, among sites and tissues (Fiala-Médioni *et al.*, in prep). Metals, with the exception of Ag, are preferentially bound to insoluble compounds. According to Rousse *et al.* (1998) the majority of the metals in Menez-Gwen are in the soluble fraction, with the exception of Cu in the gills, while in Lucky Strike they are mainly present in the insoluble fraction. Cu showed the higher affinity for soluble compounds than Cd and Zn (Rousse *et al.*, 1998). In the soluble fraction, metal partitioning is also site specific. At

Menez-Gwen, Cd in the soluble fraction is mostly present associated with heat-stable phase, bound to MT, in the gills and mantle while Zn in this phase is found mainly in the gills and Cu equally distributed among heat-stable and heat-denatured proteins between tissues (Rousse *et al.*, 1998). Cd, Cu and Zn in mussels from Lucky Strike seem to be preferentially immobilised in lysosomal systems that act as a detoxification mechanism (Rousse *et al.*, 1998).

Levels of Ag, Cd, Cu and Zn present in *Bathymodiolus* would, in their coastal counterparts, induce MT synthesis but in this species MT seems to act as an effective antioxidant defence system by scavenging preferentially hydroxyl radicals (Thornalley & Vasak, 1985; Hayes & McLellan, 1999; Viarengo *et al.*, 2000) and functionally substitute SOD in protecting from oxidative stress (Cavalleto *et al.*, 2002). The antioxidant role of MT in the mussel *M. galloprovincialis* provides evidence that MT is effective in the protection of the cells and the entire organism against oxidative stress (Viarengo *et al.*, 2000). MT levels in *B. azoricus* are 3-fold higher in the gills than in the mantle but similar among the three vent sites (Figure 2.7). Similar results were observed for the same species and tissues of mussels from Menez-Gwen and Lucky-Strike (Table 2.2) (Rousse *et al.*, 1998; Géret *et al.*, 1998). Furthermore, in vent mussels MT is not related with vent depth in any of the analysed tissues and therefore independent of the metal content in vent fields. Although present in high levels, MT synthesis does not play a metal detoxification role in this species. Vent mussels harbour symbiotic bacteria in the gills, which are suspected to have a significant role in the detoxification mechanisms of metals accumulation (Cosson-Mannevy *et al.*, 1988). However this hypothesis needs to be proved. Moreover, MT can form stable complexes with metals and precipitate afterwards being responsible for the high concentration of metal insoluble compounds (Fiala-Médioni *et al.*, in prep).

Metals, besides inducing MT synthesis, are known to enhance the production of ROS as side products of electron transfer during aerobic vent metabolism (Fridovich, 1998), a common pathway of toxicity induced by stressful environmental conditions (Regoli *et al.*, 2000). If not adequately neutralized, H_2O_2 can induce direct oxidative stress or originate hydroxyl radicals (OH•), the

most dangerous of all reactive oxygen species that arise as a product of Fenton-type reactions, which are normally catalysed by Fe and Cu (Fridovich, 1998). Antioxidant enzyme systems able to counteract the presence of ROS in the gills and mantle of *B. azoricus* revealed, like metals, tissue and site variability.

The activity of SOD, CAT, TOSC and LPO were, like metals, significantly higher in the gills than in the mantle (Figures 2.2-3, 2.5-6), while the activity of total GPx (that combine the activity of Se-GPx and glutathione-S-transferase (GST)) was the opposite (Figure 2.4). Furthermore, the activity of these enzymes, TOSC and LPO are site specific. Thus in *B. azoricus* gills, the activity of cytosolic SOD (>50% of total SOD) that catalyses the dismutation of superoxide radical into molecular oxygen and hydrogen peroxide (Figure 2.2) and GPx (total and Se-dependent), although lower than in the mantle, followed the same pattern with higher activity in mussel gills from Lucky Strike (Eiffel Tower) vent field, followed by Menez-Gwen and Rainbow (Figure 2.2A and 4) with GPx activity similar among these two vent sites. Similarly, Cu concentrations in mussel gills were at Lucky Strike the most significant of all vent fields, suggesting that the excess of this essential metal is directly responsible for the enhancement of cytosolic SOD (Cu and Zn enzyme) activity in mussel gills. A partial cDNA of Cu/Zn-SOD was obtained with only 195 bp from *B. azoricus* gills (GenBank Accession N° AY377971) corresponding to a partial ORF. The deduced amino acid sequence revealed similarities with those of other coastal molluscs (63.1% with the clam *Ruditapes decussatus* and 56.9% with the fresh water mussel *Dreissena polymorpha*) and homology with mammalian Cu/Zn-SOD (> 53%). Further comparison of *B. azoricus* Cu/Zn-SOD with mammalian counterpart indicate that the active site geometry (Gly-45, Gly-62, Pro-75, Gly-83), the metal binding sites for Cu (His-47, 49, 64, 121) and Zn (His-64, 72, 81) and cysteine residues (Cys-58 and Cys-147), involved in intra chain disulfide bridge, were conserved (Géret *et al.*, 2004).

Symbiotic bacteria were not isolated from the gills, thus SOD enzymatic activity in this tissue reflects the contribution from both symbiotic bacteria and tissue. However, SOD isoforms in chemoautotrophic sulphur-oxidising and

methanotrophic symbiotic bacteria in *B. azoricus* gills need to be further identified. Preliminary results revealed the presence of Cu/Zn-SOD, Mn-SOD and Fe-SOD reflecting an antioxidant role of symbiontic bacteria in mussel gills (Hoarau, unpublished data). A single Mn-SOD and several Cu/Zn-SODs isoforms were identified in the muscle tissue of the tubeworm *R. pachyptila* reflecting the host. In contrast, trophosomal tissues contained a single Fe-SOD and several Mn-SODs that reflect also the role of the symbiotic bacteria (Blum & Fridovich, 1984). However, the giant clam *C. magnifica* contained only a single Mn-SOD and a single Cu/Zn-SOD in the gills and muscle but the activity was less than that of coastal clam *M. mercenaria* which have similar proportions of Mn-SOD and Cu/Zn-SOD in their tissues (Blum & Fridovich, 1984). Mitochondrial SOD (mainly Mn-SOD and Fe-SOD) activity in the gills of *Bathymodiolus* decreased with depth (from Menez-Gwen to Rainbow) (Figure 2.2B) and is inversely related with Mn and Fe concentrations in mussel gill tissues from these vent sites. In contrast, catalase activity (a defence against the formation of OH• by reducing H_2O_2 to water) in the gills was the opposite, increasing with the vent depth from Menez-Gwen to Rainbow (Figure 2.3).

As referred above, besides Mn, Fe concentrations were also the highest either in Rainbow vent fluid (Douville *et al.*, 2002; Pruski & Dixon, 2003, Ventox 2003) or in mussel gills suggesting that the excess of iron undergo Haber-Weiss (formation of molecular oxygen and the extremely toxic OH• radical) and/or Fenton type reactions leading to an increase in the production of hydroxyl radicals (Table 2.2). Moreover, the TOSC balance towards peroxyl and hydroxyl radicals and peroxynitrite between antioxidant parameters and pro-oxidant factors decreased from Menez-Gwen to Rainbow (Figure 2.5) nevertheless, TOSC towards hydroxyl radicals were significantly lower.

TOSC levels in mussel gills from Menez-Gwen indicate that the vent environment at this site is less stressful and the formation of ROS in mussel gills is effectively counteracted by the antioxidant defence system. Similarly, LPO levels were lower in the gills of mussels from Menez-Gwen supporting the hypothesis of a less stressful vent environment than in those from Rainbow where TOSC levels were lower. Lower TOSC levels in mussel gills from the

other two vent sites indicate an enhancement ROS production caused by either the presence of important concentrations of toxic metals (Hussein *et al.*, 1987; Muller, 1986) or a more harmful environment in general.

In the mantle, the activity of SOD (cytosolic and mitochondrial), GPx (total and Se-dependent) and LPO were, like the gills, also highest in mussels from Lucky Strike, while catalase activity was similar among vent sites (Figures 2.2, 2.3, 2.6). Like in the gills, TOSC levels decreased from Menez-Gwen to the other two vent sites among which levels were similar, indicating that mantle tissues from Lucky Strike and Rainbow were unable to cope with ROS formation (Figure 2.5). Moreover, at Lucky Strike Fe, Zn, Cd and Ag concentrations in the mantle were the most important of all the vent sites (Table 2.2).

With the exception of catalase, the enzymatic activity of (SOD), GPx (total and Se-dependent), TOSC, MT and lipid peroxidation (Figures 2.2-7) in *B. azoricus* gills and mantle are of the same order of magnitude as from other vent and non vent molluscs, particularly from those living in pristine and metal contaminated coastal areas as can be seen in Table 1.6 (See Chapter 1). Catalase activity, however, was absent from other rift animals (*C. magnifica* and *R. pachyptila*) while the coastal clam *M. mercenaria* contained high levels (Blum & Fridovich, 1984). However, Blum and Fridovich (1984) point out that the lack of catalase activity was related to sample preservation and both species contained substantial levels of peroxidases. There are not many TOSC data available in the literature for bivalve molluscs. Those available are from bivalve species living in another extreme environment (Antarctic, Arctic and Mediterranean environments). TOSC levels from the digestive gland of *Adamussium colbecki* or *Chlamys islandicus* from Antarctic and Arctic environments and from *Pecten jacobaeus,* a related temperate species, revealed that *A. colbecki* was the most efficient scavenger for ROO• and OH• radicals indicating that this species have a greater ability to counteract the toxicity of oxyradicals in comparison with the other two. For peroxynitrite, levels were lower than the other TOSC radicals for all the three species (Regoli *et al.*, 2000). However, an interesting similarity can be pointed out with another deep-sea species (crustacean though), the giant amphipod *Eurythenes gryllus*, that is characterised by a low TOSC toward OH•

(while TOSC toward peroxyl and peroxynitrite are relatively higher, which is not the case for other shallow water bivalve species) (Camus & Gulliksen, 2004). Can this be interpreted as deep-sea feature?

Although PCA analysis revealed that mussel gills and mantle from the different vent sites were similar (Figures 2.9 and 2.11), a more detailed analysis point to the existence of three distinct mechanisms to counteract ROS production and lipid peroxidation in vent mussels reflecting the specific environmental characteristics of the three vent sites. In fact ROS production during respiration seems to be more significant at Menez-Gwen. Similarly, the variability of TOSC, measuring the overall capacity to neutralize different forms of oxyradicals (Regoli & Winston, 1999; Regoli *et al.*, 1997; 1998) similar between mussel tissues (gills and mantle) were higher in Menez-Gwen (Figure 2.5) indicating that mussels from this vent site are the most efficient scavenger of ROS. In fact, mussels collected from Menez-Gwen are exposed to significantly lower metal levels when comparing to those from Lucky Strike and Rainbow (Douville *et al.*, 2002, Ventox, 2003). Moreover, Menez-Gwen is a shallower hydrothermal vent (850 m) and might be more stable in potentially toxic parameters like metals, temperature, pH and hydrogen sulphide. However, mussels from Lucky Strike and Rainbow vent fields have significant reduced capacity to scavenge free radicals (Figure 2.5). This indicates that in mussels from these sites antioxidant defences were depleted. This is confirmed by the contribution of the soluble fraction (low molecular weight scavengers <3 kDa), which is 50 % lower at these two sites (Lucky Strike and Rainbow) for peroxyl and peroxynitrite compared to Menez-Gwen. With regard to the soluble fraction, an interesting aspect is that most of the TOSC values toward hydroxyl are related to low molecular weight scavengers. This could be a sign of adaptation to pollution pulse typical of hydrothermal vents and prolonged oxidative stress may be responsible for profound alterations of cell physiology in these two sites.

Cytosolic SOD, GPx (superoxide anion and highly reactive hydroxyl radical production) and lipid peroxidation (hydroxyl radical production) is more significant at Lucky Strike (Bairro Alto) where levels of essential (Cu and Zn) and toxic metals (Cd and Ag) were highest (Table 2.1). Significant relationships

exist in mussel gills for SOD and Mn and between GPx (total and Se-dependent) and Zn and Cd. The high Cu concentrations in mussel gills from Lucky-Strike may, if not bound to proteins or enzymes, stimulate Fenton-type reactions and cause oxidative damage to lipids, proteins and nucleic acids via the generation of OH•. In laboratory experiments with *B. azoricus* exposed separately to Cd (0.9 µM) and Cu (0.4 µM), Cd inhibits SOD and increase lipid peroxidation (See Chapters 4 and 5; Company *et al.*, 2004). Moreover, Cd can compete with essential metals in protein binding leading to the release of Fe and Cu ions causing increase production of ROS and oxidative stress (Pruski & Dixon, 2002). Furthermore, Cu exposure decreases lipid peroxidation (See Chapter 5; Company *et al.*, 2004).

In the mussels from Rainbow, catalase activity (Figure 2.3) and lipid peroxidation (Figure 2.6) seem predominant. H_2O_2 enters the cells by diffusion, where it is detoxified by catalase activity, but the excess of Fe in mussels and in Rainbow fluid (the highest of all three vent sites) alter cell physiology by reacting with H_2O_2 in the Fenton reaction; consequently there is production of hydroxyl radicals that react with molecules in the cytosol and membranes causing LPO (Cavaletto *et al.*, 2002). Significant relationship between CAT and LPO with Fe were observed at this site. Therefore at Rainbow (the deepest vent site), catalase activity in mussel gills (Figure 2.3) is not enough to counteract the formation of OH• and the excess of Fe induced LPO. This interpretation is also supported by PCA results obtained for mussel gills (Figure 2.8).

MAR vent sites not only contain high metal concentrations when compared to contaminated coastal waters, but are also characterised by a mixture of toxic compounds (metals, hydrogen sulphide and radionuclide) that do play an important role in the antioxidant defence of marine organisms by scavenging free radicals. Hydrogen sulphide is known to react spontaneously with oxygen to produce ROS (Tapley *et al.*, 1999) and in vent mussels, hydrogen sulphide are oxidised to thiosulphate before being used by symbionts in the gills. Species like *B. azoricus* which not only live in a sulphide-rich environment but depend on intracellular sulphur-oxidising symbionts for their nutrition are thus at additional risk from free radical damage (Tapley *et al.*, 1999). Oxygen- and sulphur-

centred free radicals produced during sulphide oxidation and sulphur-amino acids may well act as antioxidants in these organisms. Large quantities of sulphur-amino acids (taurine, hypotaurine and thiotaurine) present in *Bathymodiolus* seem a common feature in deep-sea symbiotic organisms (Pruski *et al.*, 1997). The induction of oxidative stress by sulphur was confirmed in another chemosynthetic autotrophic bivalve species, the solemyid clam *Solemya reidi* (Tapley, 1993), which also contains sulphur oxidising endosymbionts. However, in *B. azoricus* this hypothesis needs to be confirmed. Furthermore, surprisingly high background levels of DNA damage were detected in gill cells and haemocytes in the Atlantic vent mussel *B. azoricus* from the same sites (Pruski & Dixon, 2003). Moreover, exposure of *B. azoricus* to H_2O_2, also induces the formation of DNA strand breaks (Pruski & Dixon, 2003) which suggests that this species was not fully resistant to oxidative stress. However, the results obtained in the present study, particularly for mussels from Menez-Gwen do not confirm these results. The decrease capability to neutralize these ROS enhanced genotoxic damage. Relationships between the diminished efficiency of antioxidant defences and increased susceptibility to genetic damage were confirmed in *M. galloprovincialis* from a Mediterranean lagoon (Frenzilli *et al.*, 2001).

2.6. Conclusions

In conclusion, three distinct pathways for antioxidant enzyme systems and lipid peroxidation based on environmental metal characteristics of MAR vent fields can be proposed for *Bathymodiolus* gills. In Menez-Gwen TOSC towards peroxyl and hydroxyl radicals and peroxynitrite are predominant, while at Lucky Strike cytosolic SOD activity and GPx are the main antioxidant mechanisms. Finally at Rainbow, catalase and lipid peroxidation are dominant. However, seasonal and size variability of these antioxidant systems and lipid peroxidation need to be considered to support this hypothesis.

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Chapter 3

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

3.1. Abstract

Hydrothermal vent mussels are exposed continually to several toxic compounds, including high metal concentrations from hydrothermal fluids, as well as other substances like dissolved sulphide, methane and natural radioactivity. Fluctuations in these parameters appear to be common due to normal and frequent instability of the hydrothermal environment. Seasonal 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 were evaluated and related to the accumulated metal concentrations (Ag, Cu, Cd, Fe, Mn and Zn) in their tissues. As a general trend, maximum antioxidant enzyme activities in the gills were detected in the beginning of summer, followed by a gradual decrease throughout the following months. This cyclical pattern was repeated the following year. Lipid peroxidation (LPO) in this tissue exhibited a similar seasonal variation trend. A different pattern of seasonal 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 seasonal 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 ROS production and that these tissues responded differently to the metal accumulation. However, seasonal variation in biomarkers of defence and damage were in general similar to coastal bivalve species and can be associated to seasonal variations of the physiological status due to reproduction. These seasonal variations might also be linked to the highly instable nature of the hydrothermal environment. Moreover, the antioxidant defence system in *B. azoricus* is independent of size, in agreement with other coastal mussels.

3.2. Introduction

The Mytilid mussel *Bathymodiolus azoricus* represents a key species in Mid-Atlantic Ridge (MAR) hydrothermal vents and are among the most common organisms in the Azores Triple Junction - Menez-Gwen, Lucky Strike and Rainbow. These mussels are highly abundant compared to other organisms such as crabs (*Segonzacia mesatlantica*) and shrimps (*Rimicaris exoculata*), 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 chemoautotrophic bacteria in the gills 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 (Cavanaugh, 1983; Fisher *et al.*, 1987; Le Pennec *et al.*, 1988; Kochevar *et al.*, 1992; Nelson *et al.*, 1995). Nevertheless, these mussels also filter the surrounding water that contains bacterioplankton (Utsumi *et al.*, 1994), holoplanktonic organisms (Berg & Van Dover, 1987; Wiebe *et al.*, 1988; Burd *et al.*, 1992; Burd & Thomson, 1994; Kaartvedt *et al.*, 1994; Burd & 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 & 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), Ag (4.3 nM), high temperatures (271-284 °C), low pH (4.4 - 4.5), high CO_2 (17-20 mmol Kg^{-1}) and H_2S levels (<1.5 mM) (Desbruyères *et al.*, 2001; Douville *et al.*, 2002) (See Chapter 1).

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. The former include superoxide anion radical ($O_2^{-\bullet}$), hydroxyl radical (OH•), peroxyl radical (ROO•), alkoxy radical (RO•) and

hydroperoxyl radical (HO_2•), and the latter include hydrogen peroxide (H_2O_2), hypochlorus acid (HOCl), singlet oxygen and peroxynitrite (HOONO) (Livingstone, 2001). Peroxynitrite formed from the reaction between superoxide and nitric oxide is a potent oxidant that can also contribute to cell injury. Peroxynitrite and its decomposition products induce peroxidation of lipids, cause DNA damage, deplete antioxidants and oxidise methionine and –SH residues in proteins (Darley-Usmar *et al.*, 1995; Reist *et al.*, 1998).

Organisms are able to deal with these radicals by several mechanisms, including the production of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and 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 & Gutteridge, 1984; Halliwell & 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 in the antioxidant enzyme levels and lipid peroxidation. Several studies with coastal bivalves, including mussels, *Mytilus galloprovincialis* (Viarengo *et al.*, 1990), *Mytilus edulis* (Géret *et al.*, 2002a) and *Unio tumidus* (Doyotte *et al.*, 1997), clams, *Ruditapes decussatus* (Géret *et al.*, 2002b) and oysters, *Crassostrea virginica* and *C. gigas* (Ringwood *et al.*, 1998; Géret *et al.*, 2002b), showed the influence of some metals (Cd, Cu, Zn, Ag, Hg) in the antioxidant defence system and/or lipid peroxidation levels.

The metal content in hydrothermal vent bivalves have been followed for several years, mainly in the clam *Calyptogena magnifica* (Roesijadi & Crecelius, 1984; Roesijadi *et al.*, 1985), and mussel *Bathymodiolus* sp. (Rousse *et al.*, 1998; Smith & Flegal, 1989), as well as metal detoxification mechanisms in vent organisms (Cosson-Mannevy *et al.*, 1988; Cosson & Vivier, 1995; Cosson, 1997; Géret *et al.*, 1998).

The relation between metals and 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 focused, as these proteins bind and sequester transition metal ions, like copper, which contain unpaired electrons and strongly accelerate free radical formation (Cui *et al.*, 2004), or scavenging directly oxyradicals like hydrogen peroxide (Anderson *et al.*, 1999). Earlier studies pointed out the fact that MT expression and ROS formation might be linked processes (Roesijadi *et al.*, 1997), and more recently evidences that oxidative stress induce MT-1 gene expression in mammals were obtained (Haq *et al.*, 2003).

Biochemical parameters in coastal species can be influenced by seasonal factors, including temperature, salinity, sunlight exposure, diet and gametogenesis (Cotelle & Férard, 1999; Livingstone *et al.*, 1990; Sheehan & Power, 1999; Sleiderink *et al.*, 1995). Thus, antioxidant enzymatic enhancement or depletion may be related to seasonal effects, whether exogenous or endogenous, as well as contaminant exposure, or interactions of both. Several studies point out that seasonal variations of antioxidant defences in bivalves are related to the reproductive cycle and food availability (Viarengo *et al.*, 1991; Solé *et al.*, 1995; Power & Sheenan, 1995, 1996; Cancio *et al.*, 1999; Regoli *et al.*, 2002).

No information is available about seasonality of antioxidant enzymes in hydrothermal vent species, and the possible parameters that might control those changes. The hydrothermal vent environment is highly variable and the instability can occur at various 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 due to their dependence of continuous geochemical-based energy (Dixon *et al.*, 2002). However, seasonal patterns of particle flux variations have been identified in the MAR hydrothermal region (Khripounoff *et al.*, 2000), and the

seasonality of reproductive cycle in vent bivalves has been recently focused (Tyler & Young, 1999; Le Pennec & Beninger, 2000).

The aim of the present work was to study the natural variability of antioxidant enzymes activity (SOD, CAT, Total-GPx and Se-GPx), metallothioneins (MT) concentrations and lipid peroxidation levels (LPO) in the gills and mantle of *B. azoricus* collected over a period of several months in Menez-Gwen vent site. The size effect was also considered to determine if differences in size affect these stress related biomarkers.

3.3. Materials and Methods

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 Victor6000 (IFREMER) in the Menez-Gwen hydrothermal vent field (37º 51'N, 32º 31'W, 850 m), located in the Mid-Atlantic Ridge (MAR) near the Azores Triple Junction (ATJ) (Figure 3.1A) (Dixon *et al.*, 2001). The cages were filled with approximately six hundred vent mussels (*Bathymodiolus azoricus*) each. Afterwards these cages were recovered periodically from July to November 2001 using the R/V Arquipélago by acoustically retrievable system, a fast recovery method (approximately 20 min to surface) and therefore less stressful compared with typical submersible recovery, which in some cases can take as long as 15 hours (Figure 3.1B) (Pruski & Dixon, 2003). The mussels were maintained in temperature-controlled tanks (9±1ºC) until arrival to the laboratory (LabHorta) in the University of Azores. One year after ATOS cruise, a new mussel sampling was carried out during SEAHMA cruise (July 29 – August 14, 2002) in the same period of the year to complete an annual cycle.

Additionally, *B. azoricus* collected from Menez-Gwen (ATOS 10) with different shell length were categorized as small (2.86 ± 0.06 cm) and large mussels (7.88

± 0.12 cm) in order to compare the levels of antioxidant enzymes in mussels of these two size classes.

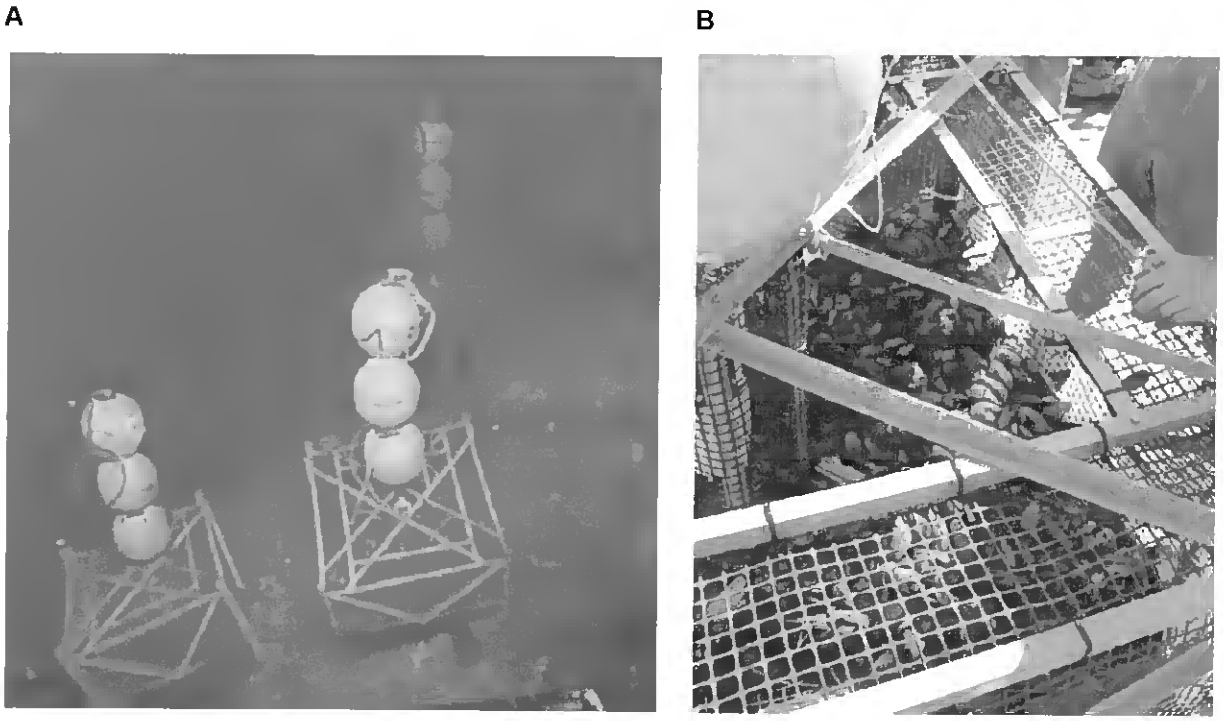

Figure 3.1 – Three cages placed on a mussel bed in Menez-Gwen vent field (A). One of the cages at the time of recovery on board of the R/V Arquipélago (B) (adapted from Dixon *et al.*, 2001).

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

Biochemical analysis

Symbiotic bacteria were not separated from the tissues, thus the enzymatic activity in tissues reflect both host and symbionts. The enzymatic activities of SOD, CAT, Total GPx and Se-GPx were determined in the gills and mantle after homogenisation in 20 mM Tris buffer pH 7.6, containing 1 mM EDTA, 0.5 M saccharose, 0.15 M KCl and 1 mM DTT as described in the previous chapter (See Chapter 2). The homogenates were centrifuged at 500 g for 15 min at 4°C. Supernatants were recentrifuged at 12,000 *g* for 45 min to precipitate the

mitochondrial fraction. SOD and CAT activities were measured in cytosolic and mitochondrial fractions. SOD activity was measured by the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm (McCord & Fridovich, 1969). CAT activity (sum of cytosolic and mitochondrial contributions) was determined by the decrease in absorbance at 240 nm due to H_2O_2 consumption (Greenwald, 1985). Total and Se-dependent GPx activities were determined exclusively in the cytosolic fraction, following the NADPH reduction at 340 nm in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (cumene hydroperoxide or H_2O_2 respectively) (Lawrence & Burk, 1976). The activities of antioxidant enzymes (SOD, CAT and GPx's) were expressed respectively as U mg^{-1}, mmol min^{-1} mg^{-1} and µmol min^{-1} mg^{-1} of total protein concentrations.

To determine MTs concentrations, the tissues of *B. azoricus* were homogenized at 4°C in a Tris buffer (100 mM), pH 8.1, containing 10 mM of β-mercaptoethanol. The soluble and insoluble fractions were separated by centrifugation (30000 *g*, 30 min, 4°C). Aliquots of the supernatants were heated (15 min, 95°C) and allowed to cool on ice. Heat-denatured proteins were separated from heat-stable proteins by centrifugation of the heated supernatants (10000 *g*, 15 min). Supernatants containing the heat-stable proteins, including MTs, were stored at -20°C until use. MT analyses from VENTOX Project samples was determined in the ISOMer Marine Biology Laboratory in the University of Nantes (France) by Dr. Richard Cosson with differential pulse polarography (DPP) using a PAR 394 analyser and an EG&G PAR 303A SMDE in accordance to the method of Olafson & Sim (1979) modified by Thompson & Cosson (1984). The samples from SEAHMA Project (2002) were homogenized over ice in 0.02 M Tris–HCl (pH 8.6). One aliquot (3 ml) was centrifuged at 30000 *g* for 45 min at 4°C. The cytosol was heat-treated at 80°C for 10 min and recentrifuged at 30000 *g* for 45 min at 4°C to precipitate the high molecular weight proteins that interfere in MT determinations. Quantification of MT concentrations in heat-treated cytosol was also determined by DPP following the method of Bebianno & Langston (1989). The standard addition method was used for calibration with rabbit liver MT (Fluka) in the

absence of *B. azoricus* MT standard. The levels of MTs are expressed as mg g^{-1} total proteins.

Total protein and lipid peroxidation levels were determined in the gills and mantle of vent mussels after homogenisation in 20 mM Tris buffer pH 8.6 containing 150 mM NaCl. The homogenates were centrifuged for 15 min at 6,000 *g* at 4°C. Total protein concentrations in the supernatants were determined according to Lowry *et al.* (1951) and expressed in mg g^{-1} wet weight. Lipid peroxidation in the cytosol was evaluated in terms of production of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) due to decomposition of polyunsaturated fatty acids (Erdelmeier *et al.*, 1998). Lipid peroxidation was expressed as nmol of MDA and 4-HNE g^{-1} of total protein concentrations.

Metal analysis

Both essential (Cu, Fe, Mn and Zn) and non-essential metals (Ag and Cd) in *B. azoricus* from the VENTOX Project (mussels collected with retrievable cages during 2001) were analysed, as mentioned in Chapter 2, by Lic. Inês Martins in the University of Paris IV (France) under the supervision of Dr. Jacques Boulègue and Dr. Aline Fiala-Médioni (Fiala-Médioni *et al.*, in prep). The same metals were determined in the gills and mantle of mussels collected in 2002 during the SEAHMA Project, on HNO_3 digests of the homogenate using flame atomic absorption spectrophotometry (FAAS). Metal concentrations were expressed as µg g^{-1} dry weight tissue.

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.

Principal component analysis (PCA) was used to discriminate the different sampling months in both tissues of *B. azoricus*. 13 variables were taken into consideration: the concentrations of Ag, Cd, Cu, Fe, Mn and Zn, the activities of cytosolic SOD, mitochondrial SOD, CAT, TGPx, Se-GPx and the levels of MT and LPO.

3.4. Results

3.4.1. Seasonal variation of antioxidant enzymes in *B. azoricus*

Figure 3.2 shows the seasonal variability of enzyme activity of SOD, CAT, GPx, in the gills of *B. azoricus*. As can be seen from the figure, seasonal changes were observed for all antioxidant enzyme activities in the gills of *B. azoricus*.

SOD was predominantly found in the cytosolic fraction (> 75%) of mussel gills. The activity of this enzyme increased linearly from July to the beginning of September (0.401 U mg^{-1} protein d^{-1}, r = 0.874, p<0.05) where a maximum activity of cytosolic SOD was found (36.4 ± 5.7 U mg^{-1} protein) (p<0.05) and significantly decreased until November (14.9 ± 1.32 U mg^{-1} protein). SOD activity in the mussels collected during SEAHMA cruise in July 2002 was similar to those found in the previous year (16.1 ± 5.2 U mg^{-1} protein) (Figure 3.2A).

Similarly, the mitochondrial SOD exhibited a maximum activity in September as well as in June (6.9 ± 3.5 U mg^{-1} protein) (p<0.05) compared with the remaining months, with a minimum SOD activity in the mussels collected during SEAHMA cruise in July 2002 (1.60 ± 0.29 U mg^{-1} protein) (p<0.05) significantly different from the SOD activity observed in July from the previous year (Figure 3.2B).

CAT activity increase from June to July (0.023 ± 0.005 mmol min^{-1} mg^{-1} protein) (p<0.05), followed by a rapid decrease in August (0.013 ± 0.004 mmol min^{-1} mg^{-1} protein). From August to November, CAT activity increased linearly (0.0001 mmol min^{-1} mg^{-1} protein d^{-1}, r = 0.995, p<0.05). In the mussels collected in November 2001, as well as in July 2002, CAT activity was close to the values reported in July 2001 (0.023 ± 0.002 mmol min^{-1} mg^{-1} protein) (Figure 3.2C).

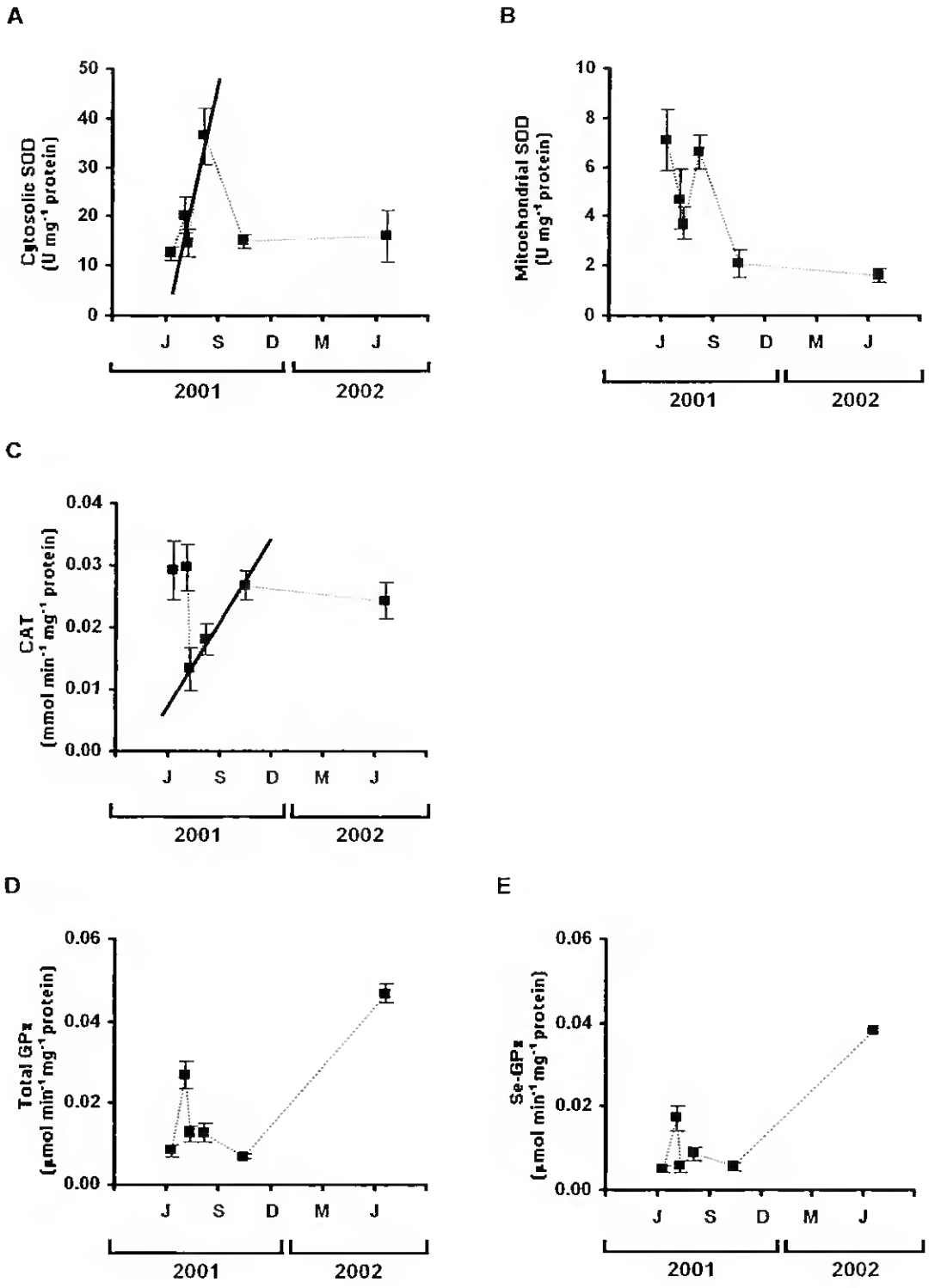

Figure 3.2 – Seasonal variation (Mean ± SD) of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills of *B. azoricus* from Menez-Gwen vent field (n = 10).

Both total GPx and Se-GPx activities in the gills of *B. azoricus* exhibited a similar seasonal variability with a rapid increase from June to July were activity of these enzymes were maximum ($p<0.05$), followed by a decrease throughout the summer period. For total GPx this inhibition was exponential (Total GPx [µmol min^{-1} mg^{-1} protein] = $0.02e^{-0.016t\ [days]}$, $r = 0.872$, $p<0.05$). Surprisingly the activities of both enzymes in the mussels collected in 2002 were 3 to 5 fold-higher than those reported in the gills of mussels from the previous year (Figure 3.2D and E).

The seasonal variability of MT and LPO levels in the gills of *B. azoricus* is presented in Figure 3.3.

LPO levels in the gills also followed the same pattern of GPx (Total and Se-dependent) with significantly higher levels in July (279.61 ± 43.71 nmol g^{-1} protein) ($p<0.05$), when a maximum activity of total and selenium dependent GPx was also reported. During the other months, LPO remained unchanged (127.88 ± 21.27 nmol g^{-1} protein) and after a year period LPO levels were similar (Figure 3.3A).

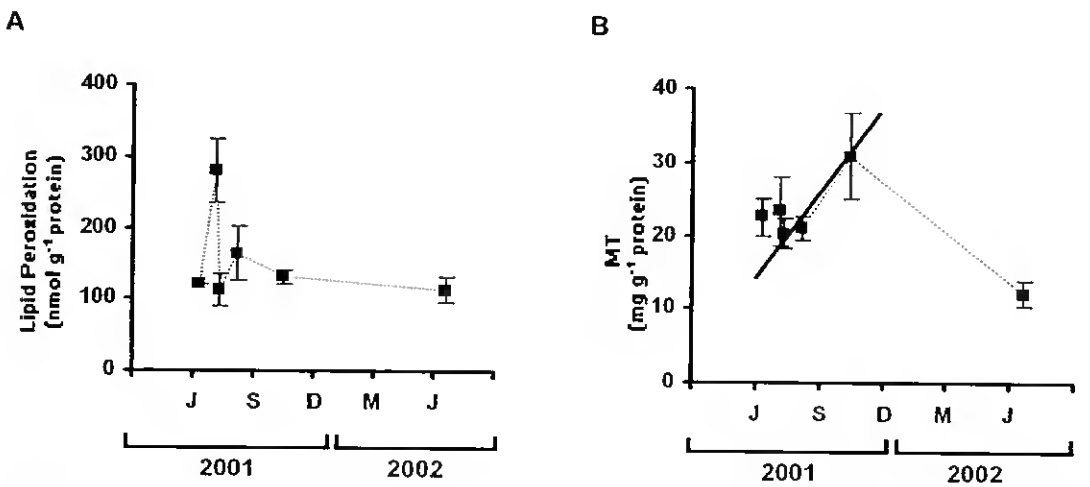

Figure 3.3 – Seasonal variation (Mean ± SD) of LPO (A) and MT concentrations (B) in the gills of *B. azoricus* from Menez-Gwen vent field ($n = 10$).

MT concentrations in the gills remained unchanged from June to July (22.92 ± 3.62 mg g^{-1} protein) and increased linearly from August to November (0.119 mg

g^{-1} protein d^{-1}, r = 0.983, p<0.05). The mussels collected in the following year, exhibited significantly lower MT concentrations (12.02 ± 1.74 mg g^{-1} protein) compared to equal period of 2001 (Figure 3.3B).

In the gills among all antioxidant enzyme activities, LPO and MT, the increase of total and Se-GPx activities were directly related with the increase of LPO levels (Total GPx = 0.0001 [LPO] – 0.004, r = 0.937, p<0.05 and Se-GPx = $7x10^{-5}$ [LPO] – 0.004, r = 0.994, p<0.05) (Figure 3.4).

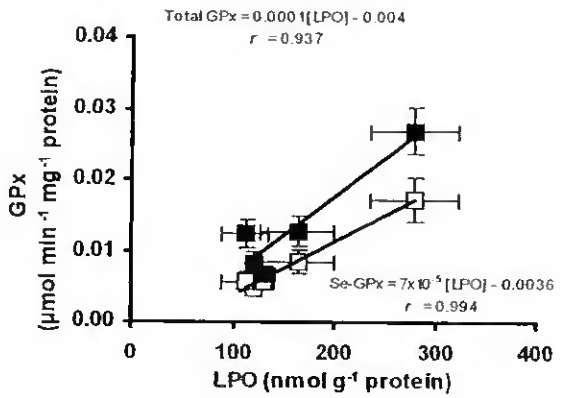

Figure 3.4 – Relationship between GPx activities and LPO in the gills of *B. azoricus*.

The seasonal variability of antioxidant enzyme activities in the mantle of *B. azoricus* is presented in Figure 3.5. As for the gills this tissue also presented seasonal variations although less pronounced in some cases than in the gills.

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 some exceptions (TGPx and Se-GPx were significantly higher in the gills in November 2001 and July 2002) (p<0.05).

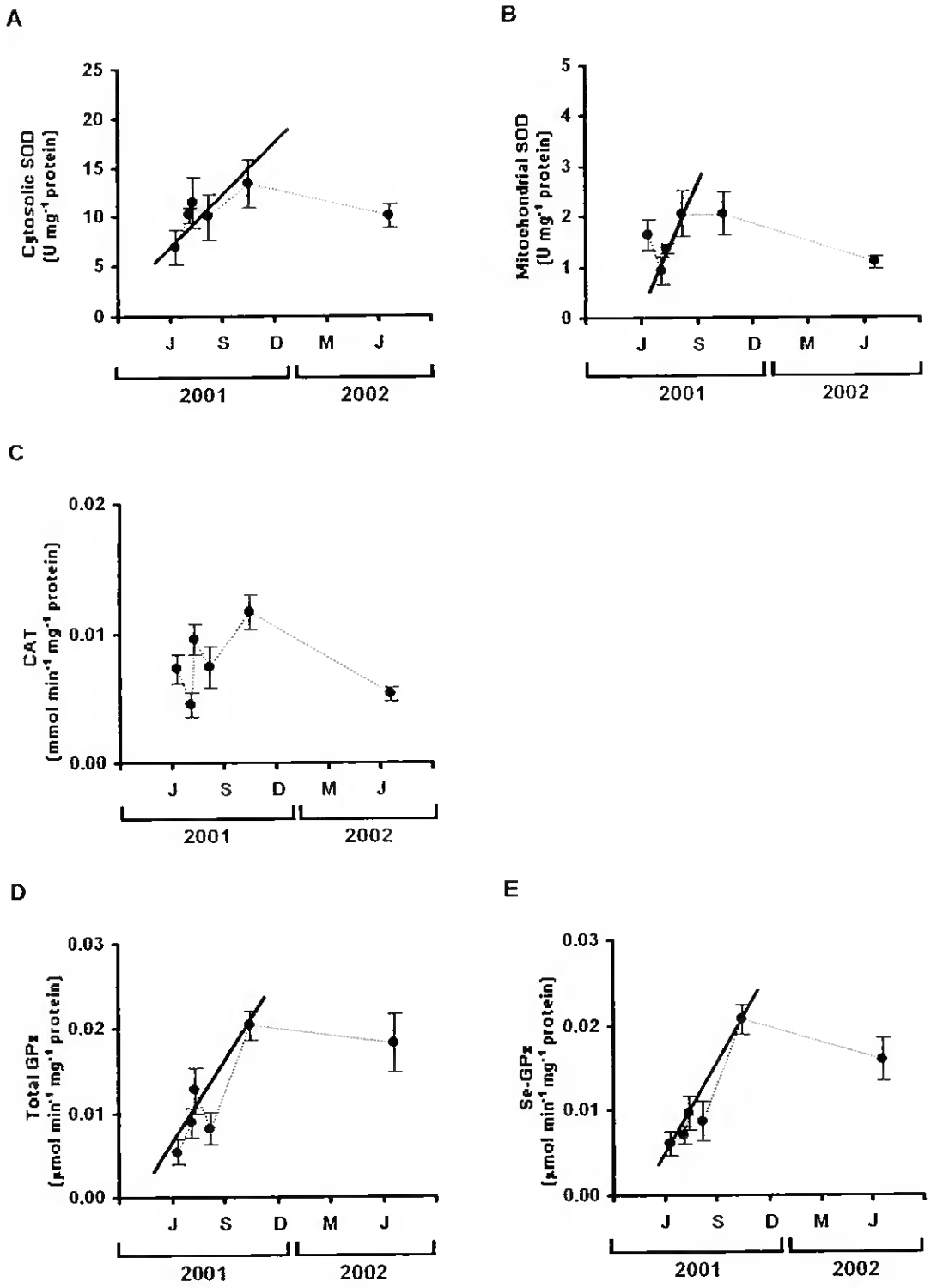

Figure 3.5 – Seasonal variation (Mean ± SD) of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the mantle of *B. azoricus* from Menez-Gwen vent field (n = 10).

The seasonal pattern of the activity of CAT in the mantle was similar to what was observed for SOD, with an increase from July to November although not linear as observed for SOD. The maximum activity of CAT in the mantle was in November (0.01 ± 0.001 mmol min^{-1} mg^{-1} protein) ($p<0.05$) and recovering in 2002 to the values observed in the previous year (Figure 3.5C).

Contrarily 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 (Total GPx [mmol min^{-1} mg^{-1} protein] = 0.0001t [days] – 4.11, $r = 0.893$, $p<0.05$; Se-GPx [mmol min^{-1} mg^{-1} protein] = 0.0001t [days] – 4.47, $r = 0.957$, $p<0.05$) and remained unchanged until July 2002 (Figures 3.5D and E).

The seasonal variability of MT and LPO levels in the mantle of *B. azoricus* is presented in Figure 3.6.

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 in the end of July (116.63 ± 9.63 mg g^{-1}) ($p<0.05$) and showed a marked seasonal variability (Figure 3.6A).

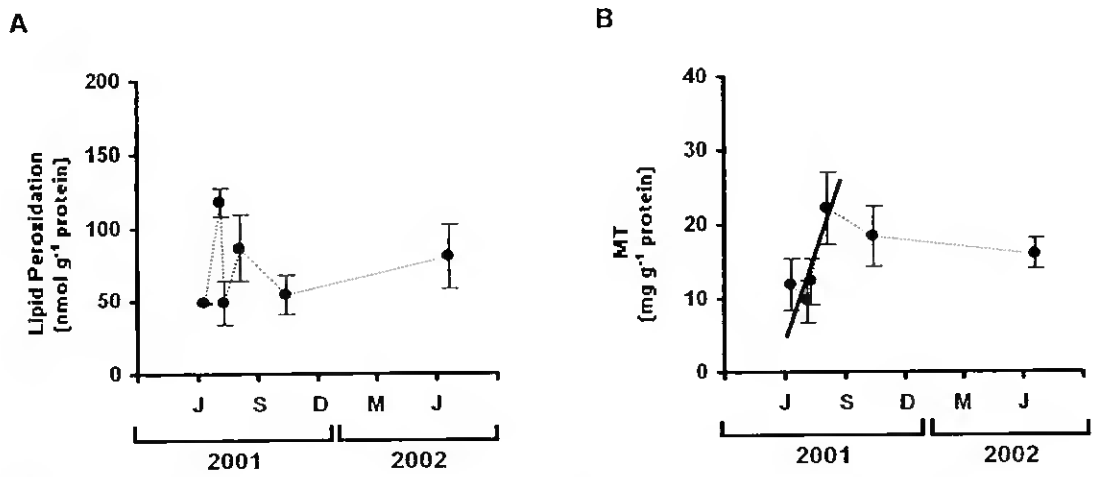

Figure 3.6 – Seasonal variation (Mean ± SD) of LPO (A) and MT concentrations (B) in the mantle of *B. azoricus* from Menez-Gwen vent field ($n = 10$).

MT concentrations in the mantle were approximately 2-fold lower than in the gills. MT levels, unlike 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 (Figure 3.6B).

3.4.2. Relationship between metal concentrations and antioxidant parameters

The total concentration and subcellular distribution of essential (Cu, Fe, Mn and Zn) and non-essential metals (Ag and Cd) in the gills and mantle *B. azoricus* obtained during the ATOS cruise (2001) and SEAHMA cruise (2002) are presented in Annexe I (Adapted from Fiala-Médioni *et al.*, in prep). The gills exhibited always significantly higher metal concentrations than the mantle throughout the months (p<0.05).

Most of the Cu in the gills and mantle is in the insoluble fraction (50-90%) and decrease during the seasonal period especially from July to August. Fe is also present mainly in the insoluble fraction in the gills (71-82%) and mantle (50-92%). Fe concentrations also decrease from July to August in both tissues. After this period, the concentration of Fe remained unchanged in the gills, while in the mantle they increased until July 2002. Subcellular distribution of Mn shows that this metal is contained predominantly in the insoluble fraction in both gills (62-68%) and mantle (53-71%). The two tissues shows similar accumulation pattern to that of Fe. The concentration of this metal decreased in the gills, while in the mantle, Mn levels decrease initially from July to August and then increase significantly until July 2002. Zn concentrations in the gills, contrarily to what was observed for the other metals, are mainly in the soluble fraction (cytosol) (56-64%). However in the mantle, Zn was found predominantly in the insoluble fraction (77-89%) (Annexe I).

The non-essential metals followed, in both tissues, a different seasonal pattern compared to the essential ones. Ag subcellular distribution showed that this metal is mainly accumulated in the soluble fraction of gills (54-86%), whereas in the mantle is found principally in the pellet (insoluble fraction) (50-72%). Ag

concentrations in the gills increased along the summer months, with a peak registered in August, followed by a decline until November and remained unchanged in the mussels collected in July 2002. Also in the mantle, Ag concentrations increased during the summer period, with a maximum concentration in September. Afterwards, the concentration of this metal strongly decreases in November. Ag levels found in the mantle of mussels collected in July 2002 are similar to that reported in the previous year (Annexe I).

A very analogous seasonal pattern was found for Cd levels. Cd was mainly found in the soluble fraction of gills (50-57%) and in the insoluble fraction of mantle (50-87%). The concentration of this metal in the gills increased significantly from July to September, with a rapid decline in November. Cd levels increased again in the gills of mussels collected in July 2002. In the mantle, Cd concentrations also increased in the beginning of summer with a maximum registered in 31 July. After this period a gradual decline was observed in Cd concentrations in this tissue until November. The mussels collected in July 2002, presented similar Cd levels to those sampled in the previous year (Annexe I).

Statistical analysis revealed that some of the metals are positively related with antioxidant enzymes in the gills and negatively related in the mantle. These relationships are presented in Figures 3.7 to 3.10.

In the gill tissue cytosolic SOD activity increase exponentially with the increase of Ag concentrations in the soluble fraction (SOD Cyt = $7.89e^{0.28[\text{Soluble Ag}]}$, $r = 0.794$, $p<0.05$) (Figure 3.7A). Moreover, mitochondrial SOD activity also increase with the enhancement of soluble Ag concentrations in the gill tissues but in this case the relationship was linear (SOD Mit = 2.40 [Soluble Ag] – 4.04, $r = 0.979$, $p<0.05$) (Figure 3.7B).

Similarly, for the essential metals, mitochondrial SOD activity also increases significantly with the accumulation increase of soluble Cu (SOD Mit = 0.10 [Soluble Cu] – 10.0, $r = 0.877$, $p<0.05$) and total Cu (SOD Mit = 0.10 [Total Cu] –

1.31, $r = 0.800$, $p<0.05$) in the gills, with the same induction rate ($p>0.05$) (Figure 3.7C).

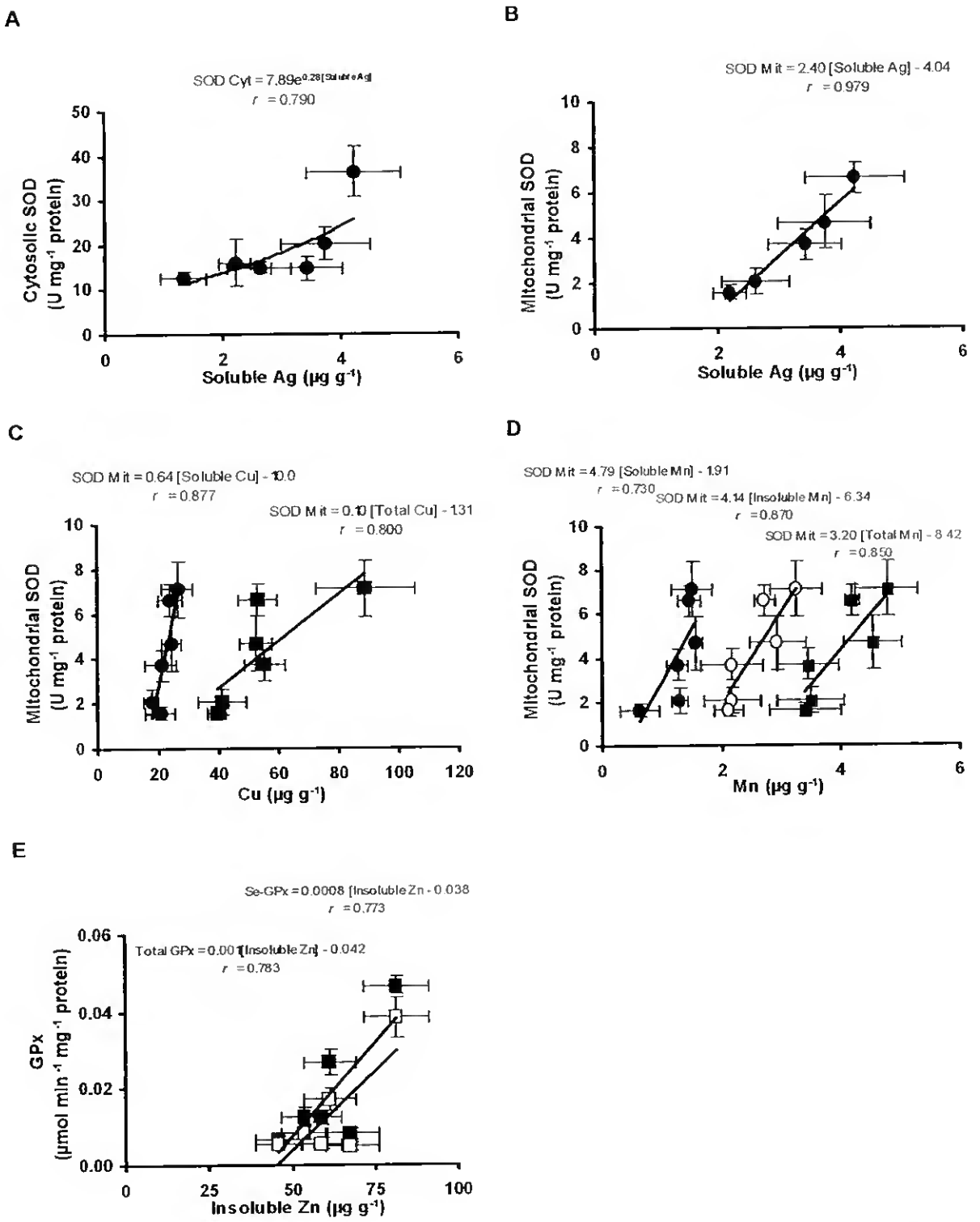

Figure 3.7 – Relationships between cytosolic SOD and soluble Ag (A), mitochondrial SOD with soluble Ag (B), Cu (C), Mn (D) and GPx with insoluble Zn (E) in the gills of *B. azoricus*.

Moreover, mitochondrial SOD, a Mn containing enzyme is also linearly induced with the increase of Mn concentrations either total or in both subcellular fractions (soluble and insoluble) accumulated 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, $r = 0.870$, $p<0.05$) (Figure 3.7D). In this case mitochondrial SOD is induced more rapidly by the increasing concentrations of soluble Mn, followed by insoluble Mn and last by total Mn concentrations ($p<0.05$).

Furthermore, both total and Se-GPx activities in the gills also increase linearly with the accumulated Zn 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; $r = 0.773$; $p<0.05$) (Figure 3.7F) with the same induction rate ($p>0.05$).

In the mantle, in contrast with the gills, the activity of some of these antioxidant enzymes were significantly inhibited by the increase of some of the metals accumulated (Cd, Cu, Mn and Zn) in this tissue (Figures 3.8 and 3.9).

Thus, CAT is linearly inhibited by the increment of total and insoluble Zn concentrations in this tissue (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 the same inhibition rate ($p>0.05$) (Figure 3.8A).

Similarly total GPx is linearly inhibited by the increase in Zn concentrations in the insoluble fraction (Total GPx = -0.0004 [Insoluble Zn] + 0.03, $r = 0.823$, $p<0.05$), as well as Mn in the soluble fraction (Total GPx = -0.029 [Soluble Mn] + 0.03; $r = 0.868$; $p<0.05$) (Figures 3.8B and C).

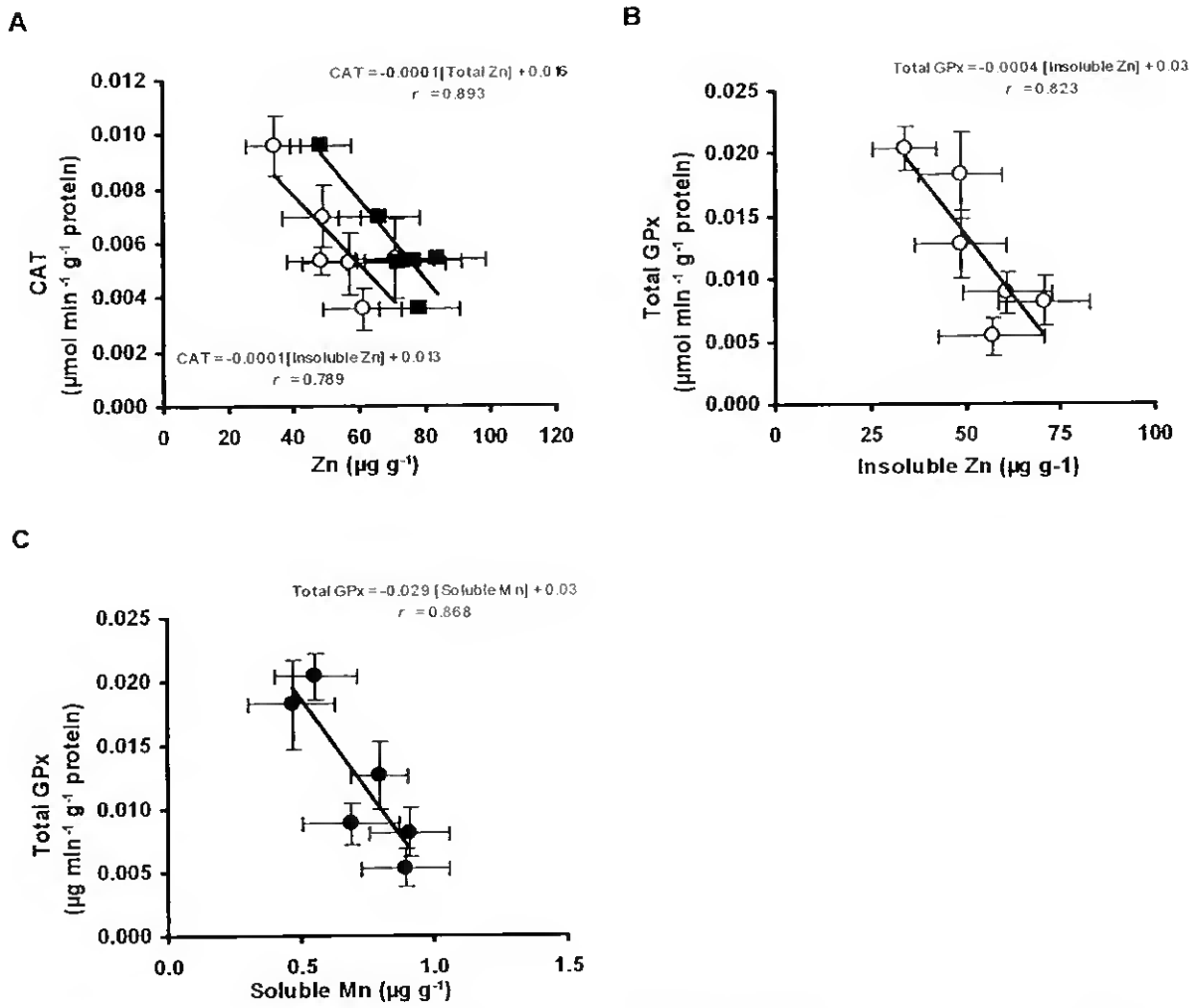

Figure 3.8 – Relationships between CAT activity with total and insoluble Zn (A) and between total GPx with insoluble Zn (B) and soluble Mn (C) in the mantle of *B. azoricus*.

Furthermore, the increase of total Cd concentrations also inhibited linearly both CAT and mitochondrial SOD activity in the mantle (CAT = -0.012 [Total Cd] + 0.001, r = 0.814, p<0.05; SOD Mit = -3.32 [Total Cd] + 2.51, r = 0.939, p<0.05) (Figures 3.9A and B), while this inhibition is exponential between Se-GPx and insoluble Cd (Se-GPx = $0.019e^{-3.14[\text{Insoluble Cd}]}$, r = 0.809, p<0.05) (Figure 3.9C).

However, changes in metal concentrations in both gills and mantle of *B. azoricus* had no effect on LPO or MT levels (p>0.05).

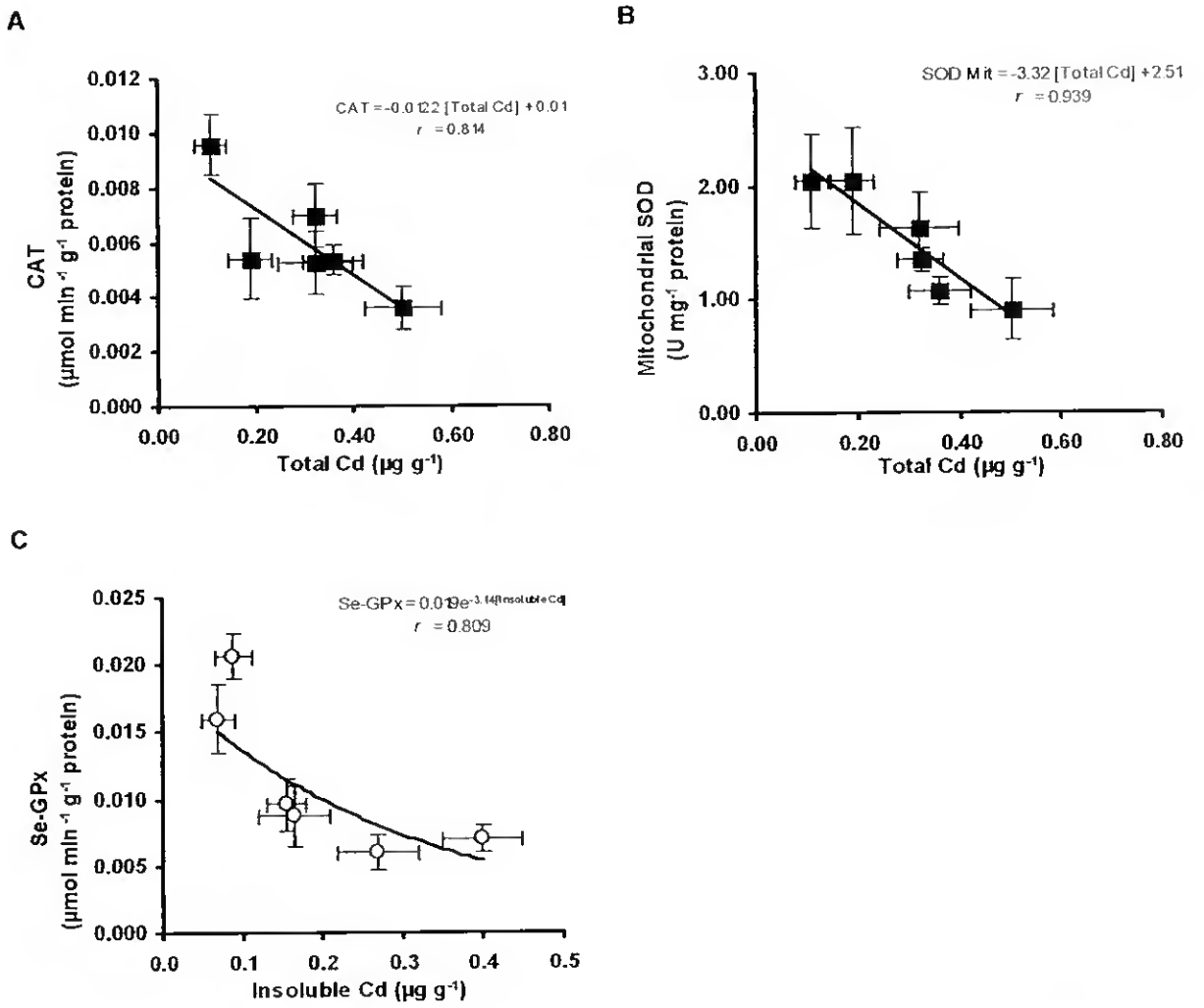

Figure 3.9 – Relationships between CAT and total Cd (A), mitochondrial SOD and total Cd (B) and Se-GPx and insoluble Cd (C) in the mantle of *B. azoricus*.

Principal component analysis (PCA) performed on untransformed antioxidant enzymatic activities, LPO, MT and metal concentrations in the gills and mantle of mussels collected both in cruises (ATOS and SEAHMA) and from cage recovery are presented in Figures 3.10-13. Significant variables in PC1 and PC2 for gills and mantle are showed in Table 3.1.

PCA in the gills shows that PC1 and PC2 explain 60% of total variance of the data (32% for the first principal component and 28% for the second principal component) (Figure 3.10).

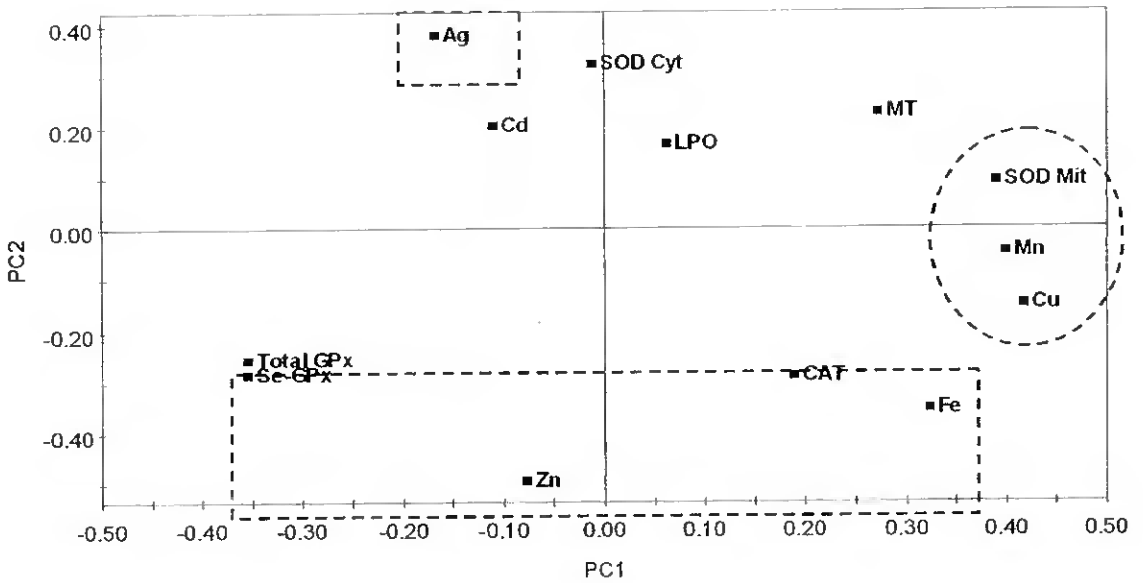

Figure 3.10 – PCA of the antioxidant enzymes activity, LPO, MT and total metal concentrations in the gills of *B. azoricus* showing the loadings of the variables on PC1 and PC2. Significant variables in PC1 are grouped with circles while those significant in PC2 are grouped with rectangles.

Mitochondrial SOD activity, Cu and Mn concentrations are the only significant variables on PC1 as observed in Table 3.1 (significant values higher than 0.226 limit). It was been previously observed that these variables are related as mitochondrial SOD is linearly induced by the increase of these metals, suggesting that Cu and Mn increase the production of superoxide radical, inducing SOD as an antioxidant mechanism with subsequent production of hydrogen peroxide. The PCA on the data scores shows the samples from ATOS cruise (July 2001) separated from the other sampling months in PC1 (Figure 3.11). In fact, the concentrations of both Cu and Mn are higher in July 2001 when compared with the other sampling months (Annexe I) and mitochondrial SOD also exhibited a maximum of activity in this month (Figure 3.2B).

Table 3.1 – Principal component model for significance level of variables for PC1 and PC2.

Variables	PC1	PC2	PC1	PC2
	Gills		Mantle	
Ag	-1.179	0.285 **	-1.042	0.306
Cd	-0.060	-0.704	0.399 *	-0.093
Cu	0.238 *	0.067	0.243	-0.912
Fe	-0.107	0.669 **	0.396	0.270
Mn	0.381 *	-0.266	-0.815	0.223
Zn	-1.803	0.564 **	0.071	0.619
SOD Cyt	-0.530	-0.319	-0.063	-0.668
SOD Myt	0.407 *	-0.598	-0.023	-1.142
CAT	-0.368	0.271 **	0.417 *	0.889
Total GPx	-0.227	0.190	0.270 *	-0.927
Se-GPx	-0.348	0.271 **	0.423 *	-0.484
MT	-0.403	-0.374	0.046	-0.576
LPO	-0.274	-0.154	-0.212	-0.788

Significant values when higher than significance limits of 0.226 in PC1 (*) and 0.262 in PC2 (**)

The concentrations of Ag, Fe and Zn as well as CAT and Se-GPx activities are significant in PC2 (significant values higher than 0.262 limit) (Table 3.1). These variables are responsible for the separation in the PC2 of the samples collected in both July 2001 and July 2002 (ATOS and SEAHMA cruises) with those collected in the other months (Figure 3.11).

Thus, PCA on the data scores shows that, in PC1 only the samples from ATOS cruise (July 2001) are distinct from the other sampling months (SEAHMA cruise (July 2002) and cages), which is explained by the concentrations of essential metals Cu and Mn and the activity of mitochondrial SOD. However, in PC2 the samples from both cruises (ATOS and SEAHMA) are considered distinct from the samples collected by acoustic release cages (Figure 3.11).

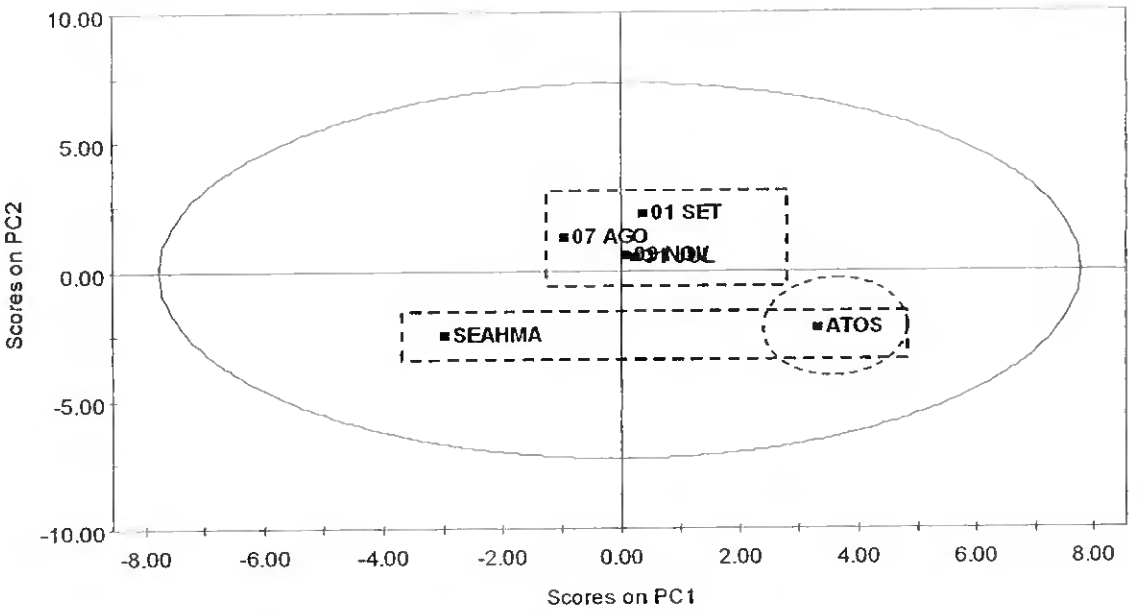

Figure 3.11 - PCA of the antioxidant enzymes activity, LPO, MT and total metal concentrations in the gills of *B. azoricus* showing the data scores labelled as cages/cruises. Months are separated by the significant variables in PC1 (circles) and PC2 (rectangles).

PCA in the mantle shows that PC1 and PC2 explain 61% of total variance of the data (41% for the first principal component and 20% for the second principal component) (Figure 3.12).

In this tissue, the activities of CAT, total and Se-GPx (positive axis) and Cd (negative axis) are significant in PC1 (Figure 3.12) (significant values higher than 0.262 limit) (Table 3.1). This confirms the inhibition of CAT and Se-GPx enzymatic activities by the increase of Cd concentrations previously observed (Figures 3.9A and C). Moreover, the activities of these three enzymes are responsible for the separation between the samples collected in November and in the other months observed in PCA on the data scores, while Cd concentration is responsible by the separation of the samples from July 2001 (Figure 3.13).

Contrarily to what was observed in the gills, no variables were found to be significant in PC2 (Table 3.1).

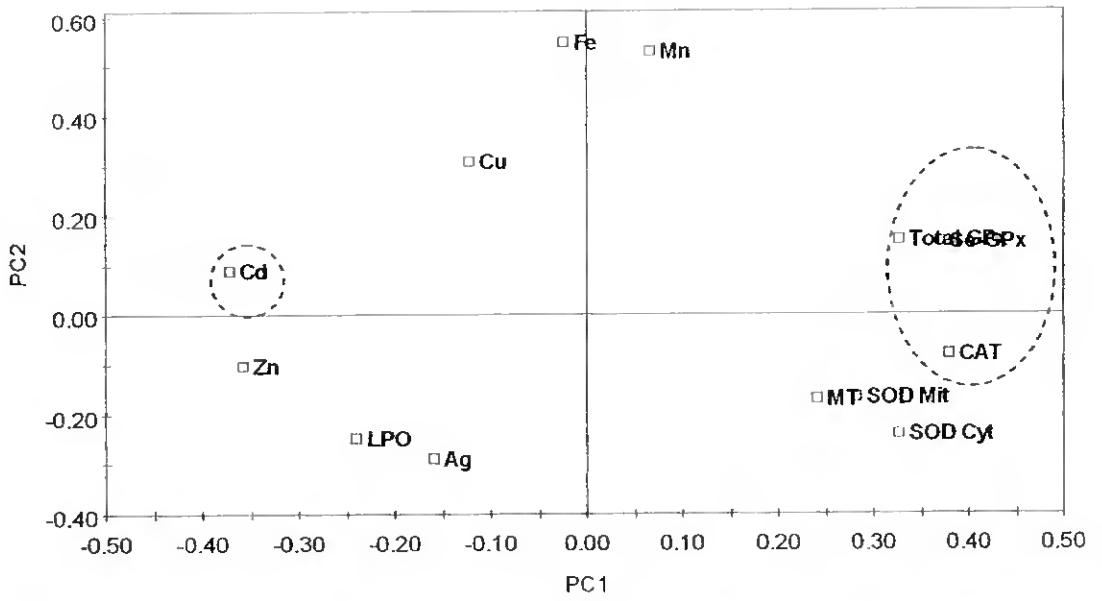

Figure 3.12 - PCA of the antioxidant enzymes activity, LPO, MT and total metal concentrations in the mantle of *B. azoricus* showing the loadings of the variables on PC1 and PC2. Significant variables in PC1 are grouped with circles.

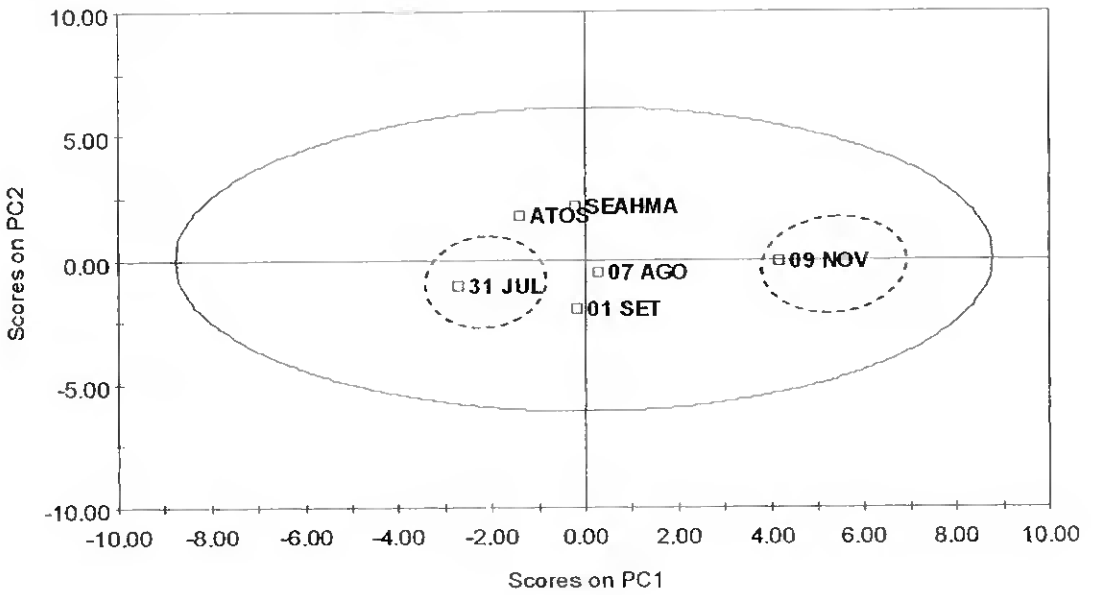

Figure 3.13 - PCA of the antioxidant enzymes activity, LPO, MT and total metal concentrations in the mantle of *B. azoricus* showing the data scores labelled as cages/cruises. Months are separated by the significant variables in PC1 (circles).

3.4.3. Variation of antioxidant enzymes and lipid peroxidation with size of *B. azoricus*

Table 3.2 presents a comparison of antioxidant enzymes activities and lipid peroxidation levels between *B. azoricus* from two size classes 2.86 ± 0.06 cm and 7.88 ± 0.12 cm respectively.

Table 3.2 – Antioxidant enzymes activities and lipid peroxidation levels (Mean ± SD) in small (2.86 ± 0.06 cm) and large (7.88 ± 0.12 cm) *B. azoricus* from Menez-Gwen vent site. The symbol (*) represents significant differences between the two size classes for each tissue (n = 10).

	Gills		Mantle	
	Small	Large	Small	Large
SOD Cyt	12.5 ± 1.04	13.3 ± 2.89	5.92 ± 1.14	6.57 ± 1.60
SOD Mit	3.87 ± 0.87	6.90 ± 1.14 *	0.48 ± 0.12	1.84 ± 0.31 *
CAT	0.023 ± 0.007	0.022 ± 0.006	0.008 ± 0.001	0.008 ± 0.001
Total GPx	0.006 ± 0.001	0.007 ± 0.001	0.010 ± 0.002	0.013 ± 0.001
Se-GPx	0.005 ± 0.001	0.006 ± 0.001	0.011 ± 0.003	0.013 ± 0.002
LPO	84.1 ± 19.2	72.8 ± 13.9 *	66.0 ± 5.9	42.2 ± 5.1 *

As a general trend, antioxidant enzymatic activities are similar between small and large mussels, with exception of the mitochondrial SOD and LPO that were significantly higher in large mussels in both tissues (p<0.05). Therefore, with the exception of SOD in the mitochondria, levels of these antioxidant enzymes are independent of size.

3.5. Discussion

The traditional methods for seasonal studies are compromised at hydrothermal vents due to technical constraints of sampling in these environments. Analyses of time-series from the deep-sea organisms are rare and have often been accomplished by sampling over many years and integrating data from different years (Gage & Tyler, 1991). Because of the difficulty of sampling in the deep sea in general and at hydrothermal vents in particular, the knowledge has to be

extrapolated from a limited number of data and by comparison with known established patterns (Tyler & Young, 1999).

The acoustically retrievable cages used in this study represent a useful and innovative approach that proved to be very promising for periodic sampling of sessile deep-sea organisms and consequently to monitor biochemical parameters over a wide period of time (Dixon *et al.*, 2001). 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 due to technical difficulties in cage recovery under adverse weather conditions.

The knowledge of antioxidant systems in hydrothermal vent organisms is still scarce. Earlier studies had put on evidence the presence of these enzymes (CAT, SOD and GPx) in two important vent organisms, the tubeworm *Riftia pachyptila* and the clam *Calyptogena magnifica* (Blum & Fridovich, 1984). Only recently antioxidant enzymes were determined in the vent mussel *B. azoricus* from different Azores hydrothermal vent sites (See Chapter 2; Bebianno *et al.*, submitted). Surprisingly the levels of antioxidant enzymes found in these mussels were of the same order of magnitude to those reported in coastal mussels like *M. galloprovincialis* (Table 1.7; Chapter 1). Considering that vent fluids are loaded with potentially toxic chemical species and the organisms exposed to extreme temperatures, pH and pressures, other levels of oxidative stress biomarkers would be expected. In this research, antioxidant enzymatic activities, LPO and MT concentrations have also been periodically analysed in two tissues of *B. azoricus*, gills and mantle. Seasonal changes in metal content of both tissues were followed as well.

From the results obtained, it is clear that both gills and mantle have significant seasonal variations of all the parameters mentioned above and in particular antioxidant enzymes at least during summer and autumn months, nevertheless, the two tissues exhibit different patterns of variability.

In the gills, SOD, CAT and GPx´s activities are higher in the summer (Figure 3.2), which also corresponds to a maximum of LPO levels (Figure 3.3A). In fact, levels of MDA and 4-HNE compounds are 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 tissue 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 (Annexe I).

In the mantle, a different seasonal pattern was observed, with a gradual increase of antioxidant enzyme activities from July to November, when a maximum for SOD, CAT and GPx´s was registered (Figure 3.5). In this tissue, similarly to what was observed in the gills, a maximum of MDA and 4-HNE levels occurred in July, but in this case the activities of the antioxidant enzymes were lower. Moreover, the variation of the metal content in this tissue was not significant during this period (Annexe I) which suggests that damage in the lipid membranes are probably related to other factors capable of inducing oxidative stress than metals, such as hydrogen sulphide.

Many aspects of the biochemistry of marine coastal bivalves are under marked seasonal control, and probably reflect variations in environmental conditions like temperature, changes in food availability and also endogenous factors like reproductive status and fluctuations in the physiological condition of the organisms. In mussels, *M. edulis* (Viarengo *et al.*, 1991; Power & Sheehan, 1996; Sheehan & 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 coastal mussels, despite some small differences, have a similar pattern, where the highest antioxidant activities correspond to spring-summer months, and lower levels occur during autumn-winter. One exception of 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 reaching a minimum in June (Solé *et al.*, 1995).

The seasonal variation of antioxidant enzymes for *B. azoricus* is thus in agreement with the majority of seasonal studies in coastal mussel species, even though the seasonality 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 & 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 & Young, 1999). However, with the increase of knowledge about these species it was concluded that *B. azoricus* have 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 seasonal 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). This information may contribute to explain the seasonal variation in antioxidant enzymes in this species. If *B. azoricus* exhibit a specific reproductive period, contrarily to a continuous reproduction during all year, this clearly indicates that, like their coastal counterparts, the physiological status of the organisms also fluctuate seasonally and consequently the susceptibility to oxidative stress and antioxidant defences display seasonal changes.

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 three mechanisms: (1) eruption of vent fluids that precipitate chemicals (specially metals) as fine particles and are carried by water currents and hydrothermal plume; (2) direct erosion of the chimneys by biological activities and/or mechanical erosion by the current; (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 areas 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* in this study.

In the gills, Ag in the soluble fraction (that represent the most part of the Ag in this tissue, 54-86%), induce both cytosolic and mitochondrial SOD activity (Figures 3.7A and B). 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 ($O_2^{-\bullet}$) with subsequent induction of Cu/Zn-SOD (cytosol) and Mn-SOD (mitochondria matrix) to detoxify this radical into hydrogen peroxide. Evidence that Ag can impair Cu/Zn-SOD function was detected in the yeast *Saccharomyces cerevisiae*, with a substitution of Cu by Ag in the metal active site (Ciriolo *et al.*, 1994).

Mitochondrial SOD activity in the gills of *B. azoricus* was also induced by the increase of essential metals. SOD present in the mitochondria uses Mn as metal cofactor (Mn-SOD) and was linearly induced by the increase of Mn concentrations (total and both subcellular fractions) in the gills of *B. azoricus* (Figure 3.7D). Mn is an essential metal and therefore required in small amounts by the organisms and is found in many enzymes, including glutamine synthetase, alkaline phosphatase, and arginase besides Mn-SOD (Zhang *et al.*, 2003). At low concentrations, Mn^{2+} can have a protective effect by reducing the highly reactive OH• radical to yield $Mn(OH)^{2+}$ (Chang & 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 Mn^{2+} and Mn^{3+} forms (Ali *et al.*, 1995; Soliman *et al.*, 1995). The levels of this metal in MAR hydrothermal vent environments (59 – 2250 µM) are extremely high when compared to the average seawater (0.0013 µM) (Table 1.1; Chapter 1) and therefore the accumulation of Mn by *B. azoricus* can largely exceed the minimum required amounts for normal cellular function. In *B. azoricus* Mn seems able to enhance superoxide radical production in the gills, since mitochondrial SOD is linearly induced by this metal either in the soluble and soluble compartments.

The increase of total and soluble Cu (29-47% of the total Cu accumulated) concentrations in the gills of *B. azoricus* also induced mitochondrial SOD (Figure 3.7C). Although Cu is an essential metal to the organisms 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 & Slater, 1993). The reduction of Cu^{2+} to Cu^{+} that occur in presence of superoxide radical ($O_2^{-\bullet}$), possible produced in this case by the Ag accumulation in this tissue, is capable of catalysing the formation of hydroxyl radicals ($HO^{\bullet}$) from hydrogen peroxide (H_2O_2) through Haber-Weiss reaction (Bremner, 1998; Kadiiska & Mason, 2002).

Moreover, the increase of Zn concentrations in the insoluble fraction (36-44% of total accumulated Zn) induced both total and Se-GPx (Figure 3.7F). 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 acts as antioxidant at different cellular levels (Bray & 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 concentrations this metal 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 the excess of peroxides.

Therefore, it seems that the increase of Ag, Cu, Mn and Zn levels in the gills of *B. azoricus* ultimately enhance the production of hydrogen peroxide although by

different mechanisms, that is preferentially detoxified by total and Se-dependent glutathione peroxidases rather than CAT. However, hydrogen peroxide seems to accumulate inside gills cells at higher rates than GPx are able to detoxify it. This can be confirmed by the direct linear relationships between GPx (both total and Se-GPx) and LPO levels (Figure 3.4). Although elevated, the activity 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 abstraction of a hydrogen atom from a methylene group of a PUFA (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 have 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 investigations. Nevertheless the role of chemoautotrophic endosymbionts in *B. azoricus* may be more diversified rather than for nutritional purpose only, as earlier supposed.

Contrarily 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 (Figures 3.8A and B respectively). 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 approximately half of the Zn concentration in the mantle than the gills (Table 1.4; Chapter 1). It seems that mantle tissue even though accumulating significantly lower Zn concentrations might be more susceptible to Zn toxicity by the inhibition of hydrogen peroxide detoxification mechanisms. In the same way, the increase of soluble Mn concentrations in the mantle also

inhibits total GPx (Figure 3.8C), which also 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) (Figures 3.9A to C). Although unable to generate ROS by Fenton-type reactions (Watanabe *et al.*, 2003, Stohs *et al.*, 2001), Cd is known to induce superoxide anion and nitric oxide (Yang *et al.*, 1997, Hassoun & Stohs, 1996) and is considered one of the most toxic non-essential metals to biological systems. Several studies showed that Cd affect the antioxidant defence system and inhibits the activity of SOD, GPx and CAT (Jurczuk *et al.*, 2004; León *et al.*, 2002, Muller 1986, 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) may inhibit the level of antioxidant protection against metal mediated ROS in this tissue comparatively with the gills.

MT concentrations showed a similar pattern of seasonal variations in both tissues, with a linear increase from July to November (gills) or September (mantle) (Figures 3.3B and 3.6B). Although these proteins have a high affinity to metals and have been largely used as metal exposure biomarkers in coastal areas using mussels *M. galloprovincialis* as bioindicator species (Bebianno & Machado, 1997), in *B. azoricus* from Menez-Gwen these proteins were not related to any accumulated metal. This may suggest that the major function of *B. azoricus* MT may not be of metal detoxification but mainly constitutive as already proposed by other authors (Fiala-Médioni *et al.*, 2000; Hardivillier *et al.*, 2004).

Moreover, although numerous studies showed that MT appears to be regulated by oxidative stress (Sato & Bremner, 1993; Andrews, 2000; Maret, 2000; Viarengo *et al.*, 2000) and free radical scavenge capacity due to their high thiol content (Chubatsu & Meneghini, 1993; Markant & Pallauf, 1996; Rossman & Goncharova, 1998), seasonal MT fluctuations are not related with antioxidant

enzyme activities or LPO. In this case, MT seasonal 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).

Finally, the effect of size and therefore age in antioxidant enzyme activities and LPO levels in these organisms was studied. Many physiological rates in the organism such as metabolic rate, feeding rate, assimilation rate, activities of many metabolic enzymes, are size dependent and the effect of age on each of these processes still remains unclear. The age of mussels are frequently determined by counting the rings of winter growth delays on the shells (Sukhotin *et al.*, 2002). Generally, small-sized organisms are younger and have higher metabolic rates. This can influence metal accumulation rates and numerous metabolic processes.

However the relationship between size and age in *B. azoricus* has been questioned since these parameters may not be directly related. In vent mussels, size is frequently the result of the relatively proximity of sulphur and methane emanations, vital no maintain the chemoautotrophic symbiotic bacteria in their gills (Colaço *et al.*, 2002). Therefore, the size of *B. azoricus* may reflect very different physiological conditions in term of health status rather than age. However, from the results obtained, size was not an important factor affecting the biochemical responses (Table 3.2). LPO levels however, were higher in smaller mussels, which may reflect poor physiological conditions in these organisms. A study on antioxidant enzymes in the mussel *Perna viridis* also reveals that size was not an important factor of variation in the antioxidant defences (Lau *et al.*, 2004). Similar results were obtained for the mussel *M. edulis*, where the activities of both SOD and CAT were found to be independent of the size and age, while LPO shows a significant negative relation with mussel size (Sukhotin *et al.*, 2002).

3.6. Conclusions

In conclusion, it seems that hydrothermal vents in the Azores area might not be an aseasonal environment, like it was supposed earlier. Vent organisms, especially the mussel *B. azoricus*, have seasonal reproduction patterns. Therefore, seasonal variation of biochemical parameters needs to be studied more systematically. Seasonal variation of antioxidant defence systems, lipid peroxidation and MT levels were described for the first time in *B. azoricus* and related with accumulated metal concentrations in the gills and mantle. Different patterns of seasonal changes were found in both tissues most likely because they have different physiologic functions. This variability must be taken into account for future studies of oxidative stress and related biomarkers in hydrothermal environments. The metal increase within tissues seems to induce antioxidant protection in the gills, while an inhibitory effect was observed in the mantle. Moreover, the size of *B. azoricus*, although not an essential factor of antioxidant enzyme variation, must be carefully used since it can influence lipid peroxidation levels.

3.7. References

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Chapter 4

Effect of cadmium on antioxidant responses and susceptibility to oxidative stress in the hydrothermal vent mussel *Bathymodiolus azoricus*

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4.1. Abstract

Hydrothermal vents are a unique environment of extreme physical and chemical characteristics and biological diversity. Cd is a toxic non-essential metal present at high concentrations in hydrothermal vent environment, contrary to those found in marine coastal areas. Cd toxicity has been related among other things with reactive oxygen species production, even though this is a non-redox metal. *Bathymodiolus azoricus* is a deep-sea Mytilid bivalve very common in Mid Atlantic Ridge (MAR) hydrothermal vent fields and very little is known about the antioxidant defence system in this species. Because lethal Cd concentration in *B. azoricus* is unknown, the aim of this study was to assess the effects of a Cd concentration higher than in the hydrothermal vents on oxidative stress biomarkers, such as antioxidant enzymes. Mussels were exposed to 100 µg l^{-1} Cd during 24, 48 and 144 hours in a pressurised aquarium (IPOCAMP) and to 50 µg l^{-1} Cd for 26 days, followed by 6 days of depuration at atmospheric pressure. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), total oxyradical scavenging capacity (TOSC), metallothionein (MT) and lipid peroxidation (LPO) were measured in the gills and mantle of *B. azoricus*. Results indicate that gills are firstly affected by Cd toxicity. This may be due to different physiological functions of the tissues, and by the presence of thio and methanotrophic symbiotic bacteria in the gills. SOD and CAT are inhibited during the first days of exposure in the gills, although TOSC and MT concentrations were the same in control and exposed mussels. In the mantle, enzymatic activation only occurred after 6 days, and no significant differences in MT concentrations were found in control and exposed mussels during the first day, as observed in the gills.

4.2. Introduction

Due to the interaction between seawater and magmatic rocks, end-member hydrothermal vent fluids are characterized by very high temperatures (300 – 350°C) elevated levels of silica (SiO_2), metals (e.g. Fe, Mn, As, Cd, Cu, etc) and dissolved gases (e.g. H_2S, CH_4, H_2, CO_2) (Sarradin *et al.*, 1998; Lowel *et al.*, 1995; Von Damm *et al.*, 1995; Von Damm 1990). Among metals, Cd concentrations in these particular marine environments can reach between 1.0 – 1.3 µg l^{-1} in Menez-Gwen, 2.0 – 8.9 µg l^{-1} in Lucky Strike and 14.6 µg l^{-1} in Rainbow hydrothermal vents sites, located in the Mid-Atlantic Ridge (MAR) (Douville *et al.*, 2002). This non-essential metal is normally present at low concentrations in uncontaminated open seawater (0.1 µg l^{-1}; EPA 2001) although in coastal and estuarine areas reach 42 µg l^{-1}, frequently as a result of anthropogenic activities (Caccia *et al.*, 2003, Langston 1990).

Cd is one of the most toxic metals to biological systems including marine organisms. The toxic effect of Cd include the interference in various metabolic processes, especially energy metabolism, membrane transport and protein synthesis and may act on DNA directly or indirectly by interference with genetic control and repair mechanisms (Yamada *et al.*, 1993; Hartwig 1994; Hassoun & Stohs 1996; Beyersmann & Hechtenberg 1997; Pruski & Dixon 2002). Also, the production of reactive oxygen species (ROS) is involved in the mechanism of Cd toxicity, although this metal is a non-redox metal unable to participate in Fenton-type reactions (Zhong *et al.*, 1990; Manca *et al.*, 1994; Kumar *et al.*, 1996; Stohs *et al.*, 2001; Watanabe *et al.*, 2003).

ROS are continually produced in biological systems, and the most important include the superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH•). Many antioxidant defence mechanisms within living organisms have evolved to limit the level of ROS and the oxidative damage they induce (Cheeseman & Slater 1993). These systems can be divided into enzymatic and non-enzymatic groups. Among the enzymatic antioxidant group, the most important include the superoxide dismutase (SOD), which can be found in the cytosol and mitochondria that catalyses the

dismutation of superoxide anion into H_2O_2, catalase (CAT) that converts H_2O_2 into water and molecular oxygen and glutathione peroxidases (GPx) that are capable of metabolising both hydroperoxides and H_2O_2 (Se-GPx) (Fridovich 1978). The sensitivity of single antioxidant parameters enables their use as rapid and easy-to-use biomarkers. However, their ecological relevance is restricted, as it is impossible to obtain a complete assessment of the oxidative stress based on a few biochemical responses. Consequently, Winston *et al.*, (1998) proposed the Total Oxyradical Scavenging Capacity (TOSC) assay, to measure the balance between antioxidant parameters and prooxidants factors. The TOSC assay measures the capability of a tissue to neutralize ROS in quantifiable terms. This provides better understanding, and predictive capacity, of the effects of environmental conditions on the redox status of the organisms and their susceptibility to oxidative stress and was successfully validated as a biomarker (Regoli & Winston 1998; Regoli 2000).

Cd interferes with antioxidant enzymatic defence system in marine bivalves, such as in the mussel *Mytilus galloprovincialis* (Viarengo *et al.*, 1990) and the clam *Ruditapes decussatus* (Géret *et al.*, 2002a). Cd also decreases glutathione content, inhibits the activity of SOD, GPx and CAT (Muller 1986; Hussein *et al.*, 1987; León *et al.*, 2002). Cd can simultaneously have an inhibitory effect in some enzymes (CAT) and an inductor effect on others (GPx) (Iszard *et al.*, 1995). This metal causes oxidative cellular damage and induces superoxide anion and nitric oxide production, which are known as potential inducers of apoptosis (Hassoun & Stohs 1996; Yang *et al.*, 1997).

Lipid peroxidation (LPO) is an important feature in cellular injury and results largely from free radical reactions in biological membranes, which are rich in polyunsaturated fatty acids (PUFA) (Chesseman 1982). Halliwell & Gutteridge (1984) pointed out that transition metals might stimulate the peroxidation of PUFA by acting as catalysts in the formation of oxygen radicals or, more likely in the decomposition of pre-formed hydroperoxides. Evidences that Cd enhance LPO in bivalves have been demonstrated, particularly in the gills of the mussels *Mytilus edulis* (Géret *et al.*, 2002b) and *Pyganodon grandis* (Giguère *et al.*,

2003) and in the gills and digestive gland of the clam *R. decussatus* (Roméo & Gnassia-Barelli 1997; Géret *et al.*, 2002a).

Mytilid mussel *Bathymodiolus azoricus* are among the most common species in MAR hydrothermal vent fields, including Menez-Gwen, Lucky Strike and Rainbow. They are known to form extensive mussels beds in high-density clusters surrounding the hydrothermal active area and covering the base and walls of vent chimneys (Desbruyères *et al.*, 2000).

Although several studies have been carried out on the effects of metals on hydrothermal vent species, including *B. azoricus*, specially regarding mechanisms of metal uptake and detoxification (Cosson-Mannevy *et al.*, 1988; Smith & Flegal 1989; Cosson 1997; Cosson & Vivier 1997; Géret *et al.*, 1998; Rousse *et al.*, 1998), little is know about the presence of antioxidant enzymatic defences and lipid peroxidation in hydrothermal vent organisms (for additional information see Chapters 2 and 3). Earlier studies identified the presence of CAT, SOD and GPx in two important vent organisms, the tubeworm *Riftia pachyptila* and the clam *Calyptogena magnifica* collected from the East Pacific Rise (Blum & Fridovich 1984). In a preliminary study the behaviour of some antioxidant enzymes following the exposure to three metals (Cd, Cu and Hg) in the gills were investigated (Company *et al.*, 2004 – Annexe II). Since lethal Cd concentration in *B. azoricus* is still unknown and the effects of 100 µg l^{-1} Cd in coastal mussels *M. edulis* and *M. galloprovincialis* are well documented, this Cd concentration was selected.

Consequently, the aim of this work was to study the effect of a Cd concentration exposure (100 µg l^{-1}) higher than in hydrothermal environments, for a short period of time (6 days), as well as the effect of 50 µg l^{-1} Cd for a longer period (25 days of exposure, followed by 6 days of depuration) in several stress related biomarkers, including the activity of antioxidant enzymes (SOD, CAT and GPx), TOSC, MT and LPO levels in two tissues (gills and mantle) of the hydrothermal vent mussel *B. azoricus*.

4.3. Materials and Methods

Sample collection

Deep-sea vent mussels *B. azoricus* were collected using the French Remote Operated Vehicle (ROV) Victor (IFREMER) and acoustically retrievable cages during the ATOS cruise in Summer 2001 (Sarradin *et al.*, 2001) from Menez Gwen hydrothermal vent site (37° 51`N, 32° 31`W, 840 m) located in the Mid-Atlantic Ridge (MAR) south-west of the Azores archipelago. The mussels were brought to the surface and acclimated in filtered seawater collected from the Azores coastal zone and maintained at 9° C for 48 hours.

Short term Cd exposure experiment

Three groups of 10 mussels each (7.10 ± 0.81 cm shell length) were exposed to a nominal concentration of 100 µg l^{-1} Cd at 9 ± 1°C and 85 atmospheres in a pressurized container IPOCAMP (*Incubateurs Pressurises pour l'Observation en Culture d'Animaux Marins Profonds*) (Shillito *et al.*, 2001) for 24, 48 and 144 hours respectively. Controls (7.21 ± 0.45 cm shell length) were maintained in the same conditions in filtered seawater collected from the Azores coastal zone. The IPOCAMP system (Figure 4.1) consists of a thermoregulated (0 to 80°C) 20 litre stainless steel vessel with an internal diameter of 20 cm, integrated into a pressurised circuit. The temperature of the flowing seawater (through a 0.4 µm filter) is measured constantly, at pressure, in the inlet and outlet lines (±1°C). Temperature regulation was powered by a regulation unit (Huber CC 240) that circulated ethylene-glycol through steel jackets surrounding the vessel and through the seawater inlet line. IPOCAMP can also allow video-observations of the re-pressurized organisms by combining an endoscope (Fort) to a CCD (charge-coupled device) colour camera (JVC, TK-C1380). The results are displayed on a TV monitor (JVC), and recorded (Sony SVO-9500 MDP videotape-recorder) (Shillito *et al.*, 2001).

Inside the IPOCAMP chamber, each organism (control and exposed) was individually placed in sealed 1L plastic bottles. Water in each container was changed after 12 hours to avoid oxygen depletion and to re-establish the Cd

concentration. Pressure was re-established after 30 minutes. Although hydrostatic pressure is known to alter the physiology and metabolism of marine organisms, as reported recently for DNA damage of *B. azoricus* (Dixon *et al.*, 2004), these alterations were not measured in the present study.

Water temperature was unaffected because the system was thermo stabilised at 9ºC. Organisms were measured and the gills and mantle dissected and immediately frozen in liquid nitrogen and stored at –80ºC until further analysis.

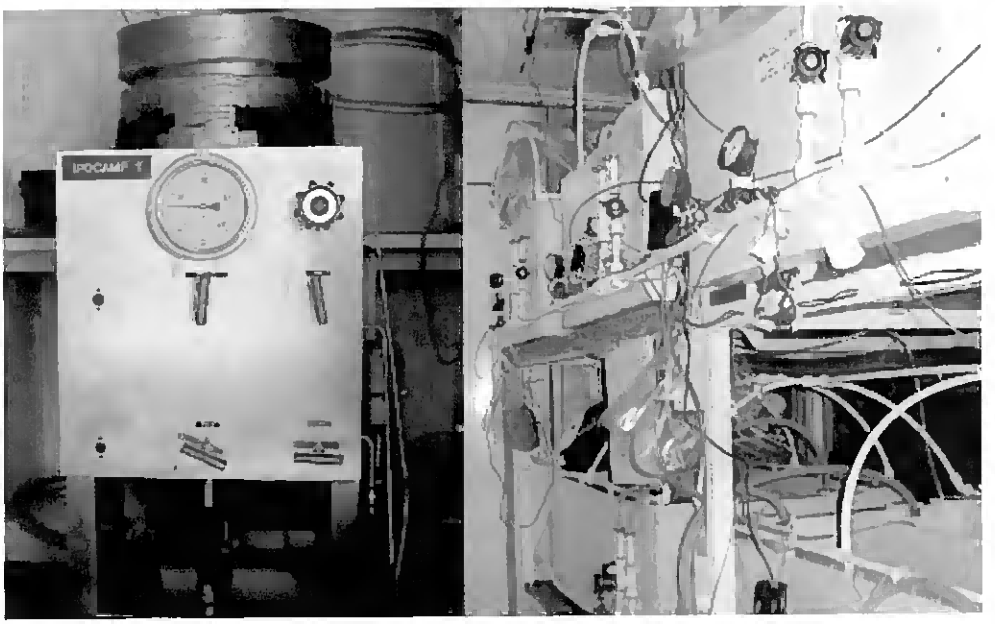

Figure 4.1 – IPOCAMP vessel (*Incubateurs Pressurises pour l'Observation en Culture d'Animaux Marins Profonds* - Universitié Pierre et Marie Curie, Paris) used for under pressure metal exposure experiments and stock and experimental aquaria (adapted from Dixon *et al.*, 2001).

Long term Cd exposure experiment

Groups of 10 mussels *B. azoricus* (7.73 ± 0.82 cm shell length) were exposed to a nominal concentration of 50 µg l^{-1} Cd during 24 days, followed by a 6 days period of depuration. The experiment was carried out on a land-based laboratory "LabHorta" (University of Azores). Another group of 10 mussels were maintained in the same conditions with clean seawater (control). The exposed and control mussels were maintained at 9 ± 1ºC at atmospheric pressure. During the experiment the water was changed every two days and Cd concentration re-established. After 24 days of experiment, the Cd treated mussels were placed in

uncontaminated seawater during 6 days for depuration. Ten mussels were sampled from control and Cd-exposed aquaria respectively after 6, 12, 18, 24 and 30 days, dissected and treated as described bellow.

Biochemical determinations

Antioxidant enzymes

Symbiotic bacteria were not separated from the gills, thus the enzymatic activities in this tissue reflect the contributions of both host and symbionts.

For the determination of antioxidant enzymatic activities, gills (tissue + symbionts) and mantle were homogenized in 20 mM Tris buffer, pH 7.6, containing 1mM of EDTA, 0.5M of saccharose, 0.15M of KCl and 1mM of dithiothreitol. The homogenates were centrifuged at 500 *g* for 15 min at 4°C to precipitate large particles. Supernatants were centrifuged again at 12000 *g* for 45 min at 4°C to precipitate the mitochondrial fraction. Supernatants were purified on a Sephadex G-25 gel column to remove low molecular weight proteins.

SOD (EC 1.15.1.1), activity was determined in the purified cytosolic and mitochondrial fractions of gills and mantle of *B. azoricus* measuring the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm (McCord & Fridovich 1969). One unit of SOD is defined as the amount of enzyme that inhibits the reduction of cytochrome c by 50%. SOD activity is expressed in U SOD mg^{-1} total protein concentrations.

CAT activity (EC 1.11.1.6) was determined in the gills and mantle of *B. azoricus* according to Greenwald (1985), by the decrease in absorbance at 240 nm due to H_2O_2 consumption. The difference in the absorbance per unit time was taken as the measure of CAT activity (ε = -0.04 mM^{-1} cm^{-1}). The CAT activity (sum of cytosolic and mitochondrial contributions) is expressed as mmoles min^{-1} mg^{-1} of total protein concentrations.

GPx activities were measured following NADPH oxidation at 340nm in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (Lawrence & Burk 1976). The Se-GPx (EC 1.11.1.9) and Total GPx activities were measured by using respectively, H_2O_2 and cumene hydroperoxide as substrates. The rate of blank reaction was subtracted from the total rate. The difference in the absorbance per unit time was taken as the measure of glutathione peroxidase activity (ε = -6.22 mM^{-1} cm^{-1}). The GPx activities are expressed as µmoles min^{-1} mg^{-1} of total protein concentrations.

Total oxyradical scavenging capacity (TOSC)

TOSC was determined by the method based on Winston *et al* (1998) and Regoli & Winston (1999), modified by adjusting the buffers used for marine bivalves (Regoli *et al.*, 2000). TOSC was only determined in the gills since this tissue exhibits significantly higher levels, compared to the mantle (See Chapter 2; Bebianno *et al.*, submitted) and for this reason could indicate more easily the changes in the oxidative stress susceptibility in *B. azoricus*. The gills were homogenised with a Potter-Elvehjem glass/Teflon homogeniser in four volumes of 100 mM KH_2PO_4 buffer, 2.5% NaCl, pH 7.5. The homogenate was centrifuged at 100 000 *g* for 1 h, and cytosolic fractions were aliquoted and stored at –80°C. Peroxyl radicals are generated by the thermal homolysis of 2-2'-azo-bis-(2 methyl-propionamidine)-dihydrochloride (ABAP) at 35 °C. The iron-ascorbate Fenton reaction was used for hydroxyl radicals, while peroxynitrite was generated from 3-morpholinosydnomine (SIN-1), a molecule that releases concomitantly nitric oxide and superoxide anion, which rapidly combine to form HOONO. Final assay conditions were: (a) 0.2 mM α-keto-γ-methiolbutiryc acid (KMBA), 20 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4 for peroxyl radicals; (b) 1.8 µM Fe^{3+}, 3.6 µM EDTA, 0.2 mM KMBA, 180 µM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4 for hydroxyl radicals; and (c) 0.2 mM KMBA and 80 µM SIN-1 in 100 mM potassium phosphate buffer, pH 7.4 with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) for peroxynitrite. Peroxyl, hydroxyl radicals and peroxynitrite can oxidize the substrate KMBA to ethylene gas, which is measured with gas chromatography. Data acquisition system was run by the software Millenium32® (Waters). Each analysis required the measurement of control (no antioxidant in

the reaction vial) and sample reactions (biological fluid in the vial). In the presence of antioxidant, ethylene production from KMBA was reduced quantitatively and higher antioxidant concentrations resulted in longer periods in which ethylene formation was inhibited relative to controls. TOSC is quantified according to Equation: TOSC = 100 – (IntSA/IntCA * 100), where IntSA and IntCA are the integrated areas from the curve defining the sample and control reactions, respectively. The specific TOSC value was calculated by dividing the experimental TOSC by the concentration of protein used for the assay. Data are expressed as TOSC unit mg^{-1} protein. These analyses were carried out in the University Centre on Svalbard (Norway) by Dr. Lionel Camus.

Metallothioneins (MTs)

To determine MTs concentrations, the tissues were homogenized at 4°C using an electric potter and a Teflon pestle in a Tris buffer (100 mM), pH 8.1, containing 10 mM of β-mercaptoethanol. The soluble and insoluble fractions were separated by centrifugation (30000 *g*, 30 min, 4°C). Aliquots of the supernatants were heated (15 min, 95°C) and allowed to cool on ice. Heat-denatured proteins were separated from heat-stable proteins by centrifugation of the heated supernatants (10000 *g*, 15 min). Supernatants containing the heat-stable proteins, including MTs, were stored at -20°C until use.

In the heat-denatured cytosol, the amount of MTs was determined by differential pulse polarography using a PAR 394 analyser and a EG&G PAR 303A static mercury drop electrode (SMDE) in accordance with the method of Olafson & Sim (1979) modified by Thompson & Cosson (1984). The electrochemical detection of MTs takes place in an ammoniacal electrolyte containing cobalt that catalyses the reduction of the cystein thiol groups (Brdicka, 1933). The standard addition method was used for calibration with rabbit liver MT (Fluka) in the absence of *B. azoricus* MT standard. The levels of MTs are expressed as mg g^{-1} wet weight. MT analysis was carried out in ISOMer Marine Biology Laboratory in the University of Nantes (France) by Dr. Richard Cosson.

Total protein concentrations

To determine the total protein content, the tissues were homogenized in 20 mM Tris buffer, pH 8.6, containing 150 mM of NaCl. The homogenates were centrifuged for 30 min at 30000*g* at 4°C. Total protein concentrations were measured on supernatants by the Lowry method (Lowry *et al.*, 1951) using BSA (Bovin Serum Albumin) as reference standard material.

Lipid peroxidation

Lipid peroxidation was determined in the supernatant used for total proteins quantification. The method described by Erdelmeier *et al* (1998) measures the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) produced during decomposition of polyunsaturated fatty acid peroxides of membrane lipids. This procedure is based on the reaction of chromogenic reagent, N-methyl-2-phenylindole (R1), where two moles of R1 react with one mole of either MDA or 4-HNE at 45°C for 60 minutes to yield a stable chromophore with maximal absorbance at 586 nm. 10 µl of 0.5 M butylated hydroxytoluene, 650 µl of diluted R1 (6 ml of methanol with 18 ml of 10.3 mM N-methyl-2-phenylindole) and 150 µl of 15.4 M methanesulfonic acid were added to 200 µl of the first cytosolic fraction. This mixture was incubated at 45°C for 60 minutes. The levels of MDA + 4-HNE were estimated at 586 nm using malonaldehyde bis (tetrametoxypropan, SIGMA) as standard. The concentration of lipid peroxidation compounds in the gills and mantle of *B. azoricus* were expressed as nmoles of MDA g^{-1} total protein concentrations.

Statistical analysis

Values were expressed as means ± standard deviation (SD). The data were previously tested for normality and homogeneity. Analysis of variance (ANOVA) to determined significant statistical differences between treatments regarding antioxidant enzymatic activity (SOD, CAT and GPx), TOSC levels, MTs and LPO concentrations. The level of significance was set at 0.05.

4.4. Results

4.4.1. Short term Cd exposure experiment

Antioxidant enzymes

SOD, CAT and GPx activities in the gills and mantle of *B. azoricus* exposed to 100 µg l^{-1} Cd in IPOCAMP container during 24, 48 and 144 hours are presented in Figure 4.2.

Superoxide Dismutase

SOD was predominantly present in the cytosolic fraction (70-85%) of both gills and mantle of control and Cd-exposed *B. azoricus*. The SOD activity in the cytosolic fraction was significantly higher in the gills when compared with mantle. Gills also exhibited significantly higher mitochondrial SOD activity compared to the mantle, except after 144 hours Cd exposure (p>0.05) (Fig. 4.2A and B).

Cytosolic SOD activity significantly decrease in the gills of Cd-exposed mussels after 24 to 48 hours of exposure compared to controls, while no significant differences in the activity of this enzyme in the mantle between control and exposed mussels were observed. After 6 days, a significant increase of cytosolic SOD activity occurred in the gills in both control and Cd exposed *B. azoricus*, although not significantly different, while in the mantle an induction of SOD activity was observed in Cd-exposed mussels (Fig. 4.2A).

The SOD activity in the mitochondria showed a very different pattern (Fig. 4.1B). A significant decrease in SOD activity was observed in both tissues, after 24 to 48 hours of exposure, in control and exposed mussels, except for the mantle of Cd-exposed mussels where no significant differences occurred between exposure times (p>0.05). After 6 days of Cd exposure, the mitochondrial SOD activity in the gills remained unchanged, while in the mantle it significantly increased in both control and Cd-exposed mussels (Fig. 4.2B).

Catalase

CAT activity was significantly higher in the gills than in the mantle at all exposure times ($p < 0.05$), and was significantly higher ($p<0.05$) in unexposed mussels except after 144 hours of experiment, where no significant differences between control and Cd exposed *B. azoricus* was found. CAT activity in the mantle of Cd-exposed mussels was only induced after 6 days of exposure (Fig. 4.2C).

Glutathione peroxidases

No significant differences ($p>0.05$) in Total GPx activity between the two tissues were observed, except after 144 hours of exposure, where Total GPx activity in the gills was significantly higher compared with the mantle ($p<0.05$).

Also, no significant variation was observed in the Total GPx activity in the gills and mantle after 24 to 48 hours of Cd-exposure. After 144 hours, a significant induction of total glutathione peroxidase activity occurred in the gills and mantle of both control and Cd-exposed mussels ($p<0.05$) (Fig. 4.2D).

The Se-GPx was the only enzyme where the activity in the mantle was significantly higher than in the gills ($p<0.05$). However this pattern changed after 6 days with the gills having significantly higher Se-GPx activity than the mantle. As observed for Total GPx, the Se-GPx activity remained unchanged in both tissues of control and Cd exposed mussels between 24 and 48 hours. After 6 days of Cd exposure, a significant induction of this enzyme activity was observed in the gills and mantle of both control and Cd-exposed *B. azoricus* ($p<0.05$) (Fig. 4.2E).

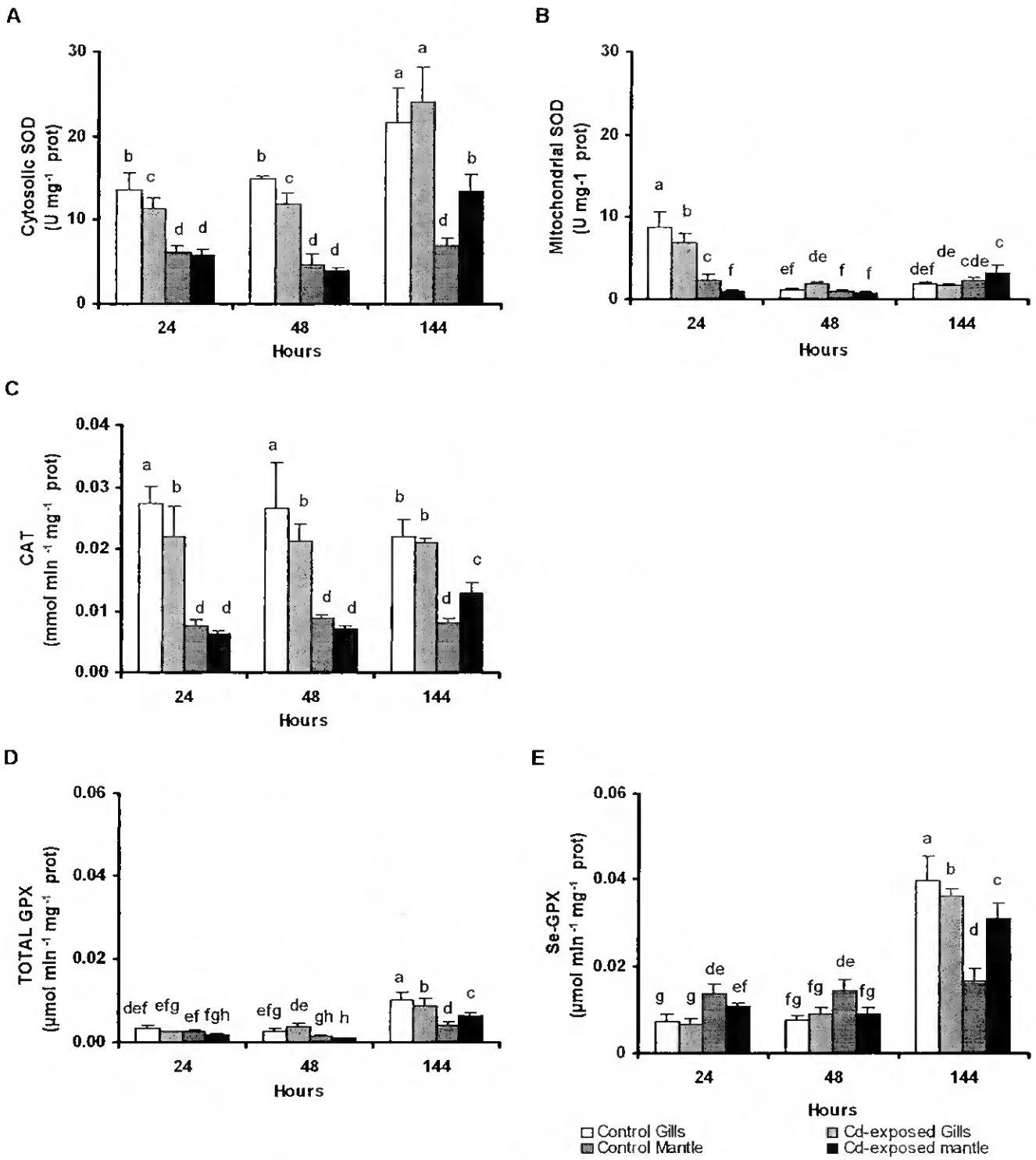

Figure 4.2 – Mean variation (± SD) of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills and mantle of control and Cd exposed (100 µg l^{-1}) *B. azoricus* for 24, 48 and 144 hours in IPOCAMP. Values followed by the same letter are not statistically different (p>0.05) (n=10).

TOSC

In order to evaluate the short-time response of scavenging capacity following Cd exposure, TOSC was determined in the gills of *B. azoricus* after 24 hours (Table 4.1).

Table 4.1 – TOSC (unit mg^{-1} protein) in the gills of *B. azoricus* exposed to 100 µg l^{-1} Cd for 24 hours in IPOCAMP

	TOSC (unit mg^{-1} protein)	
	Control	Cd-exposed
Peroxyl	200 ± 11	183 ± 28
Hydroxyl	154 ± 5	135 ±26
Peroxynitrite	218 ±20	202 ±38

The exposure to 100 µg l^{-1} Cd reduced TOSC levels toward peroxyl, hydroxyl radicals and peroxynitrite in the gills, although this decrease was not significant after 24 hours (p>0.05).

Metallothionein concentrations

MT concentrations were determined after 24 hours of Cd exposure in the gills and mantle of *B. azoricus* (Table 4.2).

Table 4.2 – MT concentrations (mg g^{-1} w.w.) in the gills and mantle of *B. azoricus* exposed to 100 µg l^{-1} Cd for 24 hours in IPOCAMP

	MT (mg g^{-1} w.w.)	
	Control	Cd-exposed
Gills	4.62 ± 0.85	5.41 ± 1.18
Mantle	1.36 ± 0.30	1.44 ± 0.61

MT concentrations were 3-fold higher in the gills compared with the mantle in both control and Cd-exposed mussels (p<0.05). Although a small increase in MT levels occurred after 24 hours of Cd exposure in both gills and mantle, it was not significant (p>0.05).

Lipid peroxidation

In Fig. 4.3 is presented the LPO levels (expressed as nmoles of MDA + 4-HNE g^{-1} total protein concentrations) in the gills and mantle of *B. azoricus* exposed to Cd for 24, 48 and 144 hours.

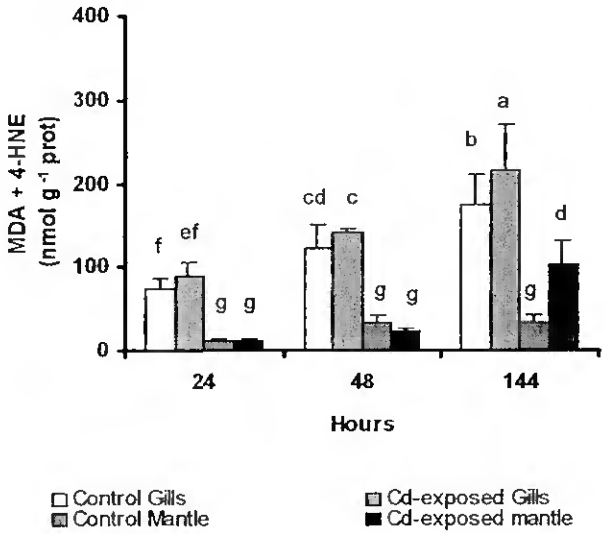

Figure 4.3 – Mean variation (± SD) of MDA + 4-HNE compounds in the gills and mantle of control and Cd exposed (100 µg l^{-1}) *B. azoricus* for 24, 48 and 144 hours in IPOCAMP. Values followed by the same letter are not statistically different (p>0.05) (n=10).

The products of LPO were significantly higher in the gills of *B. azoricus* compared with the mantle in all exposure times (p<0.05) (Fig. 4.3).

The MDA + 4-HNE levels in the gills increased linearly (p<0.05) with time, in both control (LPO [nmol g^{-1} protein] = 50.15t [hours] + 23.39, r = 0.999, p<0.05) and Cd-exposed mussels (LPO [nmol g^{-1} protein] = 62.88t [hours] + 23.36, r = 0.995, p<0.05). The increment of LPO in Cd exposed mussels is significantly higher than that of controls (p<0.05). Contrary, no significant variation was observed in MDA + 4-HNE concentrations in the mantle of control mussels. In the same tissue, although there was a slight increase in Cd-exposed mussels in LPO levels in the first hours of exposure, significant changes only occurred after 6 days (p < 0.05) (Fig. 4.3).

4.4.2. Long term Cd exposure experiment

Antioxidant enzymes

SOD, CAT and GPx activities in the gills and mantle of *B. azoricus* exposed to 50 µg l^{-1} Cd during 24 days, followed by 6 days of depuration are presented in Figure 4.4.

Superoxide dismutase

Like in the short term Cd exposure, SOD was predominantly present in the cytosolic fraction of both gills and mantle of control and Cd-exposed *B. azoricus* (70-85%), and both cytosolic and mitochondrial fractions was significantly higher in the gills than in the mantle (Figure 4.4A and B).

The cytosolic SOD activity in the gills of control mussels increased significantly ($p<0.05$) in the first 6 days of exposure, followed by a linear decrease until the end of exposure period (SOD [U mg^{-1} protein] = -33.52t [days] + 20.10, $r = 0.986$, $p<0.05$). During the depuration period the activity of this enzyme in this tissue increases significantly ($p<0.05$). In Cd-exposed mussels, the cytosolic SOD activity in the gills showed similar behaviour to controls in the first 12 days of Cd exposure. After this period the activity of this enzyme was linearly induced with the time (0.782 U mg^{-1} protein d^{-1}, $r = 0.963$, $p<0.05$) and continued even after the mussels were transferred to clean seawater.

The cytosolic SOD activity in the mantle of control mussels remained unchanged (8.1 ± 0.7 U mg^{-1} protein) during the course of the experiment, except at day 18 (3.7 ± 0.7 U mg^{-1} protein), where the SOD activity was significant lower ($p<0.05$). In the mantle of Cd-exposed mussels, this enzyme showed the same tendency of control and Cd-exposed gills. During the depuration period, in the opposite of controls, a significant increase in SOD activity was observed in this tissue (13.5 ± 4.4 U mg^{-1} protein), like in the gills. Significant differences between control and Cd-exposed mussels only occurred in the last day of the experiment, where SOD activity in Cd-exposed mussels was higher than in control mussels ($p<0.05$) (Figure 4.4A).

The mitochondrial SOD activity in the gills of control mussels showed some variability decreasing significantly in the first 6 days. After that period, SOD significantly increased reaching the same level of the beginning of the experiment. After day 12, the activity if this enzyme decrease linearly (SOD [U mg^{-1} protein] = - 0.30t [days] + 5.54, r = 0.935, p<0.05) until the end of exposure period (2.1 ± 0.6 U mg^{-1} protein) and remained unchanged during the depuration period (p>0.05). In the gills of Cd-exposed mussels, the mitochondrial SOD activity exhibited a similar pattern to control mussels in the first 12 days of Cd exposure. After this period, the activity of this enzyme remained unchanged until the 18 day (4.8 ± 0.4 U mg^{-1} protein), and significantly decreases afterwards, until the end of exposure period (1.4 ± 0.2 U mg^{-1} protein) (p<0.05). No significant changes in SOD activity were observed during the depuration period (p>0.05).

The mitochondrial SOD activity in the mantle of control mussels remained unchanged in the first 18 days of the experiment (1.4 ± 0.2 U mg^{-1} protein), and increased significantly after 24 days (2.7 ± 0.4 U mg^{-1} protein). During the depuration period the activity of this enzyme significantly decreased (1.0 ± 0.2 U mg^{-1} protein) (p<0.05). Contrary, in the mantle of Cd-exposed mussels, the mitochondrial SOD activity remained unchanged during the exposure and depuration periods (1.4 ± 0.3 U mg^{-1} protein) (p>0.05). No significant differences in the activity of mitochondrial SOD was observed in the mantle between control and exposed mussels, except at day 24.

Catalase

Like in the short term Cd exposure experiment, CAT activity was significantly higher in the gills when compared with mantle at all exposure times (Figure 4.4C). The CAT activity in the gills remained unchanged throughout the experiment in both control (0.027 ± 0.002 mmol min^{-1} mg^{-1} protein) and Cd exposed mussels (0.029 ± 0.003 mmol min^{-1} mg^{-1} protein). Moreover, no significant differences in the activity of this enzyme were observed between control and Cd contaminated mussels (p>0.05).

In the mantle of control mussels, CAT activity remained unchanged in the first 18 days of the experiment (0.006 ± 0.001 mmol min^{-1} mg^{-1} protein), followed by a significant increase in the last day of experiment (0.014 ± 0.003 mmol min^{-1} mg^{-1} protein), and remained unchanged during the depuration period (p>0.05).

In the mantle of Cd-exposed mussels the activity of this enzyme remained unchanged during the exposure period (0.007 ± 0.002 mmol min^{-1} mg^{-1} protein) (p>0.05), and a significant increase was observed during the depuration period (0.014 ± 0.004 mmol min^{-1} mg^{-1} protein) (p<0.05). As in the gills of *B azoricus*, no significant differences were found in CAT activity in the mantle between control and Cd exposed mussels (p>0.05) (Figure 4.4C).

Glutathione peroxidases

As in the Cd short term exposure in the IPOCAMP, total GPx was significantly higher in the gills compared with the mantle throughout the experiment (Figure 4.4D). Total GPx activity increased exponentially in the gills of both control (Total GPx [µmol min^{-1} mg^{-1} protein] = $0.01e^{0.07t\ [days]}$, r = 0.968, p<0.05) and Cd-exposed mussels (Total GPx [µmol min^{-1} mg^{-1} protein] = $0.009e^{0.07t\ [days]}$, r = 0.987, p<0.05) and no significant differences were observed between treatments (p > 0.05). In the mantle, total GPx activity remained unchanged in both control (0.004 ± 0.001 µmol min^{-1} mg^{-1} protein) and Cd-exposed mussels (0.005 ± 0.001 µmol min^{-1} mg^{-1} protein), and no significant differences (p>0.05) were found in total GPx between control and Cd exposed mussels (p>0.05) (Figure 4.4D).

In contrast with what was observed in the short term Cd exposure, Se-GPx activity was significantly higher in the gills, except in day 6 and 12 were no significant differences between tissues were found (Figure 4.4E). Like total GPx, Se-GPx activity in the gills of control mussels increased significantly in the first 6 days, and remained unchanged until the 18 day of the experiment (0.027 ± 0.001 µmol min^{-1} mg^{-1} protein). After this period, a linear increase in Se-GPx activity was observed until the end of the experiment (0.0012 µmol min^{-1} mg^{-1} d^{-1}; r = 0.999, p<0.05). In the gills of Cd-exposed mussels, the activity of this enzyme increased linearly during the exposure and depuration periods (Se-GPx

[µmol min^{-1} mg^{-1} protein] = 0.0013t [days] + 0.0069, r = 0.982, p<0.05). However, no significant differences (p>0.05) in Se-GPx activity between control and Cd-exposed mussels were observed during the experiment, except in the 6-day (control > Cd-exposed, p<0.05) and in the end of the depuration period (Cd-exposed > Control, p<0.05).

In the mantle of control *B. azoricus*, Se-GPx activity increased significantly (p<0.05) in the first 6 days and remained unchanged until the end of the experiment (0.015 ± 0.001 µmol min^{-1} mg^{-1} protein) (p>0.05). In Cd-exposed mussels, the activity of this enzyme in the mantle increased linearly during the first 12 days of exposure (0.0012 µmol min^{-1} mg^{-1} d^{-1}, r = 0.994, p<0.05) and decreased significantly until day 18 (p<0.05). After this period, Se-GPx activity also increased linearly until the end of the experiment, including the depuration period (Se-GPx [µmol min^{-1} mg^{-1} protein] = 0.0006t [days] + 0.0103, r = 0.940, p<0.05).

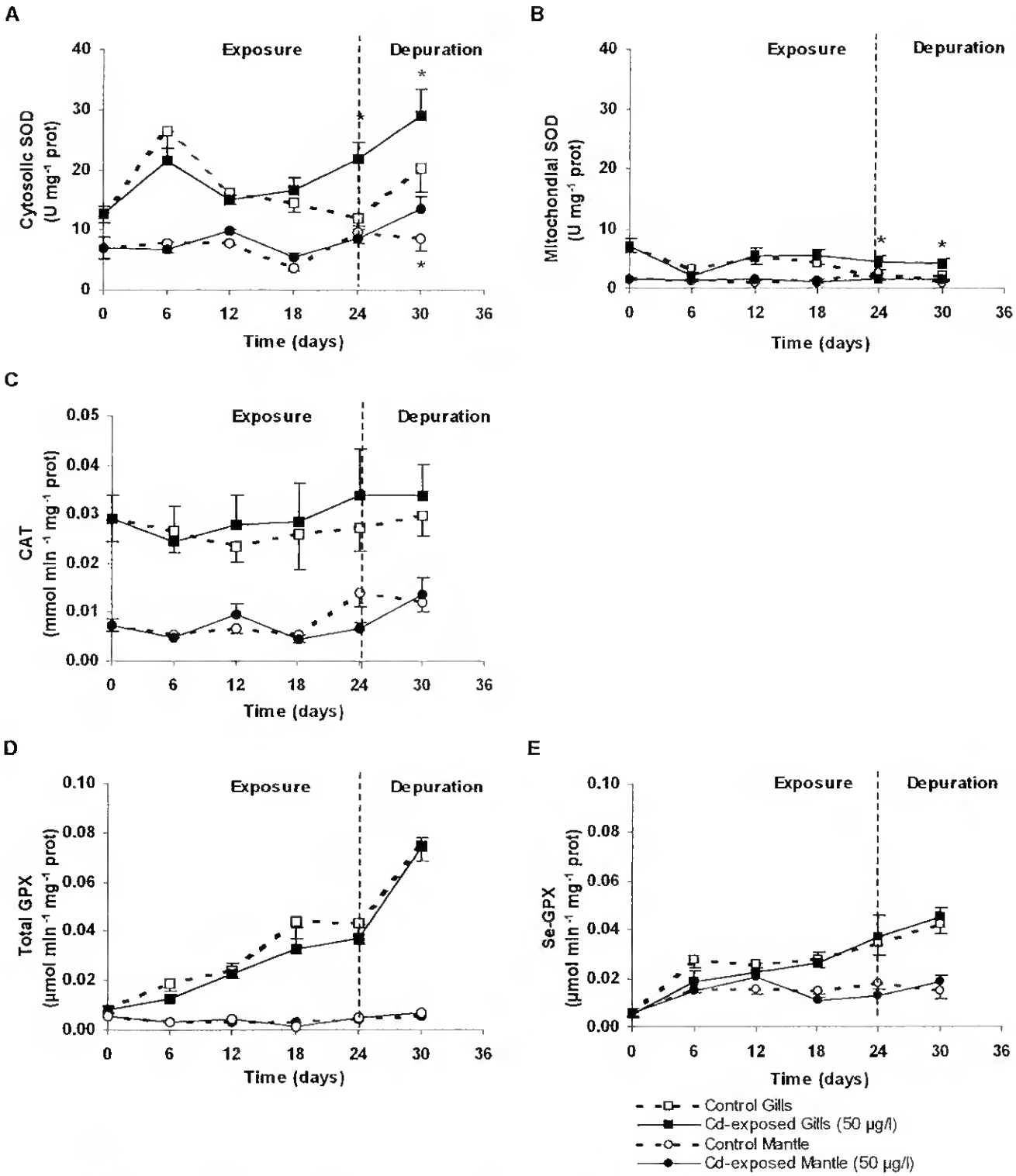

Figure 4.4 – Mean variation of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills and mantle control and Cd exposed (50 µg l^{-1}) *B. azoricus* for 24 days and 6 days of depuration. Vertical bars represent one-half of the standard deviation of the mean. Symbol * represents significant differences between control and Cd-exposed mussels.

TOSC

The TOSC assay, contrary to the measurement of antioxidant enzymes activity, represents a more integrated and holistic interpretation in terms of health condition and susceptibility to oxidative stress for exposed organisms. Therefore, TOSC was determined exclusively in pre-exposed organisms to set up baseline values for this parameter, and at the end of exposure (day 24) and depuration period (day 30) (Figure 4.5).

TOSC levels toward peroxyl radicals remained unchanged in control mussels during the experiment (711 ± 76 TOSC unit mg^{-1} protein). In Cd exposed mussels although a slight decrease in this parameter occurred after 24 days of exposure and at the end of depuration period, it was not significant compared to control (p>0.05) (Figure 4.5A). TOSC toward hydroxyl radicals also remained unchanged throughout the experiment in control mussels (282 ± 57 TOSC unit mg^{-1} protein), and no significant differences in Cd exposed mussels were observed at the end of exposure and depuration periods (p>0.05) (Figure 4.5B).

Regarding TOSC toward peroxynitrite, both control and Cd exposed mussels exhibited a significant reduction at the end of exposure and depuration periods compared to pre-exposed mussels (p < 0.05), but similar between control and Cd-exposed mussels in each day (p > 0.05) (Figure 4.5C).

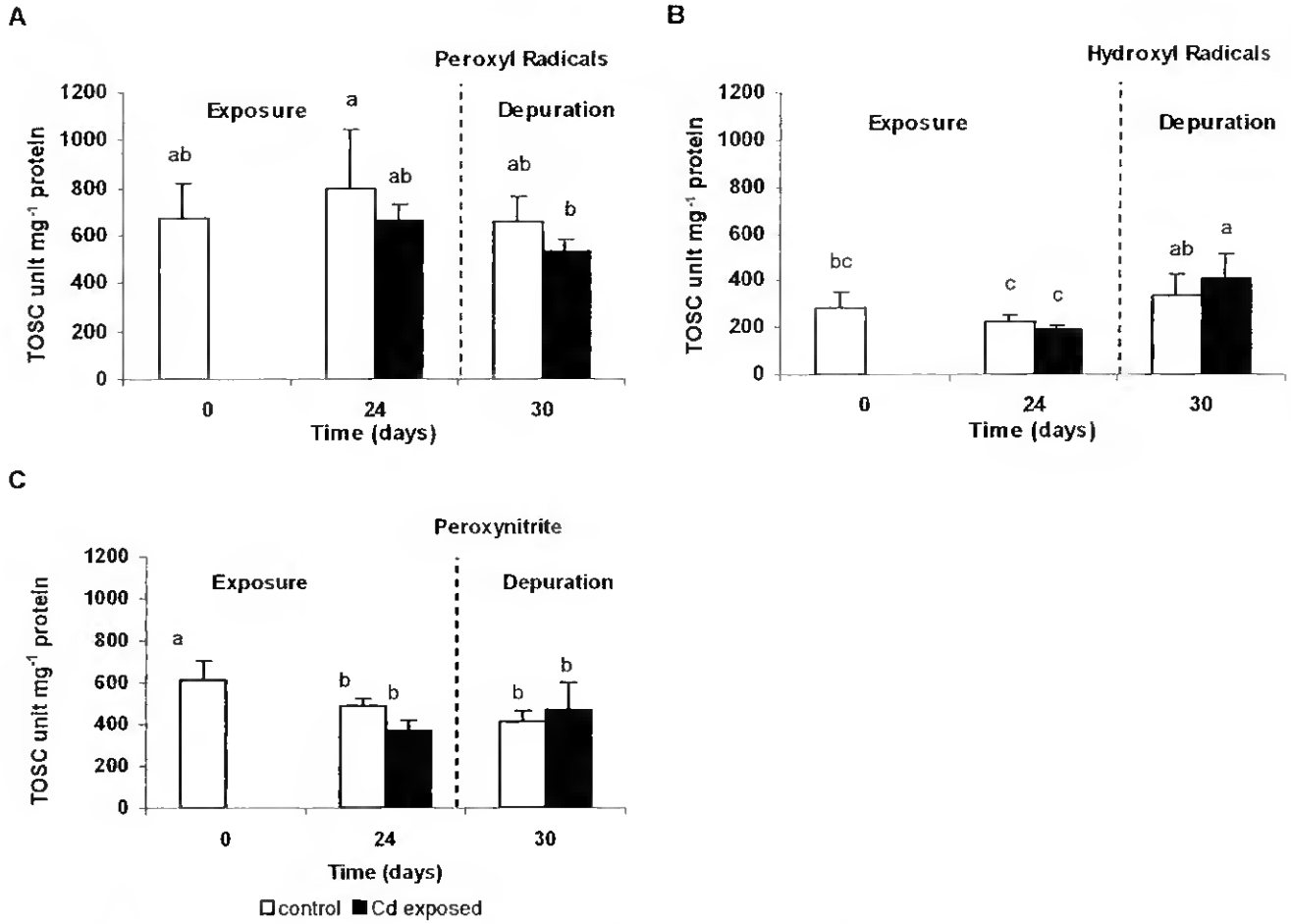

Figure 4.5 – Mean variation (±SD) of the Total Oxyradical Scavenging Capacity (TOSC) towards peroxyl (A), hydroxyl radicals (B) and peroxynitrite (C) in the gills of control and Cd exposed (50 µg l^{-1}) *B. azoricus* for 24 days followed by a depuration period of 6 days. Values followed by the same letter are not statistically different (p>0.05).

Metallothioneins

MT concentrations in the gills and mantle of control *B. azoricus* and exposed to 50 µg l^{-1} Cd for 24 days, followed by 6 days of depuration are presented in Figure 4.6A.

MT concentrations were significantly higher in the gills, compared with the mantle throughout the experiment (p<0.05). MT levels in the gills of control mussels remained unchanged during the exposure period (3.42 ± 0.08 mg g^{-1}), followed by a significant decrease during the depuration period (2.31 ± 0.15 mg g^{-1}, p<0.05; day 30). MT levels in the gills of Cd exposed mussels increased

linearly during the first 18 days of exposure (0.035 mg g^{-1} d^{-1}, $r = 0.991$, $p<0.05$). After this period, MT concentrations decreased linearly until the end of the depuration period (0.084 mg g^{-1} d^{-1}, $r = 0.996$, $p<0.05$). However, MT concentrations in the gills of exposed mussels were only significantly higher than controls at day 18 and in the end of depuration period (Figure 4.6A).

In the mantle, MT concentrations remained unchanged throughout the experiment in both control (1.60 ± 0.32 mg g^{-1}) and Cd exposed mussels (1.56 ± 0.18 mg g^{-1}), and MT concentrations were similar between control and exposed mussels ($p>0.05$) (Figure 4.6A).

Lipid peroxidation

The products of LPO in the gills and mantle of control and Cd exposed *B. azoricus* (50 µg l^{-1}) for 24 days, followed by 6 days of depuration are presented in Figure 4.6B.

Like in short term Cd experiment, LPO levels were significantly higher in the gills than in the mantle ($p<0.05$). In the gills of control mussels, MDA and 4-HNE revealed some fluctuations with concentrations increasing linearly in the first 12 days of experiment (4.96 nmol g^{-1} protein d^{-1}, $r = 0.999$, $p<0.05$), and decreasing significantly afterwards until the day 18 (138.15 ± 34.17 nmol g^{-1} protein). During the last week of the exposure period LPO levels significantly increase (213.14 ± 28.42 nmol g^{-1} protein) and remained unchanged until the end of the depuration period (199.58 ± 19.18 nmol g^{-1} protein). In Cd exposed mussels, increase in LPO in the gills in the first 12 days of Cd exposure was similar to controls (5.11 nmol g^{-1} protein d^{-1}, $r = 0.991$, $p<0.05$), and remained unchanged for another week. After this period it increase linearly again until the end of depuration period (8.21 nmol g^{-1} protein d^{-1}, $r = 0.998$, $p<0.05$). During the whole experiment no significant differences were observed in LPO levels between the gills of control and Cd exposed mussels, except in the last day of the depuration period (day 30) (Figure 4.6B).

The MDA and 4-HNE concentrations in the mantle remained unchanged during the entire experiment, in both control (37.69 ± 11.88 nmol g^{-1} protein) and Cd

exposed mussels (35.37 ± 12.23 nmol g^{-1} protein), except in day 18 where a significant decrease of LPO levels occurred. As observed for the gills, no significant differences in LPO levels were observed between control and Cd treated mussels (p>0.05) (Figure 4.6B).

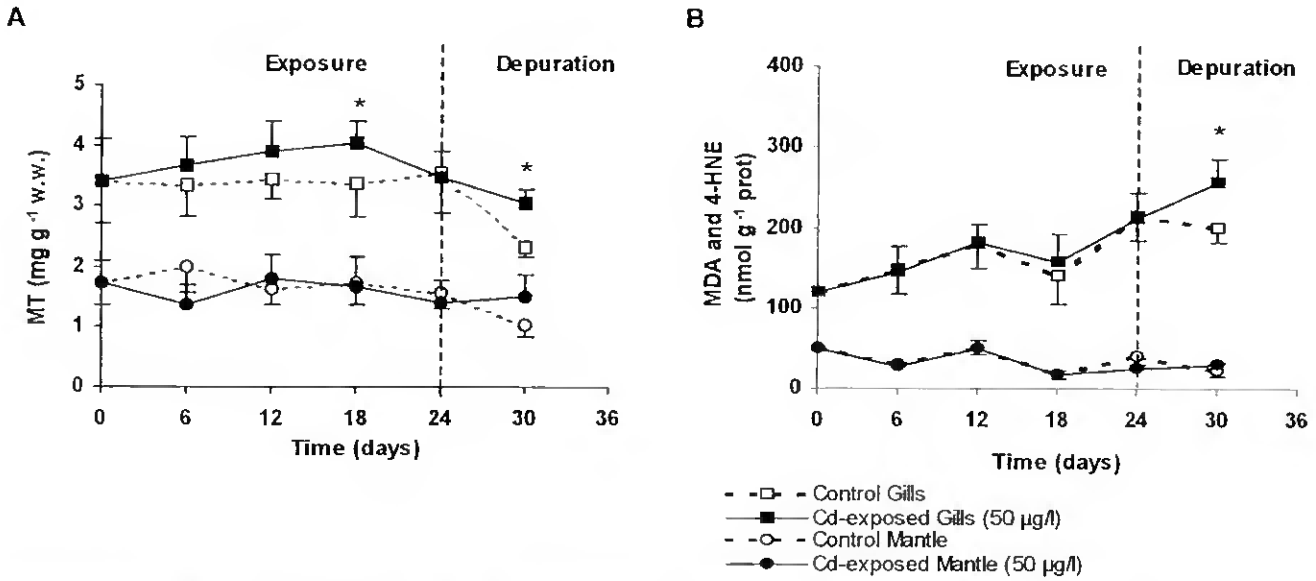

Figure 4.6 – Mean variation of MT concentrations (A) and MDA + 4-HNE compounds levels (B) in the gills and mantle of control and Cd exposed (50 µg l^{-1}) *B. azoricus* for 24 days and depurated for 6 days. Vertical bars represent one-half of the standard deviation of the mean. Symbol * represents significant differences between control and Cd-exposed mussels.

4.5. Discussion

Hydrothermal vent mussels, *B. azoricus* live in a very extreme hostile environment compared to other deep or coastal marine ecosystems (Pruski & Dixon 2002), enriched in potentially toxic species, high temperature and low pH (Desbruyères *et al.*, 2000, 2001; Douville *et al.*, 2002). However, this mussel species appears to be well adapted to such environmental conditions, since they are one of the most common species in MAR hydrothermal vents. In this context, hydrothermal vents can be used as a natural pollution laboratory to study the effects of toxic compounds in biological systems. Hydrothermal vent mussels live in this environment in symbiosis with abundant sulphur-oxidizing chemoautotrophic and/or methanotrophic bacteria in the gill tissue (Fisher *et al.*, 1987; Cavanaugh 1983; Le Pennec *et al.*, 1988; Fiala-Médioni & Felbeck 1990;

Kochevar *et al.*, 1992; Nelson *et al.*, 1995). *B. azoricus* is an example of dual symbiosis with both sulfur-oxidant and methanotrophic symbionts (Fiala-Médioni *et al.*, 2002). High metal concentrations have been detected in several bivalves living in hydrothermal vents, including the clam *C. magnifica* (Roesijadi & Crecelius 1984; Roesijadi *et al.*, 1985; Cosson-Mannevy *et al.*, 1988) and the mussels *Bathymodiolus* sp. (Smith & Flegal 1989; Géret *et al.*, 1998; Rousse *et al.*, 1998). Those organisms appear to survive and evolve through the development of a high degree of tolerance to metals generally considered to be toxic to other coastal marine species (Cosson & Vivier 1995; Cosson 1997).

Although in previous investigations the presence of antioxidant enzymes in hydrothermal bivalves, such as *C. magnifica* (Blum & Fridovich 1984) and *B. azoricus* (See Chapter 2; Bebianno *et al.*, submitted) has been demonstrated, along with preliminary studies on the effects of three metals (Cd, Cu and Hg) in antioxidant enzymes and lipid peroxidation (Company *et al.*, 2004 – Annexe II), this study assesses the effects of Cd exposure on several metal detoxification systems and stress related biomarkers in a bivalve species from hydrothermal vent and compare the toxic effects of Cd in a short term pressurized experiment (6 days) in two tissues (gills and mantle) of *B. azoricus*. Furthermore, In order to understand and isolate the effect of metal contamination the Cd concentration used in the experiments was 10 fold higher than the Cd concentrations found in the natural hydrothermal environment.

From the results obtained during short term IPOCAMP Cd experiment, the two tissues respond differently to Cd exposure. The gills are firstly affected by Cd toxicity. During the first two days of exposure (24 and 48 hours), there was a significant inhibition of cytosolic and mitochondrial SOD and CAT in the gills, while in the mantle the activity of all antioxidant enzymes remained unchanged (Figure 4.2).

Only when Cd exposure was extended to 6 days the activity of all antioxidant enzymes increased in the mantle, while SOD and CAT activities were similar between control and Cd exposed mussels in the gills and a significant inhibition of GPx activity occurred (Figure 4.2). Both gills and mantle are in direct contact

with surrounding water, and consequently exposed to 100 µg l^{-1} Cd during the experiment. Different antioxidant responses between tissues may be related to different physiological function of gills and mantle. Gills in hydrothermal vent mussels are implicated in two important functions, respiration and nutrition. *B. azoricus* is likely to depend almost entirely on symbiotic bacteria within their gills to energy supply. Recent investigations point out that symbiotic bacteria in the gills of *B. azoricus* may have significant SOD activity (Hoarau, *own data*). Thus, the antioxidant enzymatic activities present in the gills reflect the contribution of both host and symbionts.

An important source of oxidative stress in biological systems is the presence of metals, involving different ROS-generating mechanisms (Stohs & Bagchi 1995). Some metals like Fe and Cu can directly influence oxidative status of the organisms through the participation in Haber-Weiss cycle, producing the hydroxyl radical ($HO^{\cdot}$). Cd however, without redox capacity, can also enhance the pro-oxidant status by reducing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems, and affecting iron-mediated processes (Okamoto *et al.*, 2001).

Short term Cd exposure experiment

From the results obtained, Cd seems to reduce both the antioxidant capacity of SOD and CAT. Similar results where reported for other marine organisms. In the clam *Ruditapes decussatus* exposed to 100 µg l^{-1} Cd the cytosolic and mitochondrial SOD activity in the gills also decreased in the first days of Cd exposure. It was also observed that after 3 days of Cd exposure, SOD activity in the gills of Cd-exposed clams increased significantly compared to control organisms (Géret *et al.*, 2002a). Similarly, Cd effects on CAT activity in the gills of *R. decussatus* are comparable to those observed in the mussel *B. azoricus*. During the first days of Cd exposure, CAT activity was significantly inhibited in this tissue, followed by an increase after 3 days of Cd exposure (Géret *et al.*, 2002a). Contrary to what was observed in the gills of *B. azoricus*, a significant decrease in GPx activity in the first days of Cd exposure in *R. decussatus*. Contrary, in some cases high doses of Cd apparently have little effect in antioxidant systems, like in the clam *Tapes philippinarum* exposed to several Cd

concentrations (150, 300 and 450 µg l^{-1}) during 7 days, no significant effects were observed in SOD activity of hemocytes although a slight decrease was observed (Matozzo *et al.*, 2001).

The mechanisms responsible for the toxicity of Cd are not completely understood (Stohs & Bagchi, 1995), and the antioxidant enzymatic responses observed in the organisms as a result of Cd exposure may depend on the species, metal concentration and the duration of the exposure. Cd readily interacts with sulphydryl groups of amino acids, proteins and enzymes. The displacement of essential metals such as Cu and Zn by non-essential metals like Cd would tend to change the conformation of antioxidant enzymes and affect their activity (Giguère *et al.*, 2003). Although Cd exposure altered significantly the antioxidant enzymes activities in the gills of *B. azoricus* after 24 hours, TOSC results indicate that Cd exposure did not altered the scavenging capacity of this tissue (Table 4.1). Even with SOD and CAT activities inhibited, the capacity to scavenge several radicals (peroxyl, hydroxyl and peroxynitrite) remained unchanged, indicating that gills of *B. azoricus* have relatively high resistance to oxyradical toxicity after short term Cd exposure. It is well known that ROS are produced in cells submitted to environmental stress such as exposure to metals, other xenobiotics or thermal shock. The high levels of ROS induced by such environmental stressors can lead to severe cellular injury or death (Manduzio *et al.*, 2003). Despite several studies relating TOSC levels with contaminants in marine invertebrates (Regoli *et al.*, 1998, 2000; Regoli 2000; Camus *et al.*, 2002a,b; Camus *et al.*, 2003), very few are dedicated to metal toxicity (Geracitano *et al.*, 2004), and no studies relating Cd effects in total scavenging capacity in organisms exist in the literature.

Protonation of $O_2^{-\bullet}$ can produce the hydroperoxyl radicals, which can convert fatty acids in toxic lipid peroxides, destroying biological membranes. Measurement of MDA levels is commonly used as an index of LPO under stress conditions (Wu *et al.*, 2003). In the present study, an increase of MDA levels occurred in both gills and mantle of *B. azoricus* only after 6 days of Cd exposure compared with control mussels (Figure 4.3). This indicates that although most of the antioxidant enzymes were inhibited in the gills, or remained unchanged in

the mantle during the first two days of Cd exposure, no oxidative damage to the membranes occurred in *B. azoricus*, suggesting that *B. azoricus* may have developed tolerance and resistance to Cd toxicity in the first hours of exposure. When LPO levels increased in the gills, only GPx activities are significantly inhibited. Contrary, the increase in LPO in the mantle is associated with an induction of cytosolic SOD, CAT, Total and Se-GPx enzymatic activity, suggesting that in this tissue antioxidant enzymatic defence mechanisms are unable to prevent cellular damages due to Cd toxicity.

The same relation between Cd toxicity and increasing levels of LPO were observed in the gills of the clam *R. decussatus* exposed to 100 µg l^{-1} Cd, after 21 days. Moreover, MDA levels increased linearly with the increase of Cd concentrations (Géret *et al.*, 2002a). In the same clam, during an *in vitro* study, the levels of MDA in the gills and digestive gland also increased after Cd exposure, but only with the highest concentration used (500 µg l^{-1}) (Rómeo & Gnassia-Barelli 1997). In the mussel *Mytilus edulis* exposed to 200 µg l^{-1} Cd for 21 days, the LPO levels increased in the gills and digestive gland (Géret *et al.*, 2002b). During field studies with the freshwater mussel (*Pyganodon grandis*) living along a polymetallic gradient, MDA concentrations in the gills increased with increasing gill cytosolic Cd concentrations (Giguèrre *et al.*, 2003).

It is generally accepted that MTs represent an early response to selected heavy metals, such as Cd, Ag, Cu, Hg, and that MTs play a central role in cellular metal homeostasis by storing essential metals and the detoxification of some heavy metals (Lecoeur *et al.*, 2004). However, in this study MT concentrations remained unchanged in the first hours of Cd exposure (Table 4.2). Essential and non-essential heavy metal cations react in part specifically with cellular components to cause toxic effects. Mechanisms of metal sequestration and detoxification are accomplished either via the high affinity of metal cations with sulphydryl (–SH) groups of MT or via their accumulation in membrane-limited granules, representing a general strategy for metal cation homeostasis (Domouhtsidou *et al.*, 2004).

Long term Cd exposure experiment

During long term Cd exposure, antioxidant enzymatic activities in the gills and mantle were similar in both control and Cd exposed mussels, contrary to what was observed in short term experiments (Figure 4.4). Moreover, there is a general tendency to increase activity of all antioxidant enzymes with time, mainly in the gills. These results suggest that other factors than the presence of Cd may be implicated in long term enzymatic responses of *B. azoricus*. Moreover, TOSC results indicate that scavenging capacity to sequester free radicals remained unaffected by the Cd exposure, since no significant changes in TOSC levels occurred between control and Cd exposed mussels after the exposure and depuration periods. However, antioxidant enzymes are activated in response to an increase of ROS caused by stress factors.

It is well known that ROS are produced in cells submitted to environmental stress such as exposure to metals, other xenobiotics or thermal shock. Thus, in controlled experiments many other factors besides metal exposure can produce oxidative stress. Variations in temperature, salinity, pH, pressure and other parameters may be capable of modifying the enzymatic responses in organisms. During long time Cd exposure mussels were maintained at atmospheric pressure for more than 30 days. Although *B. azoricus* is capable to survive during long periods of time in environmental conditions different from their own environment (Dando, *own data*) the possible effects of maintaining *B. azoricus* at atmospheric pressure and in a chemical different environment in biological functions are unknown. Even thought methane was continuously added during Cd long term exposure, thio and methanotrophic symbiotic bacteria may have been reduced or lost from the gills of *B. azoricus*. A reduction of symbiotic bacteria in these organisms should represent a severe nutritional and physiological negative impact. In fact, LPO levels during long term Cd exposure increased significantly in both unexposed and exposed mussels (Figure 4.6B), suggesting an enhancement of ROS production and consequent membrane damage due to other factor than metal exposure in the gills. Contrary to short term Cd exposure, where no significant differences in MT levels were observed between control and Cd exposed mussels (Table 4.2), MT synthesis during long term experiments increased significantly in the gills (Figure 4.6A). Cd was also

accumulated throughout the exposure period in both the gills and mantle of *B. azoricus*. MT induction following Cd exposure in several bivalves is well established. Synthesis of these metal-binding proteins occurred in the mussels *M. galloprovincialis* and *M. edulis* (Bebianno & Langston, 1991, 1992; Serafim *et al.*, 2002; Domouhtsidou *et al.*, 2004) and the clam *R. decussatus* (Bebianno *et al.*, 1993, 1994; Bebianno & Serafim, 1998), after exposure to similar Cd concentrations for the same period. In *B. azoricus* exposed to high Cd concentrations, MT synthesis appears more important for a long term detoxification rather than a short term, sequestering the excess of metal from tissues only after the first days of exposure. Similar results were obtained for *R. decussatus* (Géret *et al.*, 2002a) and *Crassostrea virginica* (Roesijadi & Klerk, 1989) contaminated with 100 and 200 µg l^{-1} Cd respectively.

4.6. Conclusions

In conclusion, the enzymatic defence system in *B. azoricus* is affected differently by Cd exposure, during short term (with 100 µg l^{-1} Cd) and long term (with 50 µg l^{-1}) experiments. IPOCAMP vessel is an important tool in such experiments and should be considered a more accurate experimental design. The possibility to mimic the high-pressure environment of the deep sea is vital to maintain hydrothermal organisms in good physiological conditions. The two tissues analysed showed remarkable differences regarding antioxidant enzymes responses as a result of Cd exposure. Gills appear to be more affected by Cd toxicity during the experiments. This may be related to different physiological functions of the two tissues and also with the presence of symbiotic bacteria in the gills of this species. One of the most important effects of Cd during short term exposure was the inhibition of SOD and CAT in the gills, while no significant changes in the activity of the antioxidant enzymes were observed in the mantle. However, during the same period the capacity to sequester reactive oxygen species (TOSC) remained unchanged and MT synthesis was not induced in *B. azoricus*, although Cd was accumulated in the tissues (data not shown). Contrary, during long term Cd exposure all antioxidant enzymes increase their activity, suggesting an increase production of oxygen free radicals in the cells, but rarely significant differences between control and exposed

mussels were found. Consequently, enzymatic activation in this case may be an artefact of experimental design, and/or related to other factors than metal exposure. The enzymatic responses seem to represent a short term response, capable to avoid deleterious effects of Cd within the first hours of exposure. Therefore, Cd effects on oxidative stress related biomarkers in *B. azoricus* most likely depend on the tissue analysed and time of exposure, and apparently this deep sea species is very tolerant to the presence of Cd in surrounding hydrothermal vent seawaters.

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Chapter 5

Metal detoxification mechanisms in the hydrothermal vent mussel *Bathymodiolus azoricus* exposed to Copper

5.1. Abstract

Copper (Cu) is an essential metal to various physiological processes in marine organisms. However, at high concentrations this redox-active transition metal may enhance the formation of reactive oxygen species (ROS) and subsequently initiate oxidative damage. High concentrations of Cu may increase oxidative damage to lipids, proteins and DNA. *Bathymodiolus azoricus* is a Mytilid bivalve very common in hydrothermal environments near Azores Triple Junction and is continuously exposed to high metal concentration including Cu emanating from the vent fluids. The knowledge of antioxidant defence system and other stress related biomarkers in these organisms is still scarce. The aim of this work was to study the effect of Cu on the antioxidant stress biomarkers in the gills and mantle of *B. azoricus* during a short term pressurized exposure experiment (12 and 24 hours) and a long term experiment (24 days exposure; 6 days depuration) at atmospheric pressure. In both experiments mussels were exposed to 25 µg l^{-1} Cu at 8ºC. Results show that the expression of stress related biomarkers in the gills and mantle were tissue-related in both Cu experiments. In the gills there is a general inhibition of all antioxidant enzymes during the first hours of Cu exposure, while in the mantle the activities of all antioxidant enzymes increase significantly in the first 12 hours and were only inhibited after 24 hours of Cu exposure. Metallothionein (MT) levels increased in the mantle of exposed mussels and remained unchanged in the gills although Cu was accumulated in this tissue only. Lipid peroxidation (LPO) levels were higher in both tissues of control mussels, suggesting that 25 µg l^{-1} Cu does not influence cellular damage in *B. azoricus*. In the long term experiment, the results suggest that other factors than metal exposure may influence stress biomarkers, since control and Cu exposed mussels showed little variation in antioxidant enzymes activities, MT concentrations, LPO and total oxyradical scavenging capacity (TOSC). Moreover, there is a general tendency for these parameters to increase with time, in both control and Cu-exposed mussels, suggesting that reactive oxygen species (ROS) formation is not metal dependent, and may be related with poor physiological conditions of the animals after long periods in adverse conditions compared to those in hydrothermal environments.

5.2. Introduction

Hydrothermal vents result of seawater diffusion trough fractures in the sea floor, frequently close to mid oceanic ridges. Seawater is heated when comes near the magma chamber and this superheated fluid leaches the basalts and becomes enriched with a variety of mineral compounds. Because of its lower density, the hydrothermal fluid rise up and vent out into the cold oxygenated deep-sea water. This hot end-member fluid is reduced, acidic and contains high amounts of hydrogen sulphide, hydrogen, methane, carbon dioxide and various metals (e.g. Fe, Mn, As, Cd, Cu, etc). Because of hydrostatic pressure, these fluids remain liquid at temperatures up to 350°C. When hot fluids are mixed with seawater before venting out, water emission warms up with temperatures from 10 to 50°C (Von Damm, 1990; Lowel *et al.*, 1995; Von Damm *et al.*, 1995; Sarradin *et al.*, 1998). Among metals mentioned above, high amounts of Cu are present in these environments. Douville *et al* (2002) reported Cu concentrations in the fluids ranging from 127-8896 μg l^{-1} in hydrothermal vent sites [Menez-Gwen (127 μg l^{-1}), Lucky Strike (127-1906 μg l^{-1}) and Rainbow (8896 μg l^{-1})], located in the Mid-Atlantic Ridge (MAR). These Cu concentrations are relatively high when compared to those found in coastal areas (0.2 μg l^{-1}) (Douville *et al.*, 2002).

Cu is an essential metal for many biological systems and found in small concentrations in a variety of cells and tissues. Cu ion can exist in oxidized (cupric, Cu^{2+}) and reduced (cuprous, Cu^{+}) state (Linder & Hazegh-Azam, 1996). A large number of important enzymes require Cu as a cofactor for structural and catalytic properties, including cytochrome c oxidase, tyrosinase, dopamine-β-hydroxilase, alcohol dehydrogenase, prolyl and lysyl oxidase (Nath, 1997; Suzuki *et al.*, 2002). These enzymes are involved in many important biological processes required for growth, development and maintenance. Cu is also cofactor in Cu/Zn-SOD, which is unique in requiring two essential metals for catalytic function. Harris (1992) showed that Cu deficiency quickly impairs the catalytic function of Cu/Zn-SOD. Some of the pathological changes observed in Cu deficiency may be due in part to alterations in gene transcription of SOD

enzymes (Lai *et al.*, 1996). Also the presence of excess Cu can produce mutant forms of Cu/Zn-SOD, which aggregate in pore-like structures associated to neurodegenerative diseases (Chung *et al.*, 2003). Additionally, bivalves need extra Cu for the components of the respiratory pigment, hemocyanin (Rainbow, 1990).

Several mechanisms have been proposed to explain Cu induced cellular toxicity, including the capacity of free Cu ions to participate in the formation of reactive oxygen species (ROS) (Li *et al.*, 2002; Gaetke & Chow, 2003; Pourahmad *et al.*, 2003). Redox active metals ions such as Fe and Cu play an important role in the formation of ROS (Fenton reaction) in biological systems (Aust *et al.*, 1985; Cheeseman & Slater, 1993). Both cupric and cuprous Cu ions can participate in oxidation and reduction reactions. Cu^{2+} can produce the highly reactive intermediate singlet oxygen ($^1O_2$) from phosphatidylcholine hydroperoxide, the oxidized modification product of a major constituent of biomembranes and serum lipoproteins (Takayama *et al.*, 2001). Singlet oxygen is estimated to induce oxidative injuries by oxidizing relatively oxidation-resistant components or cleaving DNA, causing cell destruction (Li *et al.*, 1994). Moreover, in the presence of superoxide ($O_2^{-\bullet}$) or reducing agents such as ascorbic acid or GSH, Cu^{2+} can be reduced to Cu^{+}, which is capable of catalysing the formation of hydroxyl radicals (OH•) from hydrogen peroxide (H_2O_2) through Haber-Weiss reaction (Kadiiska *et al.*, 1993; Bremner, 1998; Kadiiska & Mason, 2002).

These reactions occur during the metabolism of oxygen and can be considered the major mechanism by which the highly reactive hydroxyl radical is generated in biological systems (Liochev, 1999; Kehrer, 2000). This radical is the most powerful oxidizing radical present in biological systems, and is capable of reacting with practically every biological molecule (Buettner, 1993).

Peroxyl radical (ROO•) is involved in the first steps of lipid peroxidation (LPO) and derives from oxygen addition to the lipid alkyl radical intermediate, which arises by reaction of the lipids with the ROS or hypervalent metal. The peroxyl radical rapidly reacts with another lipid to generate a new peroxyl radical and a

lipid hydroperoxide. Peroxynitrite (HOONO) is another potent oxidant formed by a rapid reaction between nitric oxide (NO) and superoxide anion (Millar, 2004). Despite of its non-radical nature, peroxynitrite initiates lipid peroxidation, causes DNA breakage, enzyme inhibition and reacts with thiols. Peroxynitrite-induced protein modifications include protein oxidation (on methionine, cysteine, tryptophane or tyrosine residues) and nitration (of tyrosine or tryptophane residues) (Virág, *et al.*, 2003).

Organisms have developed antioxidant defence mechanisms to scavenge ROS and consequently control the oxidative damage they induce (Cheeseman & Slater, 1993). Enzymatic antioxidant systems include the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx) (Fridovich, 1978). Total Oxyradical Scavenging Capacity (TOSC) focus on the balance between antioxidant parameters and prooxidants factors (Winston *et al.*, 1998). TOSC assay measures the capability of a tissue to neutralize ROS in quantifiable terms, predicting the effects of environmental conditions on the redox status of the organisms and their susceptibility to oxidative stress and was successfully validated as a biomarker (Regoli & Winston, 1998; Regoli, 2000).

One of the most important consequences of excess of Cu is peroxidative damage to membrane lipids. LPO occurs by the reaction of lipid radicals and oxygen to form peroxy radicals (Chow, 1979; Powell, 2000). Lipid peroxyl radicals can damage cells by changing the fluidity and permeability of the membrane or attacking directly DNA and other intracellular molecules, such as proteins (Mattie & Freedman, 2001). Hydroxyl radical causes hydrogen depletion as the initial reaction in LPO (Hayashi *et al.*, 2002). Evidences that LPO increases as consequence of Cu exposure has been observed in several bivalves from coastal zones, including the mussels *Perna perna* (Almeida *et al.*, 2004), clams *Ruditapes decussatus* (Géret *et al.*, 2002) and oysters *Crassostrea virginica* (Ringwood *et al.*, 1998; Conners & Ringwood, 2000).

Bathymodiolus azoricus is a Mytilid bivalve commonly found in Azores Triple Junction (MAR) hydrothermal vents. These mussels are frequently the dominant specie and form extensive mussel beds in high-density clusters surrounding the

hydrothermal active area and covering the base and walls of vent chimneys (Desbruyères *et al.*, 2000).

Although several studies have been carried out on the effects of metals on hydrothermal vent species, especially regarding mechanisms of metal uptake and detoxification (Cosson-Mannevy *et al.*, 1988; Smith & Flegal, 1989; Cosson, 1997; Cosson & Vivier, 1995; Géret, *et al.*, 1998; Rousse *et al.*, 1998), little is known about the presence of antioxidant enzymatic defences and lipid peroxidation in hydrothermal vent organisms. Earlier studies identified the presence of CAT, SOD and GPx in two important vent organisms, the tubeworm *Riftia pachyptila* and the clam *Calyptogena magnifica* collected from the East Pacific Rise (Blum & Fridovich, 1984). Also, previous investigations characterized baseline activity of antioxidant enzymes of *B. azoricus* in MAR vent fields (See Chapter 2 and 3; Bebianno *et al.*, submitted) and studied the effect of Cd exposure in the antioxidant defence system in this mussel (See Chapter 4; Company *et al.*, submitted).

The aim of this work was to study short and long term effects of Cu exposure in several stress related biomarkers, including the activity of antioxidant enzymes (SOD, CAT and GPx), TOSC, LPO levels and metallothionein concentrations in the hydrothermal vent mussel *B. azoricus*.

5.3. Materials and Methods

Sample collection

B. azoricus were sampled in Menez Gwen hydrothermal vent site (37°51'N, 32°31'W, 840 m depth) using acoustically retrievable cages positioned in this hydrothermal site and filled with mussels during ATOS cruise in summer 2001 (Sarradin *et al.*, 2001). The mussels were recovered and transported to a land-based laboratory (Department of Oceanography and Fisheries – University of Azores). During the 15 hours transit, the organisms were held in chilled seawater at 9°C, their natural ambient temperature, and subsequently transferred to a cold room held at the same temperature (Dixon *et al.*, 2001).

Cu exposure experiments

Mussels were exposed to 25 µg l^{-1} Cu in two separate experiments. A short term exposure at controlled pressure for 12 and 24 hours and a long term exposure at atmospheric pressure and the same temperature during 24 days, followed by a depuration period in clean water for another 6 days. In both experiments the organisms were acclimatized for one week to reduce stress after cage recovery. During both the acclimatizing and experimental periods the water was enriched with methane (17 - 54 µM), an important element in hydrothermal vent environment to maintain the symbiotic bacteria in the gills of *B. azoricus*. No sulphur was added to prevent precipitation of Cu in the water solution.

Short term Cu exposure experiment

Ten mussels (7.02 ± 0.30 cm shell length) were exposed to 25 µg l^{-1} (nominal concentration) inside the pressured IPOCAMP tank (*Incubateurs Pressurises pour l'Observation en Culture d'Animaux Marins Profonds*) for 12 and 24 hours at 9°C. At the same time another set of 10 organisms (6.95 ± 0.42 cm shell length) were maintained in similar conditions in a second pressure tank in clean filtered seawater collected from the Azores coastal zone (controls). Water in each container was changed every 12 hours to avoid oxygen depletion and Cu concentration re-established. Pressure was re-established after 30 minutes.

Water temperature was unaffected because the system was thermo stabilised at 9°C. Shell length was measured, gills and mantle dissected and immediately frozen in liquid nitrogen. Both tissues were stored at –80°C until further analysis.

Long Term Cu exposure experiment

Two groups of mussels of fifty individual each (7.73 ± 0.82 cm shell length) were exposed to 0 (control) and 25 µg l^{-1} Cu (exposed) for 24 days, followed by a depuration period of 6 days in clean seawater. Water (9°C) was changed every two days and Cu concentration re-established in exposed mussels. A group of 10 organisms were sampled from both groups at day 6, 12, 18, 24 during the exposure period and at the end of depuration period. Gills and mantle dissected, immediately frozen in liquid nitrogen and stored at –80°C until treated as described bellow.

Biochemical determinations

Antioxidant enzymes

Symbiotic bacteria were not separated from the gills, thus the enzymatic activities in this tissue reflect the contributions of both host and symbionts.

Antioxidant enzymatic activities were determined in the gills (tissue + symbionts) and mantle of *B. azoricus* after homogenisation in 20 mM Tris buffer, pH 7.6, containing 1mM of EDTA, 0.5M of saccharose, 0.15M of KCl and 1mM of DTT. The homogenates were centrifuged at 500 *g* for 15 min at 4°C to precipitate large particles and centrifuged again at 12000 *g* for 45 min at 4°C to precipitate the mitochondrial fraction. Supernatants were purified on a Sephadex G-25 gel column to remove low molecular weight proteins.

SOD activity (EC 1.15.1.1) was determined by measuring the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm (McCord & Fridovich, 1969). One unit of SOD is defined as the amount of enzyme that inhibits the reduction of cytochrome c by 50%. SOD activity is expressed in U SOD mg^{-1} total protein concentrations.

CAT activity (EC 1.11.1.6) was determined according to Greenwald (1985) by the decrease in absorbance at 240 nm due to H_2O_2 consumption. The CAT activity is expressed as mmoles min^{-1} mg^{-1} of total protein concentrations.

GPx activities were measured following NADPH oxidation at 340nm in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (Lawrence & Burk, 1976). The Se-GPx (EC 1.11.1.9) and Total GPx activities were measured by using respectively, H_2O_2 and cumene hydroperoxide as substrates. GPx activities are expressed as µmoles min^{-1} mg^{-1} of total protein concentrations.

Total oxyradical scavenging capacity (TOSC)

TOSC was determined by the method based on Winston *et al* (1998) and Regoli & Winston (1999), modified by adjusting the buffers used for marine bivalves (Regoli *et al.*, 2000). TOSC was only determined in the gills since this tissue exhibits significantly higher levels, compared to the mantle (See Chapter 2; Bebianno *et al.*, submitted) and for this reason could indicate more easily the changes in the oxidative stress susceptibility in *B. azoricus*. The gills were homogenised with a Potter-Elvehjem glass/Teflon homogeniser in four volumes of 100 mM KH_2PO_4 buffer, 2.5% NaCl, pH 7.5. The homogenate was centrifuged at 100 000 *g* for 1 h, and cytosolic fractions were aliquoted and stored at –80°C. Peroxyl radicals are generated by the thermal homolysis of 2-2'-azo-bis-(2 methyl-propionamidine)-dihydrochloride (ABAP) at 35°C. The iron-ascorbate Fenton reaction was used for hydroxyl radicals, while peroxynitrite was generated from 3-morpholinosydnomine (SIN-1), a molecule that releases concomitantly nitric oxide and superoxide anion, which rapidly combine to form HOONO. The data acquisition system was run by the software Millenium32® (Waters). Each analysis required the measurement of control (no antioxidant in the reaction vial) and sample reactions (biological fluid in the vial). Data are expressed as TOSC unit mg^{-1} protein. TOSC analyses were carried out by Dr. Lionel Camus from the University Centre on Svalbard (Norway).

Metallothioneins (MTs)

To determine MTs concentrations, the tissues were homogenized at 4°C using an electric potter and a Teflon pestle in a Tris buffer (100 mM), pH 8.1, containing 10 mM of β-mercaptoethanol. The soluble and insoluble fractions were separated by centrifugation (30000 *g*, 30 min, 4°C). Aliquots of the supernatants were heated (15 min, 95°C) and allowed to cool on ice. Heat-denatured proteins were separated from heat-stable proteins by centrifugation of the heated supernatants (10000 *g*, 15 min). Supernatants containing the heat-stable proteins, including MTs, were stored at -20°C until use.

In the heat-denatured cytosol, the amount of MTs was determined by differential pulse polarography using a PAR 394 analyser and a EG&G PAR 303A static mercury drop electrode (SMDE) in accordance with the method of Olafson & Sim (1979) modified by Thompson & Cosson (1984). The electrochemical detection of MTs takes place in an ammoniacal electrolyte containing cobalt that catalyses the reduction of the cystein thiol groups (Brdicka, 1933). The standard addition method was used for calibration with rabbit liver MT (Fluka) in the absence of *B. azoricus* MT standard. The levels of MTs are expressed as mg g^{-1} wet weight. MT analyses were performed by Dr. Richard Cosson from ISOMer Marine Biology Laboratory in the University of Nantes (France).

Total protein concentrations

The tissues were homogenized in 20 mM Tris buffer, pH 8.6, containing 150 mM of NaCl. The homogenates were centrifuged for 30 min at 30000*g* at 4°C. Total protein concentrations were measured on supernatants by the Lowry method (Lowry *et al.*, 1951) using BSA as reference standard material. Protein concentrations are expressed as mg g^{-1} wet weight tissue.

Lipid peroxidation

Lipid peroxidation was determined in the supernatant used for total proteins quantification. The method described by Erdelmeier *et al* (1998) measures the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) produced during decomposition of polyunsaturated fatty acid peroxides of membrane

lipids. This procedure is based on the reaction of chromogenic reagent, N-methyl-2-phenylindole (R1), where two moles of R1 react with one mole of either MDA or 4-HNE at 45 °C for 60 minutes to yield a stable chromophore with maximal absorbance at 586 nm. The levels of MDA + 4-HNE were estimated at 586 nm using malonaldehyde bis (tetrametoxypropan, SIGMA) as standard. The concentration of lipid peroxidation compounds in the gills and mantle of *B. azoricus* were expressed as nmoles of MDA + 4-HNE g^{-1} total protein concentrations.

Metals

Cu quantification was determined by ET-AAS (Electrothermal atomic absorption spectrometry: Z-5000 polarized Zeeman AAS Hitachi and 989QZ AAS Solaar Unicam) or FAAS (Flame atomic absorption spectrometry: Z-5000 polarized Zeeman AAS Hitachi) or ICP-AES (Inductively coupled plasma-atomic emission spectrometry: JY-238 sequential) depending on the level of concentration and the volume of the liquid aliquot available. The accuracy of the analytical procedure was checked using certified reference material (TORT-2). Defatted lobster hepatopancreas (*Homarus americanus*) (DORM-2). Dogfish muscle (*Squalus acanthias*) (DOLT-2). Dogfish liver (*Squalus acanthias*) from the National Research Council, Canada. Results were in good agreement with certified values. Cu concentrations were expressed as μg g^{-1} dry weight tissue. These analyses were carried out by Lic. Inês Martins in the University of Paris IV (France) under the supervision of Dr. Jacques Boulègue and Dr. Aline Fiala-Médioni.

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.

5.4. Results

5.4.1. Short term Cu exposure experiment

Antioxidant enzymes

In Figure 5.1 are presented the enzymatic activities of cytosolic and mitochondrial SOD, CAT, total and Se-GPx in the gills and mantle of the mussels exposed to 25 μg l^{-1} Cu in a pressured container for 12 and 24 hours.

Superoxide Dismutase

SOD was like in the previous cases (Chapter 3 and 4) predominantly present in the cytosolic fraction (70-85%) of both gills and mantle of control and Cu-exposed *B. azoricus*. The SOD activity in the cytosolic fraction was 2-fold higher in the gills when compared with mantle after 12 hours of Cu exposure. Gills also exhibit significantly higher mitochondrial SOD activity after 12 hours of Cu exposure than the mantle (4-fold). However, after 24 hours of Cu exposure both tissues have approximately the same cytosolic and mitochondrial SOD activity (Figure 5.1A and B). The cytosolic SOD activity in the gills of control mussels was significantly higher than in Cu exposed *B. azoricus* at both collection times ($p<0.05$). In the mantle, however a significant inhibition in cytosolic SOD activity occurred after 24 hours of Cu exposure ($p<0.05$) (Figure 5.1A).

The SOD activity in the mitochondria showed a very different pattern compared with the cytosolic fraction (Figure 5.1B). SOD activity was significantly higher in Cu-exposed mussels (both gills and mantle) after 12 hours of exposure, compared to controls. However, after 24 hours of Cu exposure, a significant inhibition of mitochondrial SOD activity occurred in both tissues ($p<0.05$) (Figure 5.1B).

Catalase

CAT activity was approximately 3-fold higher in the gills when compared with mantle at all exposure times ($p<0.05$). In the gills, although CAT activity was inhibited in Cu exposed mussels this inhibition was only significant after 24 hour of exposure ($p<0.05$). In the mantle however, CAT activity increased in Cu-

exposed mussels after 12 hours of exposure, but after 24 hours CAT was also significantly inhibited in this tissue (p<0.05) (Figure 5.1C).

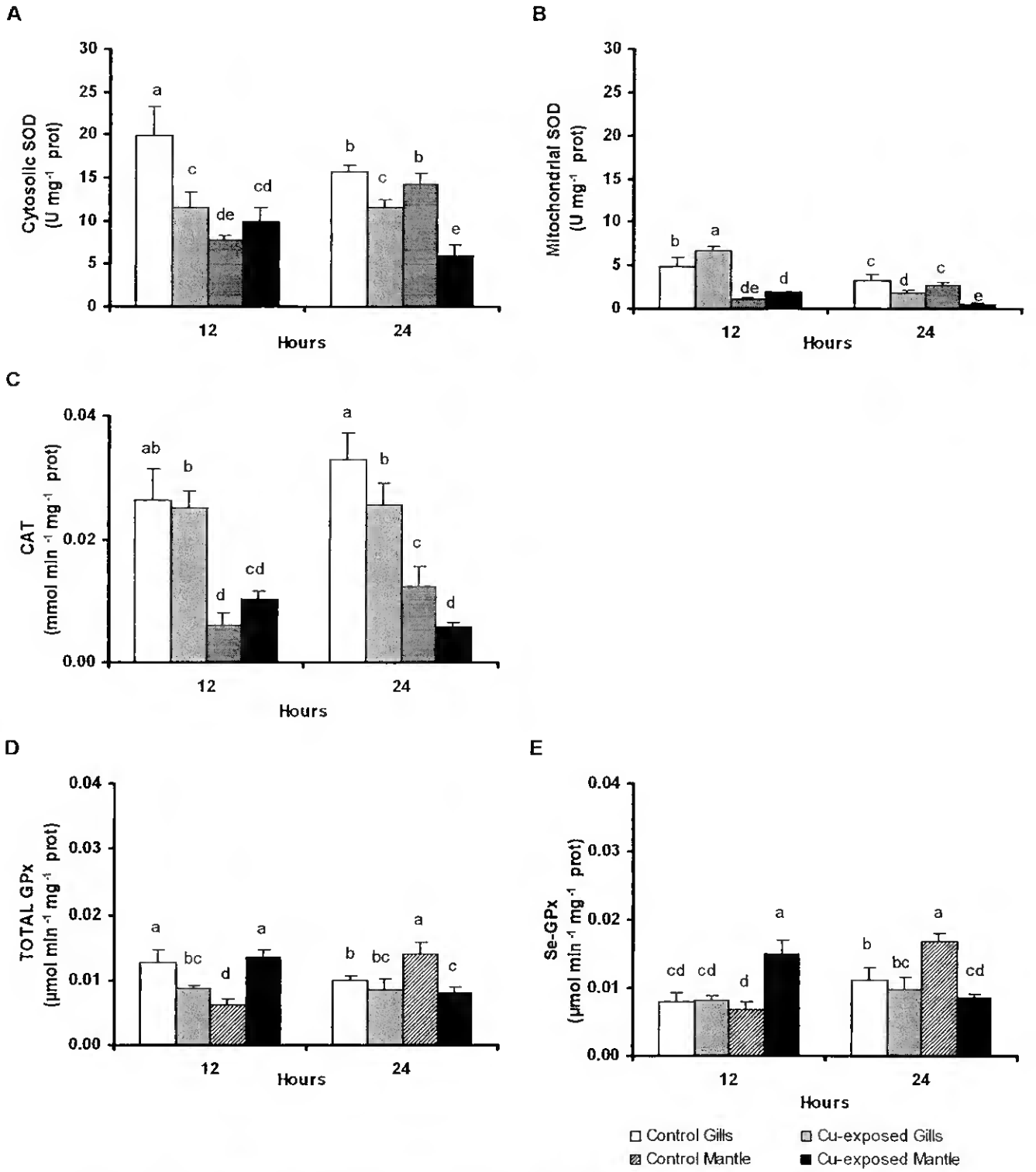

Figure 5.1 – Mean variation (± SD) of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills and mantle of control and Cu exposed (25 µg l^{-1}) *B. azoricus* for 12 and 24 hours in IPOCAMP. Values followed by the same letter are not statistically different (p>0.05).

Glutathione peroxidases

In general, levels of total GPx activities were similar between both tissues ($p>0.05$). The activity of this enzyme in the gills was inhibited at both exposure times although it was only significant after 12 hours of Cu exposure ($p<0.05$). In the mantle, as observed for SOD and CAT activities, total GPx was significantly higher in Cu-exposed mussels in the first 12 hours and was inhibited after 24 hours of exposure ($p<0.05$) (Figure 5.1D).

As observed for Total GPx levels, Se-GPx activities were similar between gills and mantle ($p>0.05$). In the gills, the activity of this enzyme remained unchanged during the whole exposure period ($p>0.05$). In the mantle, the same pattern of Total GPx was observed, with enzymatic activation in the first 12 hours of exposure and consequent inhibition after 24 hours of Cu exposure ($p<0.05$) (Figure 5.1E).

Metallothionein concentrations

MT concentrations were determined after 12 and 24 hours of Cu exposure in the gills and mantle of *B. azoricus* (Figure 5.2A) and were 3-fold higher in the gills compared with the mantle in both control and Cu-exposed mussels ($p<0.05$). MT induction was only observed in the mantle of Cu exposed mussels after 12 and 24 hours ($p<0.05$), while in the gills no significant differences occurred between control and Cu-exposed mussels during the experiment ($p>0.05$) (Figure 5.2A).

Lipid peroxidation

Figure 5.2B shows the LPO levels in both tissues of *B. azoricus* exposed to Cu for 12 and 24 hours. The products of LPO were significantly higher (between 3 to 6-fold) in the gills of *B. azoricus* compared with the mantle ($p<0.05$) (Figure 5.2B). After the mussels were exposed to Cu for 12 hours the LPO concentrations increased significantly in the gills, while in the mantle LPO levels decreased but were not significantly different from controls ($p>0.05$). Surprisingly, LPO levels significantly decreased in both gills and mantle after 24 hours of Cu exposure ($p<0.05$) (Figure 5.2B).

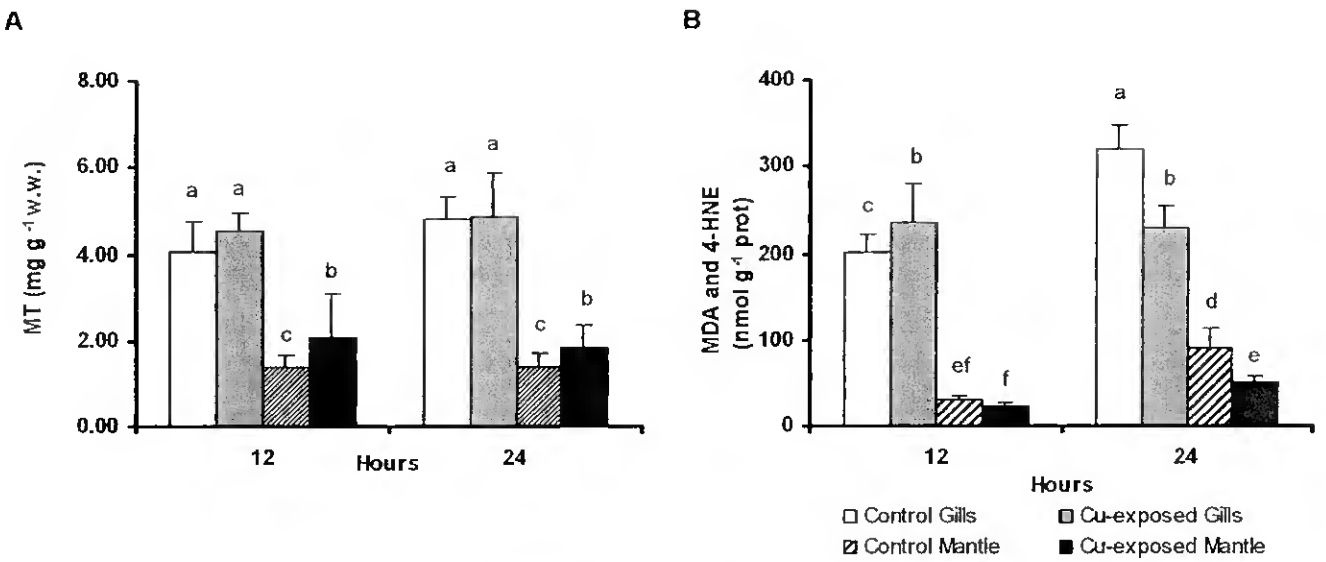

Figure 5.2 – Mean variation of MT concentrations (A) and MDA + 4-HNE compounds levels (B) in the gills and mantle of control and Cu exposed (25 µg l^{-1}) *B. azoricus* for 12 and 24 hours in IPOCAMP. Values followed by the same letter are not statistically different (p>0.05).

Metal accumulation

Figure 5.3 shows Cu accumulation in the gills and mantle of *B. azoricus* after 12 and 24 hours of metal exposure.

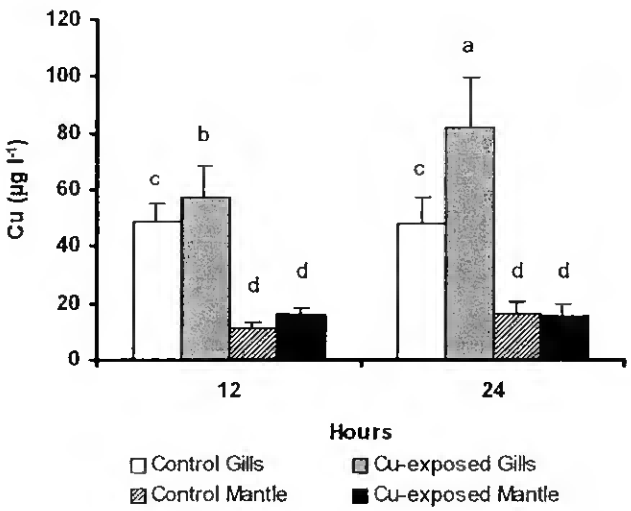

Figure 5.3 – Mean variation of Cu concentrations in the gills and mantle of control and Cu exposed (25 µg l^{-1}) *B. azoricus* for 12 and 24 hours in IPOCAMP. Values followed by the same letter are not statistically different (p>0.05).

Cu concentrations remained unchanged in both tissues of control mussels (48.3 ± 7.7 µg l^{-1} and 13.7 ± 3.3 µg l^{-1} for gills and mantle respectively) during the whole experiment. This metal was accumulated in the gills and Cu accumulation increased significantly with time (p<0.05). On the other hand, Cu remained unchanged in the mantle during the experiment (p>0.05) (Figure 5.3).

5.4.2. Long Term Cu exposure experiment

Antioxidant enzymes

Figure 5.4 shows the antioxidant enzymes activity of SOD, CAT and GPx in the gills and mantle of *B. azoricus* exposed to 25 µg l^{-1} Cu during 24 days, followed by 6 days of depuration.

Superoxide dismutase

Like in the short term Cu exposure, SOD was predominantly present in the cytosolic fraction of both gills and mantle of control and Cu-exposed *B. azoricus* (70-85%) compared to the mitochondria. SOD in the cytosolic fraction was 2-fold higher in the gills compared to the mantle, while in the mitochondria both tissues exhibited approximately the same SOD activity (Figure 5.4A and B).

The cytosolic SOD activity in the gills of control mussels increased significantly (p<0.05) in the first 6 days of exposure, and remained unchanged until day 18 (16.5 ± 0.6 U mg^{-1} protein). After this period SOD increased significantly until the end of exposure period and remained unchanged in the depuration period. In the gills of Cu-exposed mussels, cytosolic SOD activity increased exponentially throughout the exposure period (SOD $_{[U\ mg^{-1}\ protein]} = 12.04e^{0.03t}{}_{[days]}$, r = 0.979, p<0.05). After 6 days in clean seawater, the activity of this enzyme significantly decreased and reached a level similar of unexposed gills (p<0.05) (Figure 5.4A).

In the mantle the cytosolic SOD activity remained unchanged in both control (9.5 ± 1.8 U mg^{-1} protein) and Cu-exposed mussels (8.6 ± 1.4 U mg^{-1} protein) (Figure 5.4A).

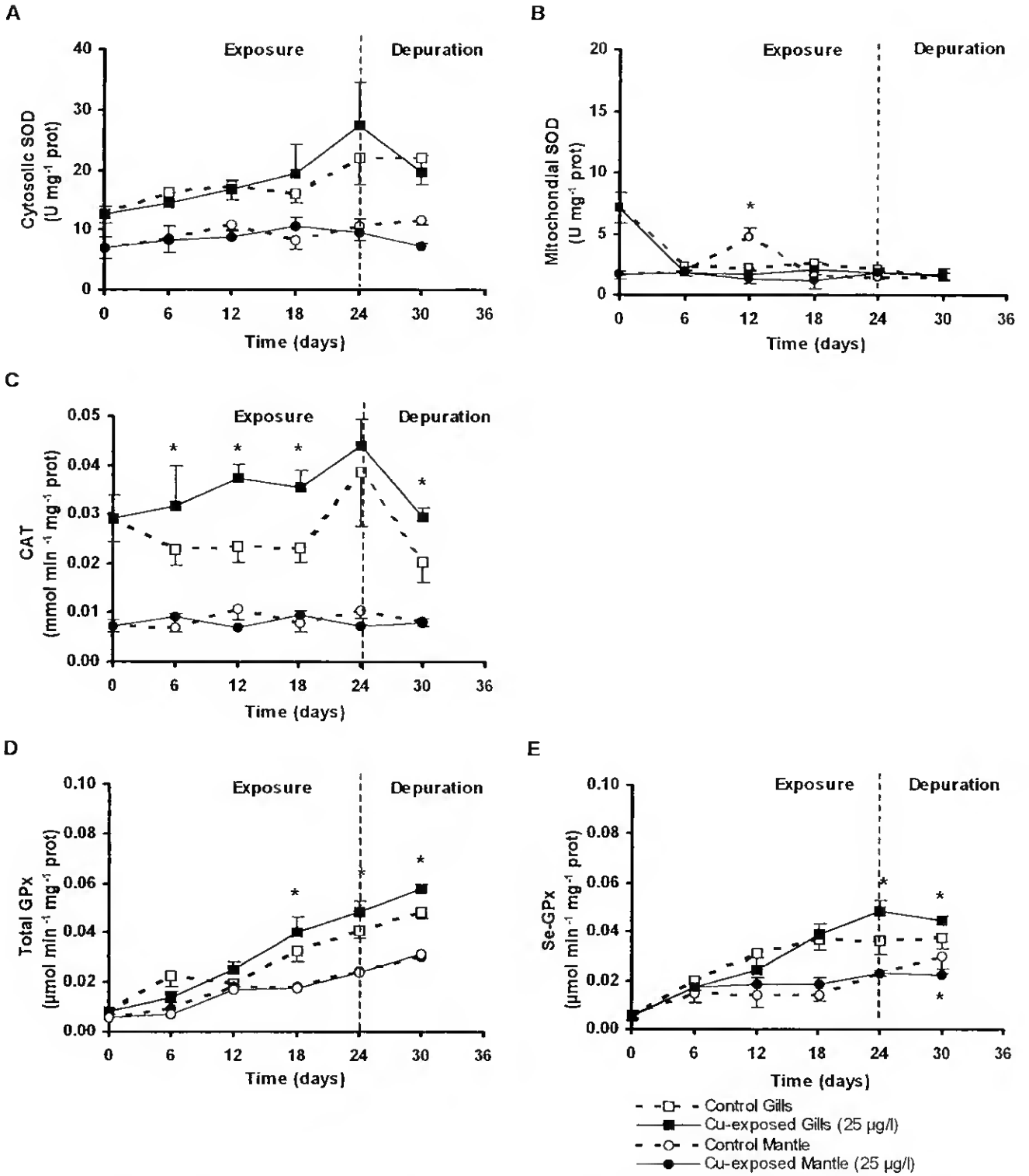

Figure 5.4 – Mean variation of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills and mantle control and Cu exposed (25 µg l^{-1}) *B. azoricus* for 24 days and 6 days of depuration. Vertical bars represent one-half of the standard deviation of the mean. Symbol * represents significant differences between control and Cu-exposed mussels.

The mitochondrial SOD activity in the gills of both control and Cu exposed mussels decreased significantly in the first 6 days and remained unchanged until the end of the experiment (control: 2.1 ± 0.4 U mg^{-1} protein and Cu exposed: 1.8 ± 0.2 U mg^{-1} protein) (p>0.05) (Figure 5.4B).

Also, in the mantle, the activity of mitochondrial SOD remained relatively unchanged throughout the experiment. In control mussels the activity of this enzyme remained unchanged (1.5 ± 0.2 U mg^{-1} protein), except in day 12 where it significantly increased reaching 4.6 ± 0.7 U mg^{-1} protein. In the mantle of Cu-exposed mussels, the mitochondrial SOD activity also remained unchanged during the exposure and depuration periods (1.5 ± 0.2 U mg^{-1} protein) (p>0.05). No significant differences in the activity of mitochondrial SOD was observed in the mantle between control and exposed mussels, except at day 12.

Catalase

Like in the short term Cu exposure experiment, CAT activity was significantly higher in the gills when compared with mantle of control and exposed mussels at all exposure times (Figure 5.4C).

CAT activity in the gills of control mussels decrease significantly in the first 6 days and remained unchanged until day 18 (0.023 ± 0.0004 mmol min^{-1} mg^{-1} protein). Afterwards, the activity of this enzyme increased significantly at day 24 (0.04 ± 0.01 mmol min^{-1} mg^{-1} protein), followed by a decrease until the end of depuration period (0.02 ± 0.004 mmol min^{-1} mg^{-1} protein). In the gills of Cu exposed mussels, CAT activity increased exponentially until the end of exposure period (CAT [mmol min^{-1} mg^{-1} protein] = $0.029e^{0.016[days]}$, r = 0.979, p<0.05), decreasing significantly afterwards when the mussels were transferred to clean seawater. Generally, Cu exposed mussels exhibited higher CAT activities in this tissue compared to controls (p<0.05), except in day 24, where the activity of this enzyme was similar between control and Cu treated mussels (p>0.05).

Contrary, CAT activity in the mantle of *B. azoricus* were similar between control and Cu exposed mussels ($p>0.05$) and levels remained unchanged throughout the experiment in both control (0.009 ± 0.002 mmol min^{-1} mg^{-1} protein) and Cu exposed mussels (0.008 ± 0.001 mmol min^{-1} mg^{-1} protein) (Figure 5.4C).

Glutathione peroxidases

Total GPx activity was significantly higher in the gills compared with the mantle except at day 12, where no significant differences occurred between the two tissues (Figure 5.4D). Total GPx activity increased linearly in the gills of both control (Total GPx [µmol min^{-1} mg^{-1} protein] = 0.0018t + 0.006, $r = 0.994$, $p<0.05$) and Cu-exposed mussels (Total GPx [µmol min^{-1} mg^{-1} protein] = 0.0013t + 0.009, $r = 0.974$; $p<0.05$). The increment of total GPx in Cu exposed mussels was significantly higher than that of controls ($p<0.05$). As observed in the gills, in the mantle total GPx activity also increased linearly with time in both control (Total GPx [µmol min^{-1} mg^{-1} protein] = 0.0009t + 0.0042, $r = 0.980$, $p<0.05$) and Cu exposed mussels (Total GPx [µmol min^{-1} mg^{-1} protein] = 0.0008 + 0.0056, $r = 0.981$, $p<0.05$), but the increase was similar between controls and Cu exposed mussels (Figure 5.4D).

Like for total GPx, Se-GPx activity was significantly higher in the gills, except at day 6 and 12 where no significant differences between tissues were found (Figure 5.4E). The activity of this enzyme in the gills of control mussels increased linearly until day 18 (Se-GPx [µmol min^{-1} mg^{-1} protein] = 0.0018t [days] + 0.007, $r = 0.982$, $p<0.05$) and after that time remained unchanged even during the depuration period. In the gills of Cu exposed mussels Se-GPx also increased linearly until the day 24 (Se-GPx [µmol min^{-1} mg^{-1} protein] = 0.0018t [days] + 0.005, $r = 0.996$, $p<0.05$). Nevertheless after transferring the mussels to clean seawater levels remained unchanged. However, the increment of Se-GPx between the two treatments was not significantly different ($p>0.05$). In fact, significant differences in Se-GPx activity between control and Cu exposed mussels only occurred in day 24 and 30 ($p>0.05$).

The activity of Se-GPx in the mantle of control mussels increased significantly in the first 6 days, and remained unchanged until the 18 day of the experiment (0.014 ± 0.0005 µmol min^{-1} mg^{-1} protein). After this period, a linear increase in Se-GPx activity was observed until the end of the experiment (0.0014 µmol min^{-1} mg^{-1} d^{-1}; r = 0.996, p<0.05). Similarly, in the mantle of Cu exposed mussels, Se-GPx activity increased significantly in the first 6 days of Cu exposure, remaining unchanged until the end of the experiment (0.020 ± 0.0025 µmol min^{-1} mg^{-1} protein).

TOSC

The TOSC assay represents an integrated interpretation in terms of health condition and susceptibility to oxidative stress for exposed organisms. Therefore, TOSC was determined exclusively in pre-exposed organisms to set up baseline values for this parameter and at the end of exposure (day 24) and depuration period (day 30) (Figure 5.5).

TOSC levels toward peroxyl radicals decreased significantly in both control and Cu exposed mussels after 24 days of Cu exposure, when compared to unexposed mussels (p<0.05) (Figure 5.5A). However, at the end of depuration period, scavenging capacity levels were similar to those found in pre-exposed *B. azoricus* (661 ± 21 TOSC unit mg^{-1} protein). In Cu exposed TOSC levels mussels were higher than in controls in day 24 and 30, although this increase was not significant (p>0.05).

A similar pattern was observed for the TOSC toward hydroxyl radicals, where TOSC decreased significantly during the course of the experiment in both control and Cu exposed mussels, and afterwards were reestablished at the end of depuration period to levels comparable to pre-exposed mussels (253 ± 61 TOSC unit mg^{-1} protein) (Figure 5.5B). Again, TOSC values were slightly higher in Cu exposed mussels, but not significantly different (p>0.05).

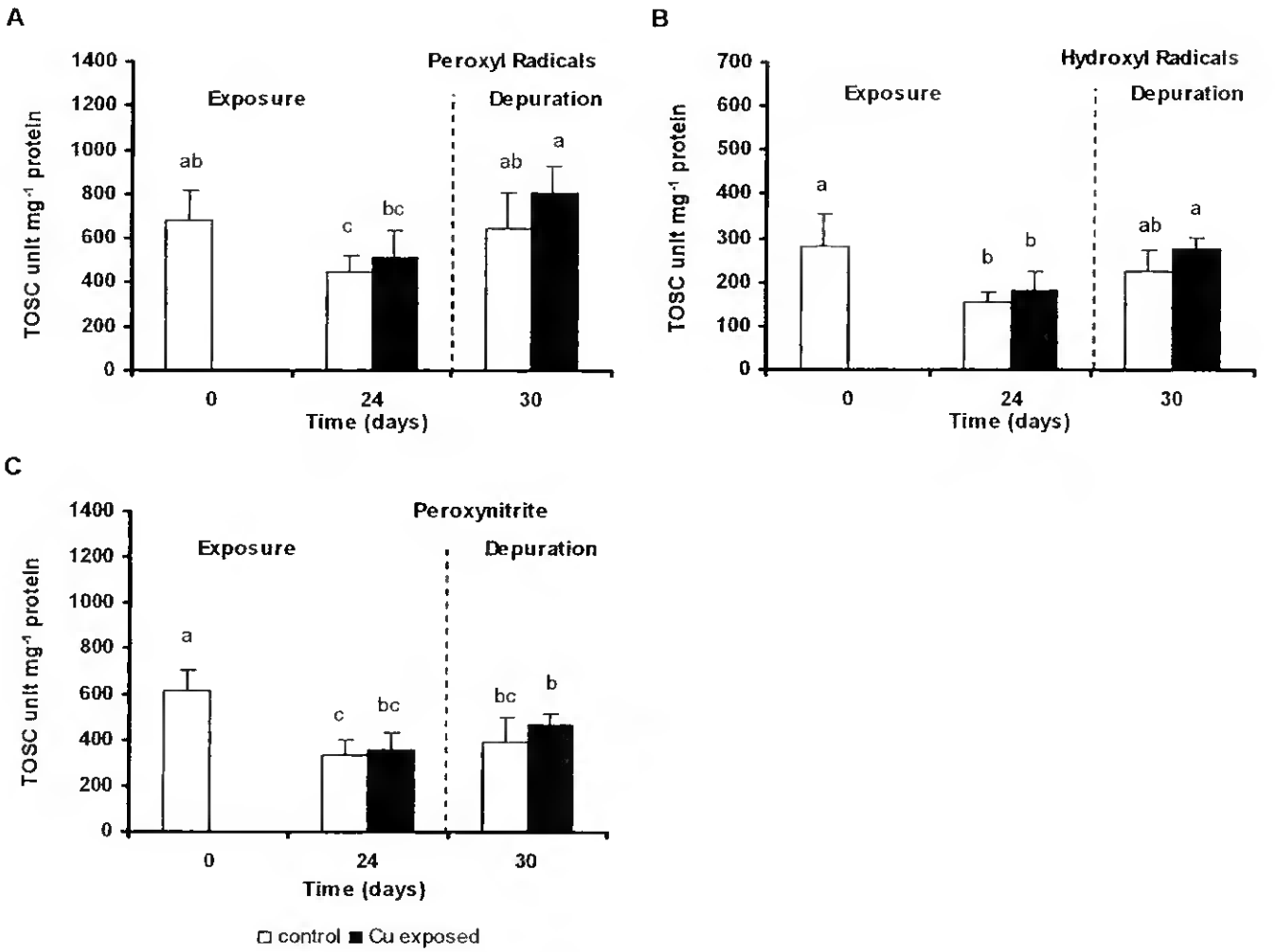

Figure 5.5 – Mean variation (±SD) of the Total Oxyradical Scavenging Capacity (TOSC) towards peroxyl radicals (A), hydroxyl radicals (B) and Peroxynitrite (C) in the gills of control and Cu exposed (25 µg l^{-1}) *B. azoricus* for 24 days followed by a depuration period of 6 days. Values followed by the same letter are not statistically different (p>0.05).

Regarding TOSC toward peroxynitrite, both control and Cu exposed mussels exhibited a significant reduction at the end of exposure and depuration periods compared to mussels collected in the field (p<0.05), but similar between control and Cu-exposed mussels in each day (p>0.05) (Figure 5.5C).

Metallothioneins

MT concentrations in the gills and mantle of control *B. azoricus* and exposed to 25 µg l^{-1} Cu for 24 days, followed by 6 days of depuration are presented in Figure 5.6A.

MT concentrations were significantly higher in the gills, compared with the mantle throughout the experiment ($p<0.05$). MT levels in the gills of control mussels remained unchanged during the exposure and depuration periods (3.06 ± 0.25 mg g^{-1}). In the gills of Cu exposed mussels, MT levels remained unchanged during the first 12 days (3.11 ± 0.22 mg g^{-1}), but increased significantly until day 18 of the experiment (4.31 ± 0.72 mg g^{-1}). After this period, MT levels decreased significantly until the end of the experiment (0.113 mg g^{-1} d^{-1}, $r = 0.994$, $p<0.05$) even during the depuration period. However, MT concentrations in the gills of exposed mussels were only significantly higher than controls at day 18 ($p<0.05$) (Figure 5.6A).

In the mantle a similar pattern was observed. MT concentrations remained unchanged throughout the experiment in control mussels (1.80 ± 0.15 mg g^{-1}). In Cu exposed mussels MT concentrations remained unchanged until the day 12, increasing afterwards in day 18. After this period, MT concentrations also decrease linearly until the end of the experiment (0.064 mg g^{-1} d^{-1}, $r = 0.936$; $p < 0.05$) including in the depuration period. As observed for the gills, MT concentrations were similar between control and exposed mussels, except in day 18 were Cu exposed mussels exhibited higher MT levels compared to controls ($p<0.05$) (Figure 5.6A).

Lipid peroxidation

The products of LPO in the gills and mantle of control and Cu exposed (25 µg l^{-1}) *B. azoricus* for 24 days, followed by 6 days of depuration are presented in Figure 5.6B.

Like in short term Cu experiment, LPO levels were significantly higher in the gills than in the mantle ($p<0.05$). In the gills of control mussels, MDA and 4-HNE revealed some fluctuations with concentrations increasing linearly in the first 12 days of experiment (14.05 nmol g^{-1} protein d^{-1}, $r = 0.981$, $p<0.05$), and decreasing significantly afterwards until the day 18 (162.86 ± 22.53 nmol g^{-1} protein). During the last week of the exposure period LPO, levels significantly

increase (401.71 ± 102.22 nmol g^{-1} protein), decreasing significantly until the end of the depuration period (310.99 ± 29.85 nmol g^{-1} protein).

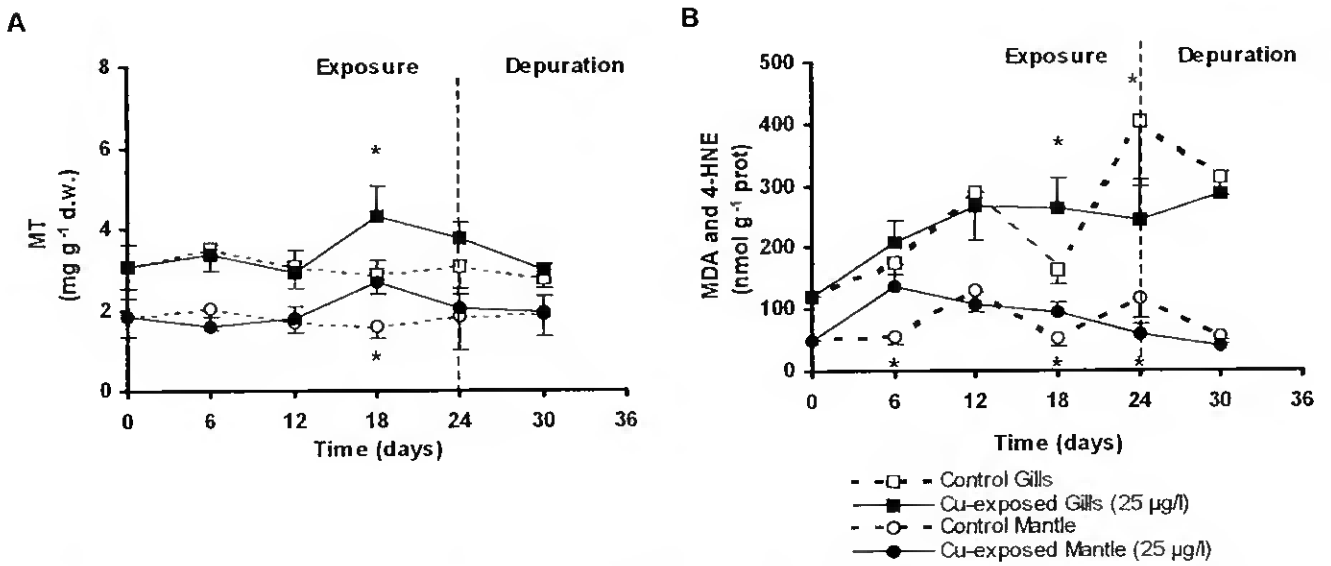

Figure 5.6 – Mean variation of MT concentrations (A) and MDA + 4-HNE compounds levels (B) in the gills and mantle of control and Cu exposed (25 µg l^{-1}) *B. azoricus* for 24 days and depurated for 6 days. Vertical bars represent one-half of the standard deviation of the mean. Symbol * represents significant differences between control and Cu-exposed mussels.

In Cu exposed mussels, increase in LPO in the gills in the first 12 days of Cu exposure was similar to controls (12.11 nmol g^{-1} protein d^{-1}, $r = 0.993$, $p<0.05$), and remained unchanged until the end of depuration period (263.45 ± 17.03 nmol g^{-1} protein). During the whole experiment no significant differences were observed in LPO levels between the gills of control and Cu exposed mussels, except at day 18 and 24 (Figure 5.6B).

The MDA and 4-HNE concentrations in the mantle of control mussels varied during the entire experiment (range between 48.71 – 130.23 nmol g^{-1} protein). In Cu exposed mussels, LPO levels increased significantly in the first week of exposure, and decreased linearly afterwards until the end of the experiment (-4.09 nmol g^{-1} protein d^{-1}, $r = 0.993$, $p<0.05$) (Figure 5.6B).

5.5. Discussion

It is unquestionable that *B. azoricus* live in a very extreme habitat for bivalve species with naturally high metal concentrations, when compared to most of the marine environments. Several hydrothermal vent bivalves show high metal concentrations in their tissues, including the clam *C. magnifica* (Roesijadi & Crecelius, 1984; Roesijadi *et al.*, 1985; Cosson-Mannevy *et al.*, 1988) and the mussels *Bathymodiolus* sp. (Smith & Flegal, 1989; Géret *et al.*, 1998; Rousse *et al.*, 1998). Those organisms appear to survive and evolve through the development of a high degree of tolerance to metals generally considered to be toxic to other coastal marine species (Cosson & Vivier, 1995; Cosson, 1997).

One of the most common mechanisms of metal detoxification, MT synthesis, has been studied in *B. azoricus* and other vent species in order to understand how these vent organisms can tolerate such amounts of metals. However, levels of these metal binding proteins in this species raised questions on the importance of MT in metal detoxification processes in *B. azoricus* mussels, and point out the importance of metal insolubilisation in lysosomes (Fiala-Médioni *et al.*, 2000). Antioxidant enzymes also play an important role in metal resistance in *B. azoricus*. Blum & Fridovich (1984) previously reported the presence of these enzymes in hydrothermal vent organisms, such as in the clam *C. magnifica* and the tubeworm *R. pachyptila*. Also, the activities of SOD, CAT and GPx were quantified in *B. azoricus* mussels collected in five hydrothermal vent sites in MAR, and results show significant spatial and seasonal differences, related to different hydrothermal vent environmental conditions (See Chapters 2 and 3; Bebianno *et al.*, submitted). Evidences that antioxidant defence system in *B. azoricus* is affected by Cd exposure similarly to coastal bivalves were also investigated (See Chapter 4; Company *et al.*, submitted). The purpose of the present study was to assess the effect of Cu exposure on the antioxidant defence system of hydrothermal vent mussel *B. azoricus* and compare the toxic effects of Cu in a short term pressurized experiment and a long term at atmospheric pressure in the gills and mantle of these mussels.

Short term Cu exposure experiment

Although *B. azoricus* live in a Cu rich environment and reported Cu concentrations for Menez-Gwen hydrothermal vent fluids are extremely high (more than 120 µg l^{-1}), no information is available about the bioavailability of Cu for these organisms. Furthermore, there is no data on the Cu toxicity in this species and thus a sub-lethal Cu concentration for coastal mussels was used (25 µg l^{-1}).

Cu was significantly accumulated in the gills following both 12 and 24 hours of exposure, while no changes in Cu concentrations were observed in the mantle between control and exposed organisms. This is probably related to different physiologic and metabolic functions of these tissues even though both tissues are in direct contact with the surrounding water, and consequently exposed to Cu. Nevertheless, gills are involved in filtration and are known to accumulate higher metal concentrations compared to the mantle.

Antioxidant enzymatic activity also varied distinctively between the two tissues suggesting different physiological functions and responses to metals as well. Studies on antioxidant enzymes in *M. galloprovincialis* exposed to Cu (60 µg l^{-1}) also reported different responses between tissues (gills and digestive gland) (Regoli & Principato, 1995). In a transplant experiment using a freshwater bivalve *Unio tumidus*, it was also suggested that gills appeared more susceptible to oxidative stress than digestive glands, indicated by the inhibition of antioxidant parameters (Cossu *et al.*, 1997).

In the gills, one of the most striking effect of Cu exposure is the enzymatic inhibition of SOD, CAT and GPx activities after both 12 and 24 hours of Cu exposure, while in the mantle, there is an increase of all scavenging enzymes in the first 12 hours of Cu exposure and a significant inhibition of these enzymes when Cu exposure is extended for 24 hours. In clams *R. decussatus* exposed to the same Cu concentration (25 µg l^{-1}) antioxidant enzymes were also inhibited during the first days of exposure in the gills (Géret *et al.*, 2002).

Different response patterns between gills and mantle were previously observed in *B. azoricus* after exposure Cd under the same pressurized conditions. When mussels were exposed to Cd (100 µg l^{-1}) a similar inhibition in antioxidant enzymes in the gills in the first hours of exposure was observed (Company *et al.*, 2004 – Annexe II; See Chapter 4; Company *et al.*, submitted). However, contrary to Cd, Cu can generate ROS by Fenton-type reactions, what would normally increase SOD, CAT and GPx activities in the organisms to prevent oxidative damage in their tissues. Nevertheless, the activities of these enzymes were in most cases inhibited in the gills and mantle of exposed mussels when compared to unexposed mussels.

Similar results were observed in the gills of the common mussel *M. galloprovincialis* exposed to 38 µg l^{-1} Cu for 1, 4 and 7 days (Canesi *et al.*, 1999) and where glutathione were depleted in Cu exposed mussels. However, some studies suggest that exposing marine bivalves to Cu not always affect stress related enzymes. In the oyster *C. virginica* exposed to two Cu concentrations (20 and 80 µg l^{-1}) the levels of glutathione were not affected by this metal (Conners & Ringwood, 2000).

Lipid peroxidation levels reflect the damages to biological membranes by reactive oxygen species. Our results show that LPO following Cu exposure only occurred in the gills in the first 12 hours of exposure. When mussels were exposed to this metal for 24 hours, LPO levels in both tissues decrease compared to unexposed organisms.

Cu however is a powerful catalyst of low density lipoprotein (LDL) oxidation. One of the most common techniques for initiating LDL oxidation in vitro involves incubation with Cu^{2+} (Steinberg, 1997). LPO occurs when Cu^{2+} reduces preformed lipid hydroperoxides to alkoxyl radicals. In the absence of lipid hydroperoxides, pro-oxidation may be initiated by the hydroxyl radical resulting from the reduction of oxygen by Cu^{+} (Burkitt, 2001). Also, high density lipoprotein (HDL) may be more susceptible to Cu-induced oxidation than LDL, because at low Cu concentrations HDL is more sensitive to oxidation due to increased tocopherol mediated peroxidation. At high Cu concentrations, HDL

has a higher concentration of bound Cu to lipoproteins lipids increasing its ability to oxidize (Raveh *et al.*, 2000).

In *B. azoricus*, short term Cu-mediated LPO does not seem significant, since unexposed mussels had higher LPO levels than Cu-exposed organisms after 24 hours in both gills and mantle. A significant decrease in LPO levels was also observed in the gills of the oyster *C. gigas* and the common mussel *M. edulis* exposed to 40 µg l^{-1} Cu during 4 and 21 days (Géret *et al.*, 2002).

Surprisingly, MT concentrations increased in the mantle of Cu exposed mussels, despite this metal was only accumulated in the gills of *B. azoricus*. This may suggest the increase in MT levels in the mantle during this experiment occurred by the presence of other factors than Cu. In view of their unusual metal binding properties toward metals, MT synthesis has been associated with the transport and storage of metal ions (Zn, Cu) and detoxification of non-essential ones (Cd, Hg). However, this protein has also been associated with other functions, like protection against reactive oxygen species (ROS) and adaptation to stress (Romero-Isart & Vasak, 2002; Suzuki *et al.*, 2002) and that physical stresses can also induce MT (Kondoh *et al.*, 2003). In *B. azoricus* the function of MT is not completely understood, but some evidences that this protein may not be the primary metal detoxification mechanism as been already proposed for this specie (Fiala-Médioni *et al.*, 2000) and seems to be confirmed by our results.

Long term Cu exposure experiment

Although no metal accumulation data is available in this long term experiment, on the basis of the short term experiment, one can assume that Cu was accumulated in the tissues of *B. azoricus* throughout the experiment. Mytilid mussels accumulate high concentrations of Cu (Nicholson, 2003). In *R. decussatus* exposed to the same Cu concentration (25 µg l^{-1}) metal accumulation was significantly higher compared to unexposed organisms (Géret *et al.*, 2002).

The results obtained show that antioxidant enzymes varied significantly during the time of experiment. The activity of most of the enzymes (SOD, total GPx and Se-GPx) increased during the course of the experiment, in either Cu exposed and unexposed mussels, and more significantly in the gills. In fact, significant differences between control and Cu-exposed organisms were only found for CAT and GPx activities at the end of exposure period and after the depuration period.

Comparable results were previously observed in *B. azoricus* exposed to Cd, where antioxidant enzymes increase indiscriminately in both control and exposed mussels throughout the entire experiment (See Chapter 4; Company *et al.*, submitted). In this case, other factors than the presence of metals seems to contribute for the enhancement of ROS production and consequently antioxidant enzymes increasing activities. The mussels were kept in the laboratory for more than 30 days, subject to different environmental conditions than those found in hydrothermal vents. This could easily increase the stressing factors in *B. azoricus*, confirmed by the enhancement of ROS in control organisms and explain the raise of enzymatic activity in both treatments.

This was also confirmed by the TOSC values. The capacity to scavenge oxyradicals remains identical in both control and Cu exposed mussels at the end of exposure and also depuration periods, and were significantly lower when compared with TOSC values in organisms prior to exposure. This suggests that *B. azoricus* is loosing progressively the capacity to detoxify ROS, although increasing the activity of antioxidant enzymes over the course of the experiment.

Moreover, MT levels found in unexposed and exposed organisms were identical throughout the experiment, except in day 18 for both tissues. Also, the concentrations of this protein remained within a narrow range during the exposure and depuration periods. This would confirm that MT may not be implicated in Cu detoxification in *B. azoricus*, either in long and short term experiments and may suggest that the metal concentration used in the experiments might not be higher enough to produce any measurable effect.

LPO data are consistent with previous explanations, since generally no significant differences between control and exposed mussels were observed, both in gills and mantle tissues. Again, other stressing factors than the presence of metal has to be implicated in ROS formation and subsequent the observed membrane level damages. Moreover, gills and mantle were affected differently during the experiment. While in the gills LPO increased significantly in the first two weeks and remained relatively unchanged until the end of the experiment, in the mantle LPO also increase in the first week and gradually the organisms recover to values close to those reported in mussels before the experiment. These results may suggest that gills are more sensitive to hydrothermal vent environmental stressors than other tissues, which are in agreement several authors for other marine bivalves (Géret *et al.*, 2002), or may reflect the loss of symbiotic bacteria in this tissue due to insufficient supply of methane and sulphite in the water.

5.6. Conclusions

In conclusion, Cu exposure experiments showed remarkable differences between short term and long term effects of Cu in the antioxidant defence system in *B. azoricus.* Also, the two tissues, gills and mantle, exhibited different responses. In general, Cu concentration used in the experiments, although toxic for coastal molluscs, might have been relatively low compared to Cu concentrations present in the hydrothermal fluids, and therefore some metal tolerance was induced in this species. Short term effects of Cu include inhibition of antioxidant defence system along with LPO decrease and little variation in MT, suggesting that ROS formation was limited and *B. azoricus* is well adapted to high metal environments. Long term Cu exposure experiment suggests that other factors than the presence of Cu influences the antioxidant enzymatic, MT and LPO responses. In this case, ROS formation is most likely due to poor physiological conditions of the organisms after long periods in artificial conditions in terms of pressure and important chemical components, such as methane and sulphide, essential for the maintenance of symbiotic bacteria in the gills and subsequently the normal metabolic functions. Nevertheless, *B.*

azoricus live in a particularly toxic environment and seems to be well adapted to high metal concentrations including Cu.

5.7. References

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Chapter 6

Short term effects of zinc, silver and mercury on stress related biomarkers in the gills and mantle of *Bathymodiolus azoricus* from Mid-Atlantic Ridge

6.1. Abstract

The deep-sea hydrothermal mussel, *Bathymodiolus azoricus*, can survive near the emission of metal rich vent fluids, of both essential and non-essential metals, but the specific adaptations they possess to deal with such high concentrations remain unknown. Metals are known to increase reactive oxygen species (ROS) production in bivalves and consequently may interfere with antioxidant enzyme protection. The effects of metals on antioxidant enzymatic system and cellular damages (lipid peroxidation – LPO) in *B. azoricus* are still largely unknown.

Therefore, the aim of this work is study the short term effects of three metals, one essential (Zn) and two non-essentials (Ag and Hg) in antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), total glutathione peroxidases (Total GPx) and selenium dependent glutathione peroxidases (Se-GPx), and LPO in deep-sea hydrothermal mussels.

The mussels were collected in Menez-Gwen vent site and exposed separately to Zn (1000 µg l^{-1}), Ag (20 µg l^{-1}) and Hg (25 µg l^{-1}) in a pressurized tank (IPOCAMP).

Results show that these metals have different effects on antioxidant parameters. The essential metal Zn in the gills, induced only mitochondrial SOD activity, while in the mantle Zn inhibited SOD, CAT, total GPx and Se-GPx. However, no changes in LPO levels occurred in both tissues, suggesting that this species can tolerate large range of Zn concentrations. Ag however, significantly induced the activity of cytosolic SOD and inhibited Total GPx activity in the gills. In the mantle, the exposure to Ag inhibited significantly mitochondrial SOD, and induced Total GPx activity and LPO occurred after Ag exposure, probably due to a reduced capacity to scavenge superoxide radicals by SOD inhibition.

Finally, Hg had little effect on both antioxidant enzymes and LPO in both tissues of *B. azoricus*, suggesting that Hg concentrations and time of exposure were either too high or too small to induce changes.

6.2. Introduction

Hydrothermal vent mussels *Bathymodiolus azoricus* are naturally exposed to a variety of metals, both essential and non-essential ones (Pruski & Dixon, 2003). Essential metals are required by the organism in small amounts for many metabolic functions. However, at high doses essential elements can be toxic and cause deleterious effects (Ballatori, 2002).

Zinc (Zn) is a classical example, since it is an essential constituent of over 300 enzymes (Vallee & Auld, 1990) and Zn finger proteins (Vallee & Falchuk, 1993), involved in numerous biological functions, including acting as a stabilizer of membranes and cellular components (Eisler, 1993; Vangen & Hemre, 2003), gene expression and intracellular and extracellular Cu/Zn-superoxide dismutase (Cu/Zn-SOD) (Olin *et al.*, 1995; Larsen *et al.*, 2000). Although the role of Zn in Cu/Zn-SOD is unclear, its removal is known to promote catalysis of peroxynitrite-mediated tyrosine nitration, resulting in protein oxidation and consequent cellular damage (Estevez *et al.*, 1999). Several authors conclude that Zn deficiency can also increase the risk for cell apoptosis (Bettger & O'Dell, 1993; Zalewski & Forbes, 1993; Zalewski *et al.*, 1993; Clegg *et al.*, 1998; Truong-Tran *et al.*, 2001). One of the proposed functions of Zn is to act as an antioxidant at different cellular levels (Bray & Bettger, 1990). Zn can induce the synthesis of metallothionein, a protein that can bind redox-active metals and scavenge hydroxyl radicals via its cysteine groups (Sato & Bremner, 1993). This metal may also act by binding to membrane sites that might otherwise bind redox-active metals (such as Cu and Fe) (Girotti *et al.*, 1985). It has been reported that chronic Zn deficiency can be characterized by tissue-oxidative damage at higher levels than normal as evidenced by increased levels of lipids (Oteiza *et al.*, 1995; Kraus *et al.*, 1997), proteins and DNA oxidation (Olin *et al.*, 1993). Although Zn is essential, excess Zn can be toxic to cells (Koh *et al.*, 1996). The mechanism of Zn toxicity is not known but this metal may bind to inappropriate intracellular ligands or compete with other metal ions for enzyme active sites, transporter protein and others (Gaither & Eide, 2001). Therefore, while maintaining adequate levels of zinc for growth, cells must also control intracellular levels when exposed to excessive zinc concentrations. Studies

point out that, at high concentrations, this metal is able to interfere with normal embryogenesis in bivalves (Beiras & Albentosa, 2004; Chen *et al.*, 2004). Although Zn is considered a redox-inert metal (Maret, 2000), and consequently does not interfere with antioxidant species, recent studies showed that an excess of Zn is able to modify the antioxidant defence systems in the clam *Ruditapes decussatus* (Géret & Bebianno, 2004).

High Zn concentrations are found in hydrothermal fluids from Mid-Atlantic Ridge sites, including in Menez-Gwen (130 µg l^{-1}), Lucky Strike (130 – 2616 µg l^{-1}) and Rainbow (10 625 µg l^{-1}). Such Zn concentrations are extremely high when compared to those found in coastal areas (1.8 µg l^{-1}) (Douville *et al.*, 2002).

On the other hand, non-essential metals such as silver (Ag) and mercury (Hg) have no recognized biological functions and therefore can be extremely toxic for organisms even at low concentrations (Bihan *et al.*, 2004).

Ag is toxic to aquatic organisms when present as ionic silver (Ag^{+}) (Grossel *et al.*, 2002). The toxicity of Ag is related to the capacity to form reversible bonds with enzymes and other active molecules at the cell surface (Wood *et al.*, 1996). Due to its sulphydryl binding propensity, biologically available Ag disrupts membranes, disables proteins and inhibits enzymes (Taylor *et al.*, 1980; Wood *et al.*, 1996). Ag can also interfere with active transport and cyclic adenosine monophosphate, as this metals binds to bases, riboses and phosphates on DNA (Klein, 1978). Adverse effects of Ag have been demonstrated in various molluscs such as the oyster *Crassostrea virginica* (Berthet *et al.*, 1992; Abbe *et al.*, 1994). Previous studies have shown that accumulation and toxicity of silver were dependent of the mollusc species and life stage (Calabrese *et al.*, 1982, 1984; Métayer *et al.*, 1990).

Ag concentration reported for MAR hydrothermal vents are exceptionally high at Menez-Gwen (0.5 – 1.8 µg l^{-1}), Lucky Strike (0.5 – 2.7 µg l^{-1}) and Rainbow (5.1 µg l^{-1}) compared to average Ag values in seawater (0.002 µg l^{-1}) (Douville *et al.*, 2002).

Hg is recognized as one of the most toxic metals to aquatic living organisms, particularly in its organometallic form (methylmercury), because of its toxicity at very low concentration (Sanfeliu *et al.*, 2003; Gatti, 2004). This metal is bioaccumulated and biomagnified in aquatic food chains (Wren & Stephenson, 1991). The toxicity of Hg is partly due to its ability to react and deplete sulfhydryl (–SH) groups. This decrease in free –SH groups may lead to the formation of oxidative stress and consequently damaging effects (Stohs & Bagchi, 1995). There are some evidences that Hg can interfere with the antioxidant defence system, causing the reduction of SOD, CAT and GPx and increasing the LPO levels (Stohs & Bagchi, 1995; Díaz *et al.*, 2004; Shanker *et al.*, 2004), although this was not confirmed in the gills of *B. azoricus* exposed to this metal (Company *et al.*, 2004 – Annexe II).

Total mercury concentration in natural water ranges from 0.2 to 100 ng l^{-1} whereas methylmercury level reaches approximately 10 ng l^{-1} (Smaele *et al.*, 1999). No data exist on Hg levels in MAR hydrothermal vent fluids, and little is known about the concentrations of this metal in vents sites in general. However, high Hg levels compared to levels in coastal waters occurred in the fluids from shallow hydrothermal vents in the Bahia Concepcion (Mexico) 1390 µg l^{-1} (Prol-Ledesma *et al.*, 2004) and also in rocks (27 to 6800 µg l^{-1}) and chimneys (6 to 21 µg l^{-1}) from hydrothermal active zones in Lake Taupo (New Zealand) (Ronde *et al.*, 2002). The Azorean archipelago is of volcanic origin and natural releases of mercury and other metals linked to hydrothermal activity occur in localised areas (Grousset & Donard, 1984; Depledge *et al.*, 1992; Andersen & Depledge, 1997). Also important Hg concentrations were found in the gills (4.96 ± 2.6 µg g^{-1}) and mantle (1.10 ± 2.0 µg g^{-1}) of the vent clam *Vesicomya gigas* (Ruelas-Inzunza *et al.*, 2003) from Guaymas basin (Pacific) and in the whole soft tissues of *B. azoricus* (2.26 to 7.41 µg g^{-1}) from Mid-Atlantic Ridge (Martins *et al.*, 2001), compared to their coastal counterparts ranging from 0.09 to 0.88 µg g^{-1} in the *Mytilus galloprovincialis* (Besada *et al.*, 2002), suggesting a high abundance of available Hg in hydrothermal environments.

Although the bioaccumulation of non-essential metals in bivalves from coastal areas was extensively studied, especially in mussels (*Mytilus edulis* and *M.*

galloprovincialis) (Bebianno & Machado, 1997; Szefer *et al.*, 1999; Saavedra *et al.*, 2004), clams (*Ruditapes decussatus*) (Bebianno & Serafim, 2003) and oysters (*Crassostrea gigas*) (Amiard-Triquet *et al.*, 1991) as well as their effects on MT induction, to the best of our knowledge no studies documenting the effects of Ag and Hg on antioxidant enzyme activity in bivalves exist.

In the present work, antioxidant enzymes, including SOD, CAT and GPx in the gills and mantle of the hydrothermal vent mussel *B. azoricus* were followed after the exposure to essential (Zn) and non-essential (Ag and Hg) metals at controlled temperature and pressure to mimic the hydrothermal environment. Furthermore, the effects of these metals in metallothioneins (MT) and lipid peroxidation (LPO) were also investigated.

6.3. Materials and Methods

Mussels *B. azoricus* used in the experiments were collected periodically with acoustically retrievable cages recovered in the Menez-Gwen hydrothermal vent site (37°51'N, 32°31'W, 850 m; Mid-Atlantic Ridge) for the seasonal study from July to November 2001 (See Chapter 3) by the French ROV Victor (IFREMER) during the ATOS cruise (Sarradin *et al.*, 2001).

The organisms were brought to the surface and acclimated for 48 hours to reduce the stress from cage recovery in filtered seawater collected from the Azores coastal zone, enrich with methane and maintained at 9°C. Although methane was added to the water (17 - 54 µM) the loss of symbiotic bacteria (thio and methanotrophic) can not be excluded.

After the acclimation period, three groups of 10 *B. azoricus* were exposed to Zn, Ag and Hg in separate experiments at controlled temperature (9°C) and pressure (85 bars). A group of 10 organisms (6.85 ± 0.62 cm) was exposed to a nominal concentration of 1000 µg l^{-1} Zn. Another set of 10 vent mussels (7.20 ± 0.45 cm) was exposed to a nominal concentration of 20 µg l^{-1} Ag. A third group

of 10 mussels (7.05 ± 0.55 cm) was exposed to a nominal concentration of 25 μg l^{-1} Hg.

Experiments were conducted at 9 ± 1°C and 85 atmospheres in a pressurized container IPOCAMP (*Incubateurs Pressurises pour l'Observation en Culture d'Animaux Marins Profonds*) (Shillito *et al.*, 2001) and the mussels were exposed to metals during 12 and 24 hours, except in Zn experiment (only 24 hours). Controls were maintained in the same conditions in filtered seawater. Organisms were measured and the gills and mantle dissected and immediately frozen in liquid nitrogen and stored at –80°C until further analysis.

The activity of superoxide dismutase (SOD) (EC 1.15.1.1) (McCord & Fridovich, 1969), catalase (CAT) (EC 1.11.1.6) (Greenwald, 1985) and glutathione peroxidases (GPx) (total and selenium depend) (Lawrence & Burk, 1976), as well as lipid peroxidation (Erdelmeier et al., 1998) and total protein concentrations (Lowry *et al.*, 1951) were determined in both tissues by methods described previously in Chapter 2.

Zn levels in *B. azoricus* were determined by Lic. Inês Martins in the University of Paris IV (France) under the supervision of Dr. Jacques Boulègue and Dr. Aline Fiala-Médioni.

MT concentrations in *B. azoricus* were determined by differential pulse polarography in accordance with the method of Olafson & Sim (1979) modified by Thompson & Cosson (1984). These analyses were conducted by Dr. Richard Cosson from ISOMer Marine Biology Laboratory in the University of Nantes (France).

Statistical analysis was performed using STATISTICA for Windows 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.

6.4. Results

Enzymatic activities of SOD, CAT and GPx in the gills and mantle of *B. azoricus* exposed to Zn for 24 hours and respective controls are presented in figure 6.1.

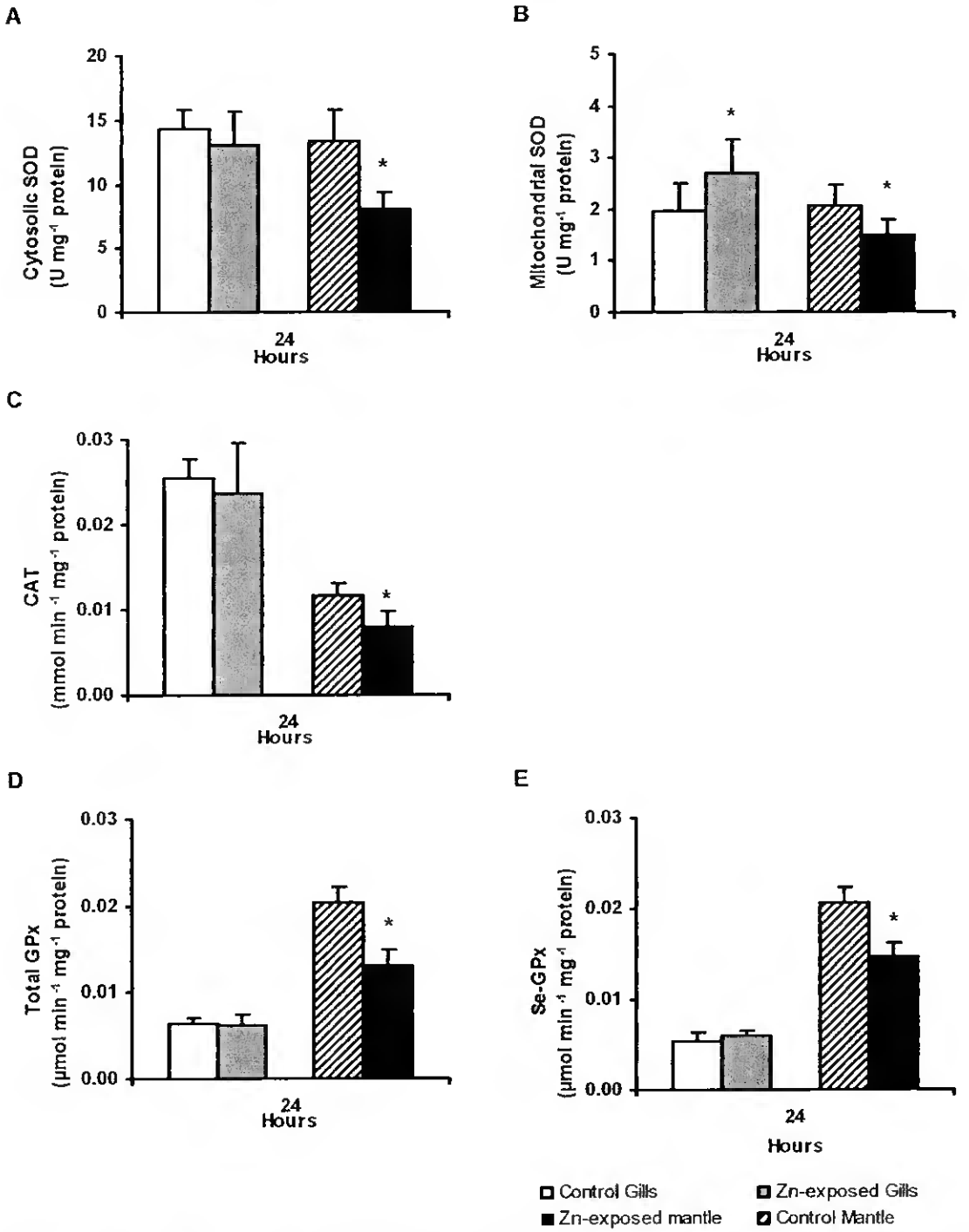

Figure 6.1 – Mean variation (± SD) of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills and mantle of control and exposed to Zn (1000 µg l^{-1}) *B. azoricus* for 24 hours in IPOCAMP. Symbol * represents significant differences between control and Zn-exposed mussels (p<0.05).

SOD is mainly present in the cytosol (approximately 75% of total SOD). SOD activity in the cytosolic fraction was significantly inhibited only in the mantle of Zn exposed mussels ($p<0.05$), while in the gills no significant differences between control and exposed mussels were observed ($p>0.05$) (Figure 6.1A).

The activity of SOD in the mitochondria, was significantly induced in the gills of exposed mussels, while in the mantle, as for cytosolic SOD, was significantly inhibited ($p<0.05$) (Figure 6.1B).

All the other antioxidant enzymes, CAT, total GPx and Se-GPx had the same pattern of cytosolic SOD. The activities of these enzymes remained unchanged in the gills of unexposed and Zn-exposed mussels ($p>0.05$), while in the mantle these antioxidant enzyme activities were inhibited after 24 hours of Zn exposure in IPOCAMP vessel ($p<0.05$) (Figures 6.1C to E).

Figure 6.2 shows the antioxidant enzymatic activity in the gills and mantle of Ag IPOCAMP experiments during 12 and 24 hours.

Cytosolic SOD activity in both tissues remained unchanged between control and exposed mussels in the first 12 hours of Ag exposure ($p>0.05$), but was significantly induced in the gills after 24 hours ($p<0.05$), while no significant changes between control and Ag-exposed mussels were observed in the mantle ($p>0.05$) (Figure 6.2A).

Contrarily, mitochondrial SOD was significantly enhanced in the gills in the first 12 hours of Ag exposure ($p<0.05$) and decreased to control levels when the exposure was extended for 24 hours ($p>0.05$). In the mantle, SOD in the mitochondrial fraction was significantly inhibited after 24 hours of Ag exposure ($p<0.05$) (Figure 6.2B).

CAT activity was approximately 3-fold higher in the gills compared to the mantle and remained unchanged between control and Ag exposed mussels in both tissues during the whole experiment ($p>0.05$) (Figure 6.2C).

Total and selenium dependent GPx showed a similar pattern, where the activity of these enzymes was significantly inhibited in the gills after 12 and 24 hours of Ag exposure ($p<0.05$), whereas in the mantle GPx was significantly induced only after 24 hours in Ag-exposed mussels ($p<0.05$) (Figure 6.2D and E).

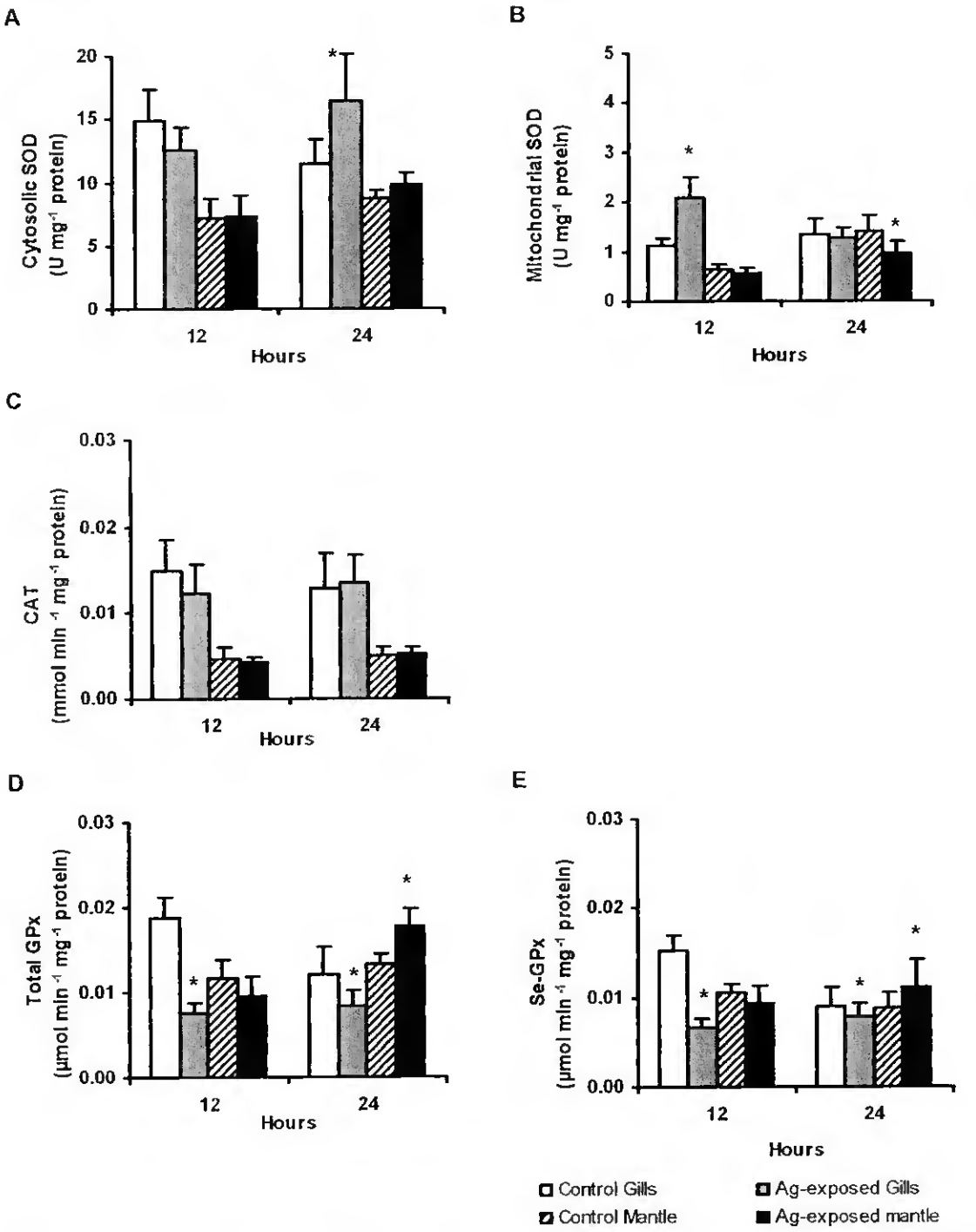

Figure 6.2 – Mean variation (± SD) of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills and mantle of control and exposed to Ag (20 µg l^{-1}) *B. azoricus* for 12 and 24 hours in IPOCAMP. Symbol * represents significant differences between control and Ag-exposed mussels ($p<0.05$).

The antioxidant enzymatic activities in the gills and mantle of Hg exposure experiments in IPOCAMP chamber during 12 and 24 hours are presented in Figure 6.3.

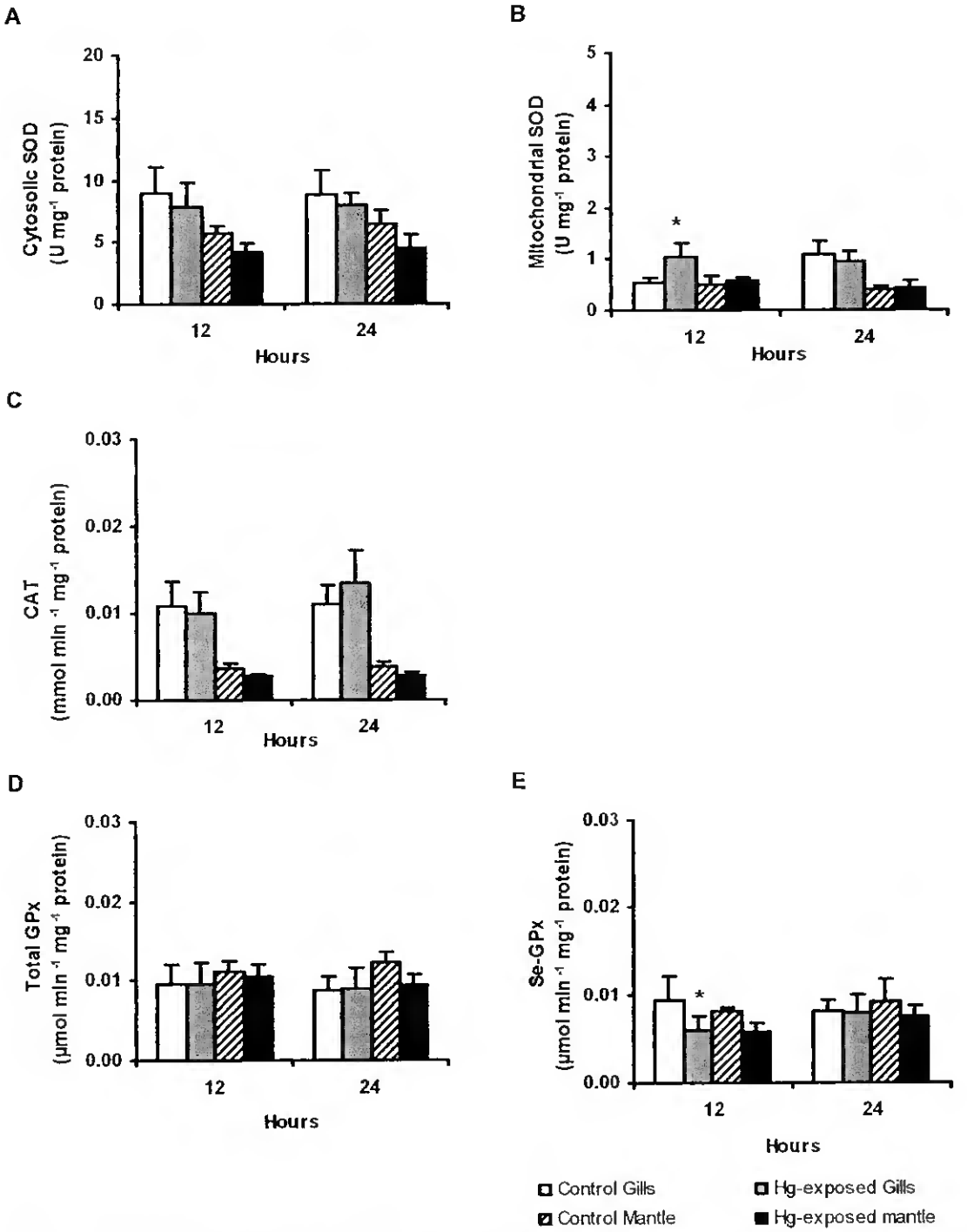

Figure 6.3 – Mean variation (± SD) of cytosolic SOD (A), mitochondrial SOD (B), CAT (C), Total GPx (D) and Se-GPx (E) activities in the gills and mantle of control and exposed to Hg (25 µg l^{-1}) *B. azoricus* for 12 and 24 hours in IPOCAMP. Symbol * represents significant differences between control and Hg-exposed mussels (p<0.05).

Most of antioxidant enzymes although showing some variability after Hg exposure (cytosolic SOD inhibited in both tissues and CAT in the mantle) these changes were not significant between unexposed and Hg-exposed mussels (p>0.05), except in the gills where mitochondrial SOD and Se-GPx were significantly inhibited and enhanced respectively after 12 hours of exposure (p<0.05) (Figures 6.3 B and E).

LPO concentrations in the gills and mantle of *B. azoricus* exposed to Zn, Ag and Hg are presented in Figure 6.4. LPO levels in the gills of Zn, Ag and Hg-exposed mussels remained unchanged compared to the respective controls, except in the gills of mussels exposed to Ag for 12 hours, where LPO levels significantly decreased in Ag exposed mussels (p<0.05) (Figure 6.4B).

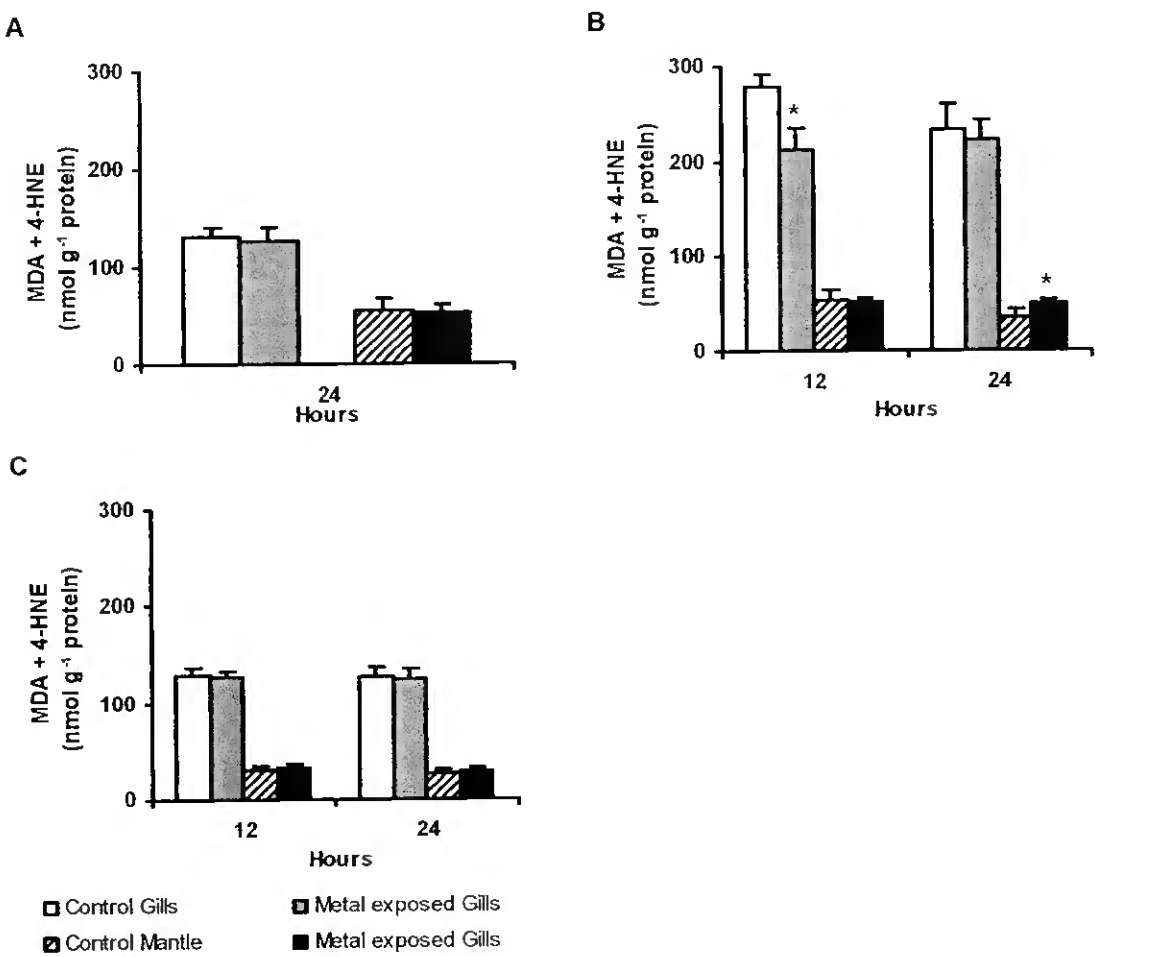

Figure 6.4 – Mean variation (± SD) of LPO levels in the gills and mantle of control and exposed to Zn (1000 µg l^{-1}) (A), Ag (20 µg l^{-1}) (B) and Hg (25 µg l^{-1}) (C) *B. azoricus* for 12 and 24 hours in IPOCAMP. Symbol * represents significant differences between control and metal exposed mussels (p<0.05).

The mantle exhibits approximately 4-fold lower LPO levels than the gills. Like in the gills, LPO levels in the mantle of *B. azoricus* exposed to Zn and Hg remain unchanged after 24 hours of exposure ($p>0.05$). Contrarily to what was observed in the gills, the concentrations of MDA and 4-HNE increased significantly in the mantle after 24 hours of Ag exposure ($p<0.05$) (Figure 6.4B).

MT concentrations were only determined in the mussels exposed to Zn and Ag (Figure 6.5). In general, the gills exhibited approximately 4-fold higher MT levels compared to the mantle, similarly to what was observed for LPO concentrations (Figure 6.4). The levels of these proteins were only significantly induced in the gills of mussels exposed to Zn for 24 hours ($p<0.05$) (Figure 6.5A) and remained unchanged in both tissues in Ag-exposed mussels ($p>0.05$) (Figure 6.5B).

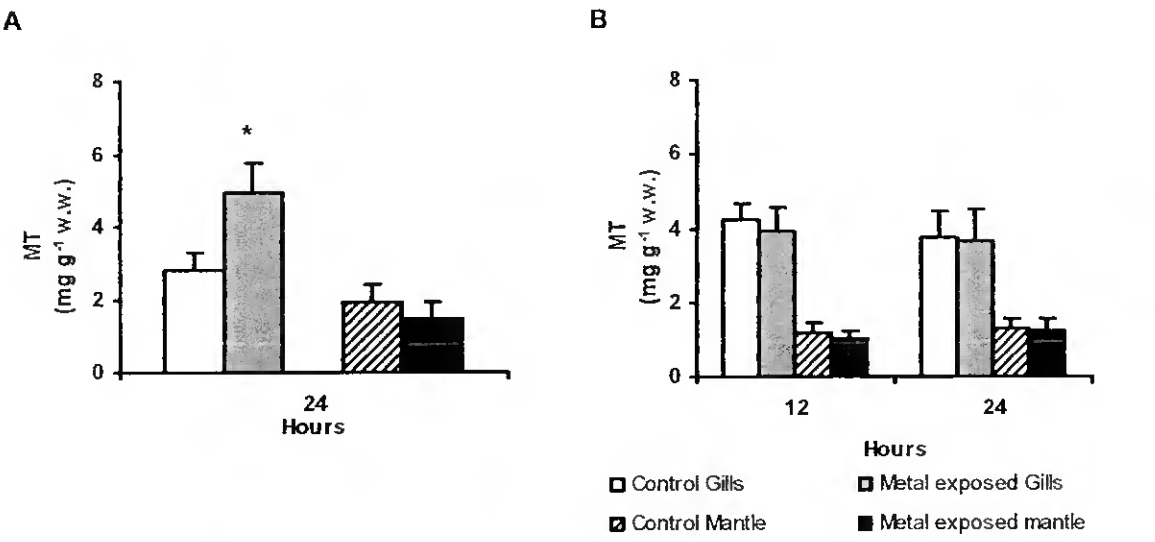

Figure 6.5 – Mean variation (± SD) of MT levels in the gills and mantle of control and exposed to Zn (1000 µg l^{-1}) (A) and Ag (20 µg l^{-1}) (B) *B. azoricus* for 24 hours in IPOCAMP. Symbol * represents significant differences between control and Ag, Hg or Zn-exposed mussels ($p<0.05$).

The metal accumulation data in the gills and mantle of *B. azoricus* exposed to Zn are showed in Figure 6.6.

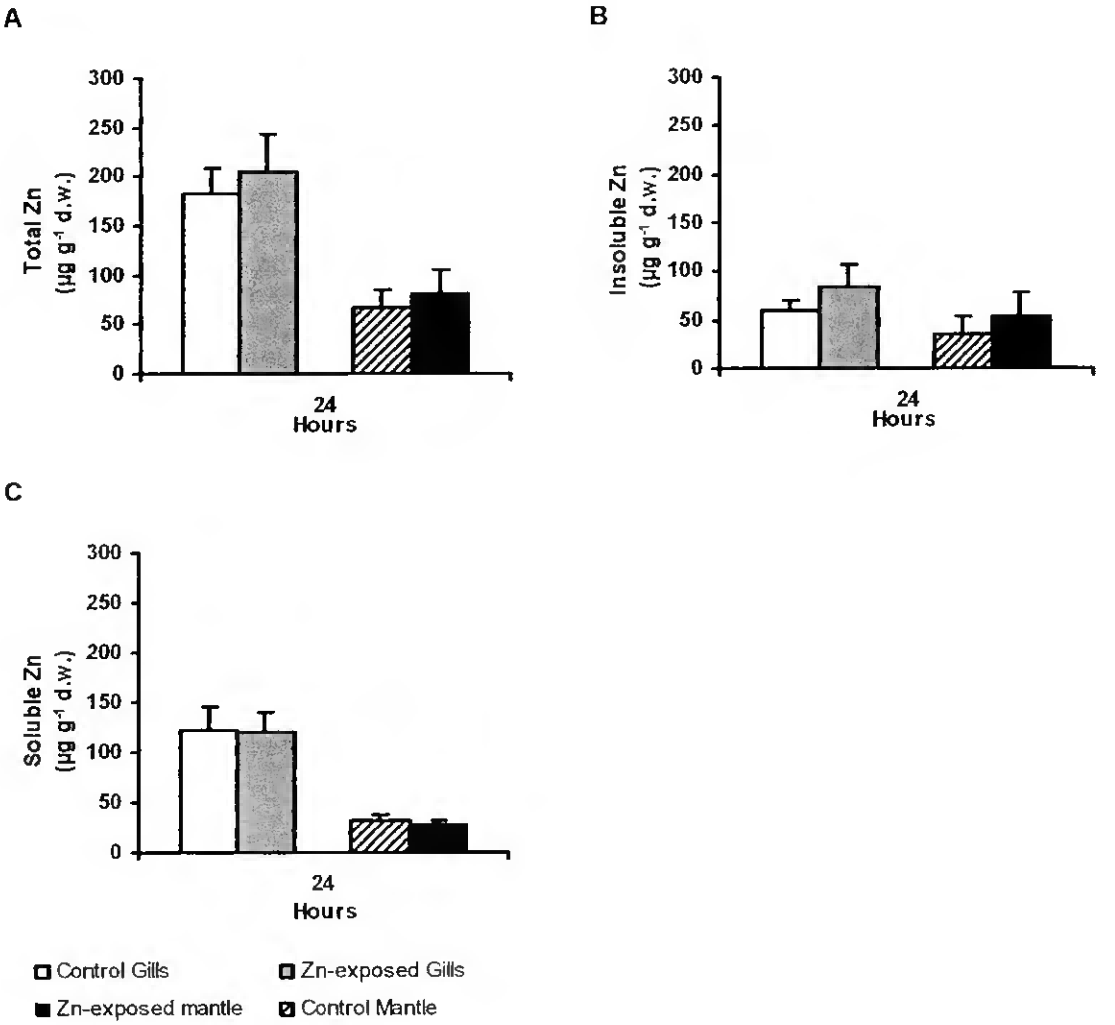

Figure 6.6 – Zn concentrations (Mean ± SD) in the gills and mantle of control and Zn-exposed (1000 µg l^{-1}) *B. azoricus* in total (A), insoluble (B) and soluble fractions (C).

Zn levels are significantly higher in the gills for total (2.5-fold) and subcellular fractions, i.e. insoluble (1.7-fold) and soluble (4-fold) Zn concentrations ($p<0.05$), compared to the mantle. Although total and insoluble Zn concentrations slightly increase in both gills and mantle of *B. azoricus* exposed to 1000 µg l^{-1} Zn, it was not significant ($p>0.05$) (Figure 6.6A and B). Soluble Zn concentrations between control and exposed mussels were also similar ($p>0.05$) (Figure 6.6C).

6.5. Discussion

High density populations of *B. azoricus* colonize active hydrothermal vent chimneys in MAR emanating metal-rich fluid emissions and seems well adapted to such extreme environment. Consequently these bivalves have to deal with high metal concentrations (Rousse *et al.*, 1998). Several studies showed that metals can interfere with DNA, lipids and proteins (Hartwig & Schwerdtle, 2002; Kasprzak, 2002) and may consequently lead to death of the organisms that are exposed to high metal concentrations (Hartwig *et al.*, 2002). Metal toxicity in bivalves can produce numerous damaging effects at cellular and sub-cellular levels (Cajaraville *et al.*, 2000; Kakkar & Jaffery, 2005).

The toxic effects of metals depend on the time and concentration to which organisms are exposed. Most of the effects are related to their interaction with carboxyl and thiol groups of proteins and to their ability to direct or indirectly generate free radicals and therefore induce oxidative stress (Vallee & Ulmer, 1972; Nieboer & Richardson, 1980; Kamiski, 1992). Metals like Cu and Fe (redox-active) can increase the production of reactive oxygen species (ROS) inside the cells directly by participating in Fenton-like reactions producing hydroxyl radicals (Dean *et al.*, 1997; Ercal *et al.*, 2001). *B. azoricus* exposed to 25 µg l^{-1} Cu in IPOCAMP vessel had short term inhibition of most of antioxidant enzymes in the gills and mantle. However, long term exposure to the same Cu concentration, antioxidant enzymes had a tendency to increase indiscriminately in both unexposed and exposed mussels suggesting that ROS formation is not Cu dependent (See Chapter 5). Other metals (e.g. Cd, Ag, Hg or Zn) are redox-inert but can also increase ROS within cells (Ercal *et al.*, 2001; Géret & Bebianno, 2004). In fact, *B. azoricus* exposed to 100 µg l^{-1} Cd showed a significant inhibition of antioxidant enzymes after 24 and 48 hours of exposure, beside a slight reduction of ROS scavenging capacity. However, LPO levels in these mussels increased in both tissues only after 6 days of Cd exposure (See Chapter 4; Company *et al.*, submitted).

In this work, the effects on antioxidant enzymes activity, LPO and MT levels of three metals (Zn, Ag and Hg) in higher concentrations than those found in their natural hydrothermal environment (Menez-Gwen) were investigated separately in the gills and mantle of the deep-sea mussel *B. azoricus*.

From the results obtained, it becomes clear the importance of having separate control groups for each experiment. The variability found both in antioxidant enzymes activity and LPO levels between control groups in the different experiments were important. Part of this variability can be explained by the fact that mussels used in these experiments were collected in different periods of time by retrievable cages. As observed previously, *B. azoricus* collected along several months in Menez-Gwen exhibited a clear seasonal variation in SOD, CAT and GPx activities (Chapter 3). Therefore, *B. azoricus* should be collected in the same time of the year to reduce natural fluctuation of antioxidant parameters.

The exposure of *B. azoricus* to 1000 μg l^{-1} Zn influences differently the antioxidant enzyme activities in the two tissues. In the gills, Zn enhances antioxidant enzyme activities, while an inhibitory effect was observed in the mantle, similarly to what was observed in the seasonal study (Chapter 3). In fact, this metal enhanced mitochondrial SOD activity in the gills of Zn exposed mussels (Figure 6.1B). However, during the seasonal study the increase in insoluble Zn concentrations in this tissue enhanced both total and Se-GPx activities (Chapter 3). Therefore, in *B. azoricus*, exposure to high concentrations of Zn is likely to enhance the production of superoxide radical and hydrogen peroxide in the gills that will consequently enhance the activity of SOD and GPx in order to detoxify the oxyradicals to less reactive oxygen species and reduce the possibility of oxidative damages to occur.

On the other hand, Zn exposure inhibited the activity of SOD, CAT, total GPx and Se-GPx in the mantle (Figure 6.1A-E). The inhibition of CAT and GPx activity by increasing total and insoluble Zn concentrations in the mantle has been previously observed during the seasonal study (Chapter 3). The inhibition of antioxidant enzymes in the mantle contrasting with the gills may reflect the

absence of chemoautotrophic endosymbionts in this tissue that might have a protective antioxidant role against ROS production.

Because Zn is an essential metal with antioxidant properties, very few studies address the effects of excess Zn in antioxidant enzymes. In *R. decussatus* exposed to the same Zn concentration for 28 days, the activities of cytosolic and mitochondrial SOD in the gills increased significantly in the first days of exposure, contrasting with what was observed for *B. azoricus*. Moreover, after the first day of Zn exposure a significant inhibition of CAT, total GPx and Se-dependent GPx was observed in the gills of *R. decussatus* (Géret & Bebianno, 2004). Although *B. azoricus* were exposed to a Zn concentration 10-fold higher than that reported for Menez-Gwen hydrothermal vent fluid and superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) production is likely to occur, no evidence of oxidative stress was found. In fact, MDA + 4-HNE levels, which reflect ROS induced damages to membrane lipids, were similar in control and Zn-exposed vent mussels in both tissues (gills and mantle), suggesting that antioxidant defences were able to neutralize ROS production. Comparable results were obtained for the non-vent clam *R. decussatus* exposed to much lower metal concentrations in their natural environment (Géret & Bebianno, 2004). *B. azoricus* from Lucky Strike (0.5 – 2.7 µg l^{-1} Zn) and especially Rainbow (5.1 µg l^{-1} Zn) are exposed to higher Zn concentrations than those from Menez-Gwen (Douville *et al.*, 2002), suggesting a long-term adaptation to a large range of metal levels. Therefore, *B. azoricus* seems able to accumulate extremely high Zn concentrations, and although some enzymes were inhibited in the mantle, no oxidative damage was observed in the tissues analyzed.

The biological essentiality of Zn implies the existence of homeostatic mechanisms that regulate its absorption, distribution, cellular uptake and excretion (Kondoh *et al.*, 2003; Vallee & Falchuk, 1993). This Zn regulation is largely done by the induction of MT, a metal binding intracellular protein (Kondoh *et al.*, 2003). Since Zn binds primarily to MT under physiological conditions and MT releases Zn under oxidative stress conditions and since this metal is an antioxidant element, it is possible that Zn mediates the protective action of MT (Zhou *et al.*, 2002; Powell, 2000). In this study MT significantly

increased only in the gills of *B. azoricus* exposed to 1000 µg l^{-1} Zn after 24 hours, while no significant differences were observed in the mantle (Figure 6.5A). Similar results were obtained in the clam *R. decussatus* exposed to the same Zn concentration (Géret & Bebianno, 2004). There are evidences of Zn regulation in other mussel species, such as in *M. edulis* (Amiard-Triquet *et al.*, 1986; Phillips & Rainbow, 1988) and *Perna viridis* (Chan, 1988). *B. azoricus* seems to be tolerant to a broad range of Zn concentrations in MAR hydrothermal vents suggesting that Zn regulation in their tissues may occur.

The exposure of *B. azoricus* to 20 µg l^{-1} Ag also affected differently the two tissues. Ag exposure significantly increased the activity of cytosolic SOD and had an inhibitory effect on Total GPx in the gills, whereas in the mantle it was the opposite. The exposure to Ag did not interfere with CAT and Se-dependent GPx in both tissues. These results confirm the relationships between metal concentration in the tissues and antioxidant enzymes during the seasonal study, where increasing Ag concentrations in the soluble fraction in the gills significantly induce both cytosolic and mitochondrial SOD (Chapter 3).

The Ag ion (Ag^{+}) is a metal ion considered much more toxic than Zn to aquatic biota (Hogstrand *et al.*, 1996; Mayer *et al.*, 2003). This metal can also interfere with other metallic elements such as Cu. Kurasaki *et al.* (2000) showed evidences that Ag administration affected a Cu transporting mechanism in mice, as Cu increases drastically after Ag injection in rat kidneys. In bivalves, Ag concentrations ranging from 20 to 50 µg l^{-1} can interfere with the normal larvae development in *Mercenaria mercenaria* (Calabrese & Nelson, 1974), *Crassostrea virginica* (Calabrese *et al.*, 1973) and *M. edulis* (Martin *et al.*, 1981; Calabrese *et al.*, 1984). One of the best known effects of Ag exposure is the induction of small cytosolic proteins (MT) that bind the excess of Ag ions and remove them from active sites, acting as a detoxification mechanism of Ag (Bofill *et al.*, 1999). In coastal bivalves, MT induction occurs after 20 µg l^{-1} Ag exposure in the oyster (*C. gigas*) and mussel (*M. edulis*) (Géret *et al.*, 2002). However, although Ag subcellular distribution of *B. azoricus* showed that this metal is mainly present in the soluble fraction in the gills (54-86%) (Chapter 3) in contrast to what is observed in coastal species where this metal is mainly in

the insoluble fraction the exposure to 20 μg l^{-1} Ag did not induced MT synthesis, at least in the first 24 hours.

In the present case Ag only induced oxidative damage in the mantle. Generally, gills of *B. azoricus* are more affected by metal toxicity compared to the mantle, in short term exposure to 100 μg l^{-1} Cd (Chapter 4), 50 μg l^{-1} Cu (Chapter 5) and 1000 μg l^{-1} Zn. However, the exposure to 25 μg l^{-1} Ag for 24 hours only increase the MDA + 4-HNE compounds in the mantle. *B. azoricus* may thus possess a higher protection of antioxidant activity in the gills, mainly SOD and CAT due to the presence of symbiotic bacteria that as mentioned previously contain antioxidant defences (Hoarau, unpublished data). Moreover, cytosolic SOD activity in the gills increased after Ag exposure, suggesting an attempt to capture superoxide anion ($O_2^{-\bullet}$) a precursor of the highly reactive hydroxyl radical (OH•), the major responsible for LPO induction. The increase of LPO in the mantle may also be linked to the mitochondrial SOD inhibition in this tissue, and consequently a reduction of the capacity to scavenge $O_2^{-\bullet}$. Similar LPO increase by Ag exposure was observed in the gills of *M. edulis* (Géret *et al.*, 2002).

The exposure of *B. azoricus* to 25 μg l^{-1} Hg was surprising, since no effects on antioxidant enzymes and LPO occur. Preliminary results already pointed out for the lack of antioxidant responses in the gills after Hg exposure (Company *et al.*, 2004 – Annexe II). Also in the mantle, although enzymatic inhibition of cytosolic SOD, total-GPx and Se-GPx occurs, it was not significant. Reported Hg concentrations for the whole soft tissues in *B. azoricus* collected in Menez-Gwen ranged from 2.26 to 7.41 μg g^{-1} d.w. (Martins *et al.*, 2001). Also, Hg levels were determined in the gills (4.96 ± 2.6 μg g^{-1}) and mantle (1.10 ± 0.2 μg g^{-1}) of the vent clam *Vesicomya gigas* (Ruelas-Inzunza *et al.*, 2003) from Guaymas basin located in the mid-ocean mountain ridge of the eastern Pacific. These levels are significantly higher than those found for *M. galloprovincialis* (range from 0.09 to 0.88 μg l^{-1}) in the Spanish coast (Besada *et al.*, 2002) confirming that hydrothermal vent organisms accumulate higher amounts of Hg than their coastal counterparts. It is also known that Hg readily deposits in mitochondria, and selective disruption of the mitochondrial electron transport chain has been

suggested as the specific mechanism by which this metal induces the formation of ROS and lipid peroxidation stress (Yee & Choi, 1996; Zaman & Pardini, 1996; Konigsberg *et al.*, 2001). However, the exposure of *B. azoricus* to a high Hg concentration (25 µg l^{-1}) does not seem to significantly induce the production of reactive oxygen species, or the short time of exposure (24h) was not enough to induce antioxidant defences responses or LPO damages in *B. azoricus*.

6.6. Conclusions

In conclusion, the antioxidant defence system and LPO damages in *B. azoricus* are metal dependent as previously detected in vent mussels collected from the field. Exposure to 1000 µg l^{-1} Zn increased mitochondrial SOD activity in the gills and inhibited all antioxidant enzymes in the mantle. However no LPO in both tissues occurred suggesting a long-term adaptation to a large range of Zn levels or that *B. azoricus* may be able to regulate Zn concentrations in their tissues. Ag also interfered with antioxidant enzymes in both tissues and LPO damages occurred in the mantle, probably due to an increase in superoxide anion caused by reduction of SOD activity in this tissue. Surprisingly, Hg had little effects on antioxidant defence system and no LPO damages were found. This suggests that *B. azoricus* can cope with high Hg levels in MAR hydrothermal vent sites and probably the Hg concentration used and exposure length was inadequate to observe any measurable antioxidant responses.

6.7. References

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Chapter 7

General Discussion

7. General Discussion

Since the discovery of hydrothermal vents much effort was done to understand the specific adaptations that organisms possess to live in such particular environment. The knowledge of hydrothermal fluids composition revealed that vent organisms are exposed to extremely high metal concentrations, much higher than those found in polluted coastal areas. However, contrary to polluted coastal areas, where biodiversity and population density are naturally reduced, hydrothermal vents, although with a low biodiversity, show high density populations around vent emissions. This obvious contrast triggered scientists to understand how vent species could survive in hydrothermal vents and if they possess any specific adaptations to deal with this natural pollution in a new ecotoxicological perspective of these environments (Pruski & Dixon, 2003). The present thesis emerges in this context, following other studies that tried to clarify the metal defence mechanisms of vent organisms. Therefore, this work aimed to study the adaptations of metal detoxification mechanisms in the mussel *Bathymodiolus azoricus*, the dominant species of hydrothermal vent sites near Azores in the Mid-Atlantic Ridge, focusing the antioxidant defence systems and the effect of metals in these systems. As already focused in Chapter 1, the antioxidant defence systems include, among other mechanisms, the action of three important enzymes, superoxide dismutase, catalase and glutathione peroxidases able to detoxify reactive oxygen species within cells. When this enzymatic protection fails, several damages may occur, one of the most studied is the lipid peroxidation of cellular membranes. In coastal bivalves, both antioxidant defences and oxidative damages were extensively studied over the last decades (Viarengo *et al.*, 1991; Livingstone *et al.*, 1992; Gamble *et al.*, 1995; Solé *et al.*, 1995; Cossu *et al.*, 1997; Regoli *et al.*, 1998). However, little is know about species living in hydrothermal vents. As referred earlier, a single study had already point out for the presence of antioxidant enzymes, which provide a defence against oxygen toxicity in two hydrothermal vent species (the giant clam *Calyptogena magnifica* and the tubeworm *Riftia pachyptila*) (Blum & Fridovich, 1984). However, for the vent mussel *B. azoricus* the existence of such protection mechanisms was still unknown. Consequently, one of the primary tasks of this investigation was to conduct a field study to screen

antioxidant enzymes in *B. azoricus*. The field study was carried out in five MAR hydrothermal vent sites Menez-Gwen (ATOS8 and ATOS10), Lucky Strike (Bairro Alto and Eiffel Tower) and Rainbow, to determine the existence of spatial variability of antioxidant parameters (Chapter 2). Moreover, to conclude about a possible seasonal variation of the antioxidant defence systems, antioxidant enzymes, LPO and MT were determined in mussels from one hydrothermal vent site (Menez-Gwen) (Chapter 3).

7.1. Spatial and seasonal variability of antioxidant defence system in *B. azoricus*

Earlier studies where the metal concentration in several tissues of *B. azoricus* were determined showed that metal levels in this mussel species are relatively high compared to coastal mussels from polluted areas (Table 1.4; Chapter 1). That could easily be explained by the long term exposure to metal rich hydrothermal vent fluids. Moreover, spatial metal concentrations found in mussels from different vent sites show significant differences among vent sites, suggesting that these mussels are exposed to considerable different metal levels. In fact, hydrothermal fluid analysis showed that there are significant differences between the metals emanated by different hydrothermal vents. In Table 1.1 (Chapter 1) it is possible to identify which metals are more abundant in end-member fluids from the three MAR hydrothermal vent fields Menez-Gwen, Lucky Strike and Rainbow. In this context, it was necessary to confirm if the antioxidant defences and damages of *B. azoricus* display any spatial variability and if these differences were related to the metal concentrations of the tissues.

From this field study, results showed that *B. azoricus* exhibited important activities of all antioxidant enzymes (SOD, CAT, TGPx and Se-GPx), TOSC and membrane damage (LPO) in the gills and mantle, and that these parameters are tissue dependent (Figures 2.2 to 2.6; Chapter 2). While SOD and CAT activities and TOSC are significantly higher in the gills, both TGPx and Se-GPx are mainly present in the mantle. Gills also showed higher LPO damage than

the mantle. This tissue specificity was also associated with the metal accumulation pattern observed for both *Bathymodiolus* tissues (Table 2.1; Chapter 2). Although both gills and mantle are in direct contact with the surrounding water, metal concentrations are higher in the gills when compared to the mantle. This may reflect different tissue functions, as the gills are involved in respiration and nutrition (due to the presence of symbiotic bacteria) while the mantle is associated with accumulation of reserves and secretion for shell formation. Therefore, it seems that antioxidant defences and damages in both tissues followed their pattern of metal accumulation and consequently must be analysed separately. However, an important question came out from these results and is related to the presence of symbiotic bacteria in the gill tissue. Because no separation techniques were applied to differentiate between host and symbionts, the antioxidant enzymatic activities and LPO damages reflect the contribution of both *B. azoricus* and bacteria in the gills. As preliminary experiments with bacterial SOD expression gene pointed out for the existence of significant SOD activity in such symbionts (Hoarau, unpublished data), the role of these bacteria in antioxidant protection may be considerably relevant. Symbiotic bacteria in *B. azoricus* are definitely involved in nutrition supply (Fiala-Médioni & Felbeck, 1990; Raulfs *et al.*, 2004), but may also have an important role in the protection of these mussels against reactive oxygen species and metal toxicity. This will reinforce the importance of symbiotic association in hydrothermal vent organisms, not only for the well-established nutritional purpose but also for protection and detoxification.

Besides tissue dependent, antioxidant defences and oxidative damage also showed to be site specific. In fact, the activities of SOD, CAT and GPx, TOSC and LPO levels varied significantly in mussels collected from different MAR hydrothermal sites (Figures 2.2 to 2.6; Chapter 2). In *B. azoricus* gills, the activity of cytosolic SOD and GPx (total and Se-dependent) followed the same pattern with higher activity in mussel gills from Lucky Strike (Eiffel Tower) vent field, followed by Menez-Gwen and Rainbow. This can be associated to the metal content in hydrothermal fluids and consequently to metal accumulation in this tissue, such as Cu concentrations, that was higher in Lucky Strike, suggesting that the excess of this essential metal is directly responsible for the

enhancement of cytosolic SOD (a Cu and Zn containing enzyme) activity in mussel gills (Table 2.1; Chapter 2).

The results of TOSC analysis support the spatial variability of antioxidant defence systems. In mussels from Menez-Gwen, the total capacity to capture reactive oxygen species (peroxyl, hydroxyl and peroxynitrite) in the gills is significantly higher than in mussels from the other sites, suggesting that this site is less stressful and the formation of ROS in mussel gills is effectively counteracted by the antioxidant defence system at this vent site (Figure 2.5; Chapter 2). Similarly, LPO levels were lower in the gills of mussels from Menez-Gwen supporting the hypothesis of a less stressful vent environment than in those from Rainbow where TOSC levels were lower (Figure 2.6; Chapter 2).

The mantle showed a similar spatial variability of antioxidant enzymes. In this tissue, the activity of SOD (cytosolic and mitochondrial), GPx (total and Se-dependent) and LPO were also highest in mussels from Lucky Strike, while CAT activity was similar among vent sites (Figures 2.2 to 2.5; Chapter 2). Like in the gills, TOSC levels decreased from Menez-Gwen to the other two vent sites among which levels were similar, indicating that mantle tissues from Lucky Strike and Rainbow had reduced capacity to deal with ROS formation (Figure 2.6; Chapter2).

Establishing a spatial variability of antioxidant defence systems and damages in the hydrothermal vent mussel was very important to understand that although vent species are well adapted to one of the most toxic environments, their tolerance may differ from site to site. Although antioxidant defence systems can be activated by a variety of compounds able to increase ROS production besides metals, i.e. they are non-specific biomarkers, significant relationships between metals (in both hydrothermal vent fluids and mussel tissues) and antioxidant enzyme activities occur in *B. azoricus*. However, MAR vent sites not only contain high metal concentrations when compared to contaminated coastal waters, but are also characterised by a mixture of toxic compounds (hydrogen sulphide, methane and radionuclides) that do play an important role in the antioxidant defence of marine organisms by scavenging free radicals. The

survival capacity of these vent mussels living in a metal rich environment, depends not only on metal induced proteins (MT), but also in enzymatic protection capable to scavenge reactive oxygen species produced in their tissues as a result of normal respiration but probably enhanced by the presence of toxic compounds. Nevertheless, one of the most surprising results was that antioxidant enzymatic activities in the gills and mantle of hydrothermal vent mussel *B. azoricus* are of the same order of magnitude from those in coastal areas, living in a very different environment in terms of chemical toxic compounds (Table 1.7; Chapter 1).

After the characterization of basal antioxidant defence systems in *B. azoricus* from five different MAR hydrothermal sites, where tissue and spatial variability was recognized and related to metal concentrations in the environmental fluids, it was necessary to understand if other parameters, such as size and season influence the activities of antioxidant enzymes and membrane damages in this mussel. Hydrothermal vents are typically considered an aseasonal environment due to their dependence of continuous geochemical-based energy and their relatively isolation from the rest of the oceanic ecosystems (Dixon *et al.*, 2002). However, these environments are also highly variable and unstable in terms of vent emission and consequently variability of available chemical species occurs. This raised the question if some biochemical parameters such as antioxidant enzymes are influenced in a seasonal way. Thus, *B. azoricus* were periodically collected from Menez-Gwen by acoustic retrievable cages (Figure 3.1; Chapter 3). This site was selected mainly because it was the shallowest hydrothermal site considered in this study (850 m), easier to sample and therefore cage recovery would be less stressful for the organisms guaranteeing that *B. azoricus* were in the best physiological condition possible.

The seasonal study revealed that, contrary to what was previously thought, hydrothermal vent organisms are under marked seasonal influence (Chapter 3). This is reflected not only in the amount of metal accumulated in both gills and mantle tissues of *B. azoricus* (Annexe I), but also in the antioxidant defences and damages studied (Figures 3.2 to 3.6; Chapter 3). The tissue dependence already observed for metal concentrations and antioxidant enzymes activities

among sites also occurred in the seasonal study, confirming that gills and mantle have different functions and that is reflected by the seasonal variations of the antioxidant defence systems. Therefore, the results of the two tissues were independently interpreted. In the gills, SOD, CAT and GPx are significantly elevated in the summer, corresponding to a maximum of LPO damage suggesting that the antioxidant defence system was unable to protect gill tissue from ROS mediated damages (Figures 3.2 and 3.3A; Chapter 3). Metal concentrations in the gills during the summer (July and August months) are also significantly higher compared to other periods, especially Ag, Fe, Mn and Zn (Annexe I). The increase of metal concentrations (Ag, Cu and Mn) in this tissue induced SOD activity (Figures 3.7A-D; Chapter 3), suggesting that these metals may enhance superoxide radical production inside cells with subsequent production of hydrogen peroxide although through different mechanisms depending on the metals concerned. Similarly, the increase of Zn in the insoluble form in the gills induces both TGPx and Se-PGx, which are important enzymes responsible for the detoxification of hydrogen peroxide (Figure 3.7F; Chapter 3). Furthermore, the activities of these enzymes were significantly related to LPO levels (Figure 3.4; Chapter 3), suggesting a more important involvement of both GPx in LPO protection, compared to CAT, and that although antioxidant defence system in *B. azoricus* was induced by metals in the gills, it may not be completely effective in detoxifying the excess of hydrogen peroxide.

In the mantle, a different seasonal pattern was observed, with a gradual increase of antioxidant enzyme activities from summer to autumn, with a maximum of SOD, CAT and GPx activities. Similarly to what was observed in the gills, a maximum of LPO damage in the mantle tissue occurred in the summer, but the activities of the antioxidant enzymes were significantly inhibited. Moreover, contrary to what was observed in the gills, metal concentrations did not increase during this period, which suggests that damages in membranes are probably related to other hydrothermal compounds capable of inducing oxidative stress, as mentioned earlier (hydrogen sulphide, methane and others). Interestingly and contrasting with the gills, antioxidant enzyme activities in the mantle were inhibited by increasing metal

concentrations. CAT and total GPx were inhibited by the increase of Zn concentrations (Figure 3.8; Chapter 3), while most of the antioxidant defence system was inhibited by the toxic metal Cd (Figure 3.9; Chapter 3). Therefore, these metals seem to inhibit most of the antioxidant capacity in the mantle, in contrast to the gills where an enhancement of enzyme activity by metals was consistently observed. This may also reflect that the bacteria present in higher amounts in the gills compared to the mantle may in fact contribute to the antioxidant protection of *B. azoricus*. This study also concluded that size of *B. azoricus* do not affect the antioxidant enzymatic responses with the exception of mitochondrial SOD. LPO levels however, were higher in smaller mussels. Although the relationship between size and age of these mussels are still not completely clear, the LPO results may reflect poor physiological conditions in small organisms.

7.2. Effects of metal exposure in the antioxidant defence systems of *B. azoricus*

After the characterization of antioxidant defence systems in *B. azoricus* in the field, where both spatial and seasonal variations were identified, it was necessary to study the effects of metal exposure in these antioxidant defence systems. The metals used are essential (Cu and Zn) to the normal function of the organisms while others are non-essential (Ag, Cd and Hg), and they exist at high concentrations in hydrothermal vent fluids. Moreover, these metals are able to interfere with antioxidant defence systems causing antioxidant damages in coastal bivalves and therefore it would be interesting to understand the mechanisms of antioxidant enzymes in species living in these extreme environments. Laboratory experiments were designed to understand and evaluate the isolated effect of metals. Although impossible to reproduce precisely the hydrothermal environment, the experimental design tried to replicate some of the abiotic conditions of the hydrothermal environment where *B. azoricus* live. Thus, all the experiments were conducted at 9 ± 1ºC the temperature measured in the mussel beds from Menez-Gwen hydrothermal

vent field. Moreover, the mussels were maintained and tested in seawater enriched with methane in an attempt to preserve the presence of methanotrophic symbiotic bacteria in the gills. On the other hand, because hydrogen sulphide could precipitate the tested metals, this compound was not added to the seawater. This may have caused the loss of sulphur-oxidizing bacteria in the gills during the experiment period and explain some of the changes observed in unexposed and metal exposed mussels.

The short term exposure experiments try to mimic the hydrostatic pressure of hydrothermal environments as well. These experiments were conducted for short periods of time (ranging from 12 to 144 hours) in a pressurized container (IPOCAMP) at 85 atmospheres, the same pressure that affects *B. azoricus* in Menez-Gwen at 850 m deep. In these conditions the metals tested were Cd (100 µg l^{-1}), Cu (25 µg l^{-1}), Zn (1000 µg l^{-1}), Ag (20 µg l^{-1}) and Hg (25 µg l^{-1}) and the main results on the antioxidant defence systems and damage in the gills and mantle of *B. azoricus* are summarized in Table 7.1.

Table 7.1 – Effects on antioxidant enzyme activities and lipid peroxidation in the gills (G) and mantle (M) of *B. azoricus* after short term metal exposures.

Metal	Hours	SOD cyt		SOD myt		CAT		TGPx		Se-GPx		LPO	
		G	M	G	M	G	M	G	M	G	M	G	M
Cd	24	↓	=	↓	↓	↓	=	=	=	=	=	=	=
	48	↓	=	=	=	↓	=	=	=	=	↓	=	=
	144	=	↑	=	=	=	↑	↓	↑	↓	↑	↑	↑
Cu	12	↓	=	↑	=	=	=	↓	↑	=	↑	↑	=
	24	↓	↓	↓	↓	↓	↓	=	↓	=	↓	↓	↓
Zn	24	=	↓	↑	↓	=	↓	=	↓	=	↓	=	=
Ag	12	=	=	↑	=	=	=	↓	=	↓	=	↓	=
	24	↑	=	=	↓	=	=	↓	↑	↓	↑	=	↑
Hg	12	=	=	↑	=	=	=	=	=	↓	=	=	=
	24	=	=	=	=	=	=	=	=	=	=	=	=

↑ significant induction/increase; ↓ significant inhibition/decrease; = unchanged

Cd pressurized short term exposure experiments showed that the two tissues are differently affected by this metal exposure and the effects on antioxidant responses and oxidative damages is time dependent as well (Chapter 4). Thus, in the gills of *B. azoricus*, Cd (100 µg l^{-1}) inhibited most of the antioxidant enzymes, particularly SOD and CAT in the first hours of exposure and TGPx and Se-GPx were inhibited only when exposure was prolonged to six days (Table 7.1). In the mantle, antioxidant defence system in general remained unchanged during the first hours of Cd exposure and contrary to what was observed in the gills, most antioxidant enzymes were induced when the exposure is extended for 6 days (Table 7.1). Although Cd is a non-redox metal, is one of the most toxic non-essential metals recognized. The type of damages involved in Cd cellular toxicity include interference with antioxidant enzymes (Hussein *et al.*, 1987; Shukla *et al.*, 1987), alterations in thiol proteins (Chan & Cherian, 1992; Li *et al.*, 1993), inhibition of energy metabolism (Muller, 1986), alteration in DNA structure (Coogan *et al.*, 1992) and altered membrane structure/function (Muller, 1986; Shukla *et al.*, 1987). The inhibitory effect of Cd on antioxidant enzymes is well documented (Muller 1986; Hussein *et al.*, 1987; León *et al.*, 2002; Jurczuk *et al.*, 2004). In the mantle though, cytosolic SOD, TGPx and Se-GPx were induced in the longest Cd exposure time (6 days), contrary to what was previously observed during the seasonal field study, where increasing Cd concentrations in this tissue caused significant inhibition of several antioxidant enzymes (mitochondrial SOD, CAT and Se-GPx) (Chapter 3). Lipid peroxidation only occurred after 6 days of Cd exposure in both tissues (Table 7.1). This may indicate a high resistance to oxyradical toxicity after short term Cd exposure, even though antioxidant enzymes, particularly in the gills were significant altered. This can be confirmed in the gills by TOSC results, which remained unchanged although SOD and CAT activities were inhibited (Table 4.1; Chapter 4). When excessive Cd concentrations occur by longer periods, antioxidant protection in *B. azoricus* seems to be unable to prevent ROS mediated lipid peroxidation damages. This was also observed for coastal bivalve species like the mussel *Mytilus edulis* and the clam *R. decussatus* exposed to 200 and 100 µg l^{-1} Cd respectively, during 21 days (Géret *et al.*, 2002a,b).

The exposure of *B. azoricus* to 25 µg l^{-1} Cu in IPOCAMP pressurized system also affected gills and mantle of *B. azoricus* in a different way (Chapter 5). In the first 12 hours of Cu exposure, cytosolic SOD and TGPx were inhibited, while mitochondrial SOD and Se-GPx were induced in the gills, while in the mantle for the same period, only total and Se-GPx were significantly induced (Table 7.1). Surprisingly, when Cu exposure is extended from 12 to 24 hours, with the exception of total and Se-GPx activities in the gills, a general inhibition of antioxidant defence system occurs in both tissues of *B. azoricus* (Table 7.1), which would decrease the ability to scavenge ROS within the cells. Contrarily to Cd, this metal is able to generate reactive oxygen species by Fenton-like reactions (Aust *et al.*, 1985; Cheeseman & Slater, 1993) so it would be expected that antioxidant defences were induced in order to counteract the ROS formation. Nevertheless, comparable antioxidant inhibition by Cu exposure was also obtained for coastal species, such as *R. decussatus* exposed to similar Cu concentrations (Géret *et al.*, 2002c) and *M. galloprovincialis* exposed to 38 µg l^{-1} Cu (Canesi *et al.*, 1999). Event though Cu is known to be powerful catalyst of low density lipoprotein oxidation and therefore may be involved in the initiation steps of lipid peroxidation (Steinberg, 1997; Raveh *et al.*, 2000; Burkitt, 2001) a significant decrease in LPO levels in both tissues of Cu exposed *B. azoricus* was registered, along with the general antioxidant system inhibition. This may be suggestive of a small oxidative risk due to Cu accumulation to this hydrothermal mussel well adapted to metal rich environments. Surprisingly, the levels of lipid peroxidation also decreased in the coastal species *M. edulis* and *C. gigas* exposed to 40 µg l^{-1} Cu (Géret *et al.*, 2002c).

The exposure of *B. azoricus* to 1000 µg l^{-1} Zn for 24 hours (Chapter 6) only induced cytosolic SOD activity in the gills (Table 7.1). Contrarily, this metal inhibited all antioxidant defences (cytosolic and mitochondrial SOD, CAT, total and Se-GPx) in the mantle (Table 7.1). Therefore, as occurred with the other metals (Cd and Cu), also the exposure to the essential metal Zn affects differently the two tissues. The same tissue specificity was previously observed for Zn during the seasonal study, where the antioxidant enzymes were induced in the gills and inhibited in the mantle (Chapter 3). Most part of cellular Zn is generally bound to intracellular sites such as proteins and therefore in normal

physiological conditions there is very little free ionic zinc (Zn^{2+}) in the cytoplasm (Dineley *et al.*, 2005). However, because the excessive elevation of intracellular free Zn^{2+} is considered very toxic, the regulation of free Zn is very important (Weiss *et al.*, 2000). Zn is mainly regulated by the induction of metal binding intracellular proteins like MT (Kondoh *et al.*, 2003). Moreover, although there are evidences that Zn is a non-redox metal and consequently unable to produce reactive oxygen species by Fenton-type reactions (Maret, 2000), other studies suggest that Zn causes ROS accumulation in the cells, possibly from multiple mechanisms including inhibition of mitochondria (Sensi *et al.*, 1999) and activation of NADPH oxidase (Noh *et al.*, 1999). In *B. azoricus* the exposure to Zn although decreasing ROS detoxification mechanisms by the inhibition of SOD, CAT and GPx in the mantle, no oxidative damages (LPO) occurred in both gills and mantle tissues, suggesting that MT levels may effectively regulate free Zn in the cells (Figure 6.5A; Chapter 6).

The short term exposure to 20 µg l^{-1} Ag showed that, similarly to what has been observed for other metals, the antioxidant responses of *B. azoricus* are tissue and time dependent (Chapter 6). After 12 hour of Ag exposure only the induction of mitochondrial SOD occurred, while both total and Se-GPx were inhibited in the gills (Table 7.1). When exposure to 20 µg l^{-1} Ag is extended from 12 to 24 hours, both tissues are affected but with contrary antioxidant responses, mostly for GPx. While in the gills both total and Se-GPx are inhibited, in the mantle they were induced (Table 7.1). Also, LPO damages only occurred in the mantle what suggests that in this tissue, the antioxidant defence system of *B. azoricus* was unable to efficiently detoxify ROS production and prevent antioxidant damage. Although Ag is recognized as extremely toxic to aquatic organisms (Hogstrand *et al.*, 1996; Mayer *et al.*, 2003), little is known about how Ag affects antioxidant defences in marine bivalves. From the results obtained it is possible to assume that the excess of Ag accumulated in *B. azoricus* tissues may increase the levels of reactive oxygen species and that the mantle is more susceptible since LPO only occurred in this tissue. As postulated before, this is possible due to the relatively low concentration of symbiotic bacteria in this tissue, comparatively with the gills (Hoarau, unpublished data). The oxidative damages in the mantle may also result from

the mitochondrial SOD inhibition in this tissue, and consequently a reduction of the capacity to scavenge $O_2^{-\bullet}$. Increase in LPO levels as a result of Ag exposure was also observed in the mussel *M. edulis* although in this case the damages occurred in the gills (Géret *et al.*, 2002d).

The exposure of *B. azoricus* to 25 µg l^{-1} Hg unexpectedly had little effect on antioxidant defence system in both gills and mantle tissues (Chapter 6). In fact, all antioxidant enzymes remained unchanged, except mitochondrial SOD (induced) and Se-GPx (inhibited) in the gills (Table 7.1). Moreover, Hg did not increased LPO in any tissue (Table 7.1). This was very surprising since this metal is one of the most toxic elements known to marine species and the Hg concentration used is the experiments was extremely high, even considering that *B. azoricus* live in a metal rich environment. Moreover, several authors showed that Hg is able to generate ROS and cause lipid peroxidation (Yee & Choi, 1996; Zaman & Pardini, 1996; Konigsberg *et al.*, 2001). Consequently in *B. azoricus* it would be expected a response from ROS scavenging enzymes to avoid deleterious effects in the tissues. Unfortunately, there are no data of Hg levels or MT concentrations in the tissues to establish if this metal was actively accumulated in *B. azoricus* after 12 or 24 hours of exposure and to determine if MT is able to bind the excess of Hg.

Along with the preceding short term experiments in pressurized IPOCAMP device, long term experiments were also considered (Cd and Cu) to study the response of antioxidant defence system of *B. azoricus* during exposure and their recovery in the depuration period (Chapters 4 and 5). Unlike short term experiments, these were carried out at atmospheric pressure. Unfortunately, the results of these long term experiments were not very promising in highlight the effects of Cd and Cu in the antioxidant defences in this hydrothermal vent mussel. During the exposure time (24 days) and elimination period (6 days) very few differences were observed in antioxidant enzymatic responses between unexposed and metal exposed (Cd and Cu) mussels. In fact, SOD, CAT and GPx increase continuously throughout the experiments in both groups and in both tissues and therefore the effect of metal toxicity was impossible to distinguish (Figure 4.4; Chapter 4 and Figure 5.4; Chapter 5). Such results are

indicative that reactive oxygen species are being produced not only in metal exposed mussels but also in mussels maintained in clean seawater. In these experiments it is reasonable to assume that antioxidant responses are not reflecting the effects of Cd and Cu contamination but the progressive physiological deterioration of *B. azoricus*. This may be consequence of the loss of symbiotic bacteria in the tissues of *B. azoricus* as a result of the long-lasting maintenance of these mussels in seawater whose chemical composition was substantially different from the hydrothermal environment. Also the fact of these mussels were kept at atmospheric pressure instead of 85 bars as in their natural environment for a extended period of time is likely to have altered the physiological conditions of this species. Changes in hydrostatic pressure were recently proven cause DNA damages in *B. azoricus* (Dixon *et al.*, 2004). In addition, TOSC levels from unexposed and metal exposed (Cd and Cu) *B. azoricus* are the same, confirming that other variables than metals might be responsible for the antioxidant responses in long term atmospheric exposure experiments. Consequently, it is prudent to assume that laboratory experiments using vent organisms must be carefully interpreted, since hydrothermal environmental conditions due to their complexity are difficult if not impossible to mimic in the laboratory with the present knowledge and technology.

Nevertheless, *B. azoricus* appears to be one of the most successful and widespread species in MAR hydrothermal vents. Although further investigations are still needed to understand how *B. azoricus* can live in such high metal concentrations, it is unquestionable that these mussels are able to survive in these potentially toxic environments. The antioxidant defence mechanisms in this deep-sea mussel also appears well adapted to such hydrothermal conditions.

7.3. Final conclusions

The final conclusions of this dissertation on the antioxidant defence systems in the deep-sea mussel from Mid-Atlantic Ridge hydrothermal vents can be summarized as following:

I *Bathymodiolus azoricus* indeed possess antioxidant defence systems to deal with reactive oxygen species production and this enzymatic protection can be measured by the specific activities of superoxide dismutase, catalase and glutathione peroxidases and by total oxyradical scavenging capacity.

II The activities of these enzymes and lipid peroxidation levels in *B. azoricus* are in the same order of magnitude of their coastal counterparts of the genus *Mytilus*, even though hydrothermal environments are completely different from coastal areas in terms of hydrostatic pressure, pH and available toxic chemical species like metals, hydrogen sulphide and methane.

III Antioxidant defences and oxidative damage depend largely on the tissues analysed, probably reflecting their different physiological functions, while it seems to be relatively independent on the size of *B. azoricus*.

IV Significant spatial and seasonal variability of antioxidant enzymes activity and lipid peroxidation levels was established, suggesting that ROS production in vent organisms is not constant and depends on the hydrothermal fluids chemistry, which are likely to vary significantly from site and time of the year.

V Antioxidant enzymes and lipid peroxidation in mussels from Lucky Strike and Rainbow fields revealed that these two vent sites are probably more stressful for *B. azoricus* than Menez-Gwen environment.

VI The antioxidant defence mechanisms in *B. azoricus* depend among other things on the bioavailable metal concentrations present in the hydrothermal fluids.

VII Metals like Ag, Cu, Mn and Zn are capable of enhancing ROS production in *B. azoricus* resulting in the induction of antioxidant enzymes activities in the gills, while Cd and Zn inhibit these enzymes in the mantle. Some of these interactions between metals and antioxidant enzymes observed in the field studies were later confirmed by laboratory exposure experiments.

VIII Laboratory manipulations with *B. azoricus* and probably other vent species should mimic as much as possible the hydrothermal vent conditions, otherwise results would be misleading and difficult to interpret. Experiments using IPOCAMP pressurized tanks are therefore a better technological approach since it is possible to control the hydrostatic pressure that affect vent organisms, in comparison to simplified unrealistic atmospheric pressure manipulations.

IX The time of laboratory experimentation seems crucial when using hydrothermal vent species. Long lasting experiments may result in poor physiological conditions of the organisms and make impossible to access some biochemical changes like antioxidant defences.

X Symbiotic bacteria present in the gills of *B. azoricus* are likely to have an important role in antioxidant protection and therefore should be isolated from the tissues and studied for systematic antioxidant enzymatic screening.

XI Because antioxidant defence mechanisms respond to a variety of chemicals besides metals that enhance ROS production, it is likely that *B. azoricus* use also this defence system against hydrogen sulphide, methane and other harmful hydrothermal chemical species.

XII Nevertheless, *B. azoricus* seems able to cope with high metal levels in their natural environment and its antioxidant defence mechanisms appears well adapted to these potentially toxic hydrothermal conditions.

7.4. References

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Annexes

Annexe I

Seasonal variation of metal concentrations (Ag, Cu, Cd, Fe, Mn and Zn) in total, soluble and insoluble fractions (μg g^{-1} d.w.) in the gills and mantle of *B. azoricus*. Data are mean ± SEM and percentage (n = 10).

		Gills			Mantle		
Metal	**Date**	**Total**	**Soluble**	**Insoluble**	**Total**	**Soluble**	**Insoluble**
Ag	**7-Jul**	1.92 ± 0.50	1.32 ± 0.39 (74)	0.47 ± 0.13 (26)	0.59 ± 0.18	0.19 ± 0.05 (34)	0.40 ± 0.10 (66)
	31-Jul	6.10 ± 1.13	3.75 ± 1.04 (61)	2.35 ± 0.47 (39)	0.66 ± 0.12	0.21± 0.07 (40)	0.46 ± 0.11 (60)
	7-Aug	6.36 ± 1.09	3.43 ± 1.07 (54)	2.94 ± 0.47 (46)	1.03 ± 0.18	0.31 ± 0.07 (28)	0.72 ± 0.19 (72)
	1-Sep	4.92 ± 1.35	4.24 ± 1.40 (86)	0.63 ± 0.18 (14)	1.37 ± 0.33	1.17 ± 0.40 (95)	0.09 ± 0.02 (5)
	9-Nov	3.36 ± 0.53	2.61 ± 0.55 (80)	0.64 ± 0.16 (20)	0.25 ± 0.06	0.18 ± 0.05 (67)	0.11 ± 0.03 (33)
	10-Jul	3.27 ± 0.50	2.20 ± 0.26 (67)	1.08 ± 0.31 (33)	0.79 ± 0.15	0.46 ± 0.11 (58)	0.33 ± 0.06 (42)
Cu	**7-Jul**	88.9 ± 16.4	26.3 ± 5.1 (29)	63.4 ± 13.7 (71)	49.0 ± 14.5	5.3 ± 1.6 (10)	42.6 ± 13.6 (90)
	31-Jul	52.5 ± 5.3	24.5 ± 3.2 (47)	28.0 ± 4.6 (53)	8.2 ± 2.1	3.9 ± 1.1 (44)	4.4 ± 1.4 (56)
	7-Aug	55.3 ± 6.8	20.9 ± 5.3 (38)	34.4 ± 5.2 (62)	13.2 ± 2.1	5.6 ± 1.4 (51)	7.6 ± 1.4 (49)
	1-Sep	53.1 ± 6.6	23.9 ± 4.1 (45)	29.2 ± 6.0 (55)	4.3 ± 1.4	6.2 ± 1.4 (57)	3.8 ± 1.0 (43)
	9-Nov	41.0 ± 7.9	17.8 ± 2.4 (43)	23.3 ± 6.8 (57)	8.4 ± 1.8	4.1 ± 0.8 (41)	4.4 ± 1.2 (59)
	10-Jul	39.3 ± 2.8	20.6 ± 4.9 (53)	18.6 ± 2.1 (47)	6.61 ± 0.9	3.7 ± 0.02 (56)	4.4 ± 0.9 (67)
Cd	**7-Jul**	2.77 ± 0.68	1.64 ± 0.50 (57)	1.22 ± 0.24 (43)	0.32 ± 0.08	0.04 ± 0.01 (13)	0.27 ± 0.07 (87)
	31-Jul	2.46 ± 0.35	1.39 ± 0.35 (57)	1.07 ± 0.26 (43)	0.50 ± 0.08	0.10 ± 0.02 (20)	0.40 ± 0.09 (80)
	7-Aug	3.24 ± 0.56	1.45 ± 0.38 (45)	1.79 ± 0.48 (55)	0.32 ± 0.05	0.16 ± 0.04 (60)	0.16 ± 0.03 (40)
	1-Sep	5.07 ± 0.87	2.67 ± 0.31 (53)	2.39 ± 0.75 (47)	0.19 ± 0.04	0.02 ± 0.01 (14)	0.17 ± 0.04 (86)
	9-Nov	2.82 ± 0.58	1.62 ± 0.25 (57)	1.20 ± 0.34 (43)	0.11 ± 0.03	0.02 ± 0.01 (19)	0.09 ± 0.02 (81)
	10-Jul	3.58 ± 0.20	2.38 ± 0.16 (66)	1.19 ± 0.04 (33)	0.36 ± 0.06	0.29 ± 0.04 (81)	0.07 ± 0.02 (19)
Fe	**7-Jul**	206.1 ± 42.5	37.0 ± 10.4 (18)	169.1 ± 39.3 (82)	50.8 ± 12.6	2.9 ± 0.8 (6)	47.8 ± 13.0 (94)
	31-Jul	107.5 ± 24.3	22.8 ± 1.8 (21)	84.9 ± 25.1 (79)	6.9 ± 1.0	4.9 ± 0.9 (56)	2.3 ± 0.7 (44)
	7-Aug	89.9 ± 17.4	25.7 ± 3.3 (29)	63.9 ± 14.3 (71)	6.2 ± 1.4	5.2 ± 1.5 (86)	2.4 ± 0.3 (14)
	1-Sep	125.3 ± 30.5	20.9 ± 2.9 (17)	104.4 ± 29.2 (83)	11.4 ± 2.3	5.2 ± 1.6 (54)	5.1 ± 1.3 (46)
	9-Nov	119.7 ± 24.4	21.1 ± 3.4 (18)	98.6 ± 22.8 (82)	21.0 ± 6.0	4.0 ± 1.1 (18)	16.9 ± 5.0 (82)
	10-Jul	134.0 ± 9.9	14.0 ± 0.1 (10)	120 ± 9.8 (90)	98.9 ± 23.7	13 ± 0.01 (13)	85.0 ± 23.72 (86)
Mn	**7-Jul**	4.77 ± 0.63	1.51 ± 0.34 (32)	3.26 ± 0.43 (68)	1.97 ± 0.25	0.89 ± 0.16 (35)	1.04 ± 0.3 (65)
	31-Jul	4.53 ± 0.49	1.58 ± 0.10 (35)	2.94 ± 0.47 (65)	1.50 ± 0.40	0.69 ± 0.18 (44)	0.81 ± 0.23 (56)
	7-Aug	3.44 ± 0.62	1.26 ± 0.17 (37)	2.18 ± 0.51 (63)	1.76 ± 0.32	0.80 ± 0.10 (29)	0.97 ± 0.25 (71)
	1-Sep	4.19 ± 0.12	1.46 ± 0.19 (35)	2.73 ± 0.18 (65)	1.73 ± 0.50	0.90 ± 0.26 (47)	0.88 ± 0.29 (53)
	9-Nov	3.50 ± 0.56	1.31 ± 0.13 (38)	2.18 ± 0.46 (62)	1.79 ± 0.40	0.56 ± 0.16 (33)	1.24 ± 0.31 (67)
	7-Jul	3.39 ± 0.99	0.63 ± 0.33 (19)	2.12 ± 0.24 (63)	2.25 ± 0.26	0.47 ± 0.16 (21)	1.87 ± 0.30 (83)
Zn	**7-Jul**	173.2 ± 24.0	103.6 ± 19.0 (60)	68.0 ± 17.2 (40)	71.4 ± 14.8	14.9 ± 1.8 (11)	58.8 ± 14.0 (89)
	31-Jul	138.6 ± 10.6	77.1 ± 12.9 (56)	61.5 ± 11.6 (44)	78.3 ± 17.8	17.4 ± 4.7 (21)	61.0 ± 17.4 (79)
	7-Aug	142.9 ± 5.1	83.8 ± 7.2 (59)	59.2 ± 5.9 (41)	66.2 ± 17.7	17.3 ± 3.5 (19)	48.8 ± 14.9 (81)
	1-Sep	125.4 ± 7.6	71.5 ± 5.4 (57)	53.9 ± 6.9 (43)	84.0 ± 14.8	14.8 ± 3.6 (18)	70.8 ± 16.2 (82)
	9-Nov	128.6 ± 10.7	82.7 ± 8.0 (64)	45.86 ± 9.0 (36)	48.5 ± 9.2	14.5 ± 2.5 (23)	34.1 ± 8.4 (77)
	10-Jul	186.9 ± 40.5	105.1 ± 6.0 (56)	81.4 ± 9.8 (44)	76.6 ± 14.9	41.50 ± 2.5 (54)	48.6 ± 10.9 (63)

Annexe II

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Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the hydrothermal vent mussel *Bathymodiolus azoricus*

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Abstract

Metals are known to influence lipid peroxidation and oxidative status of marine organisms. Hydrothermal vent mussels *Bathymodiolus azoricus* live in deep-sea environments with anomalous conditions, including high metal concentrations. Although *B. azoricus* are aerobic organisms they possess abundant methano and thioautotrophic symbiotic bacteria in the gills. The enzymatic defences (superoxide dismutase (SOD), catalase (CAT), total glutathione peroxidase (Total GPx) and selenium-dependent glutathione peroxidase (Se-GPx)) and lipid peroxidation were determined in the gills of *B. azoricus* exposed to Cd (0.9 μM), Cu (0.4 μM) and Hg (0.1 μM) with different times of exposure. The experiments were performed in pressurized containers at 9 ± 1 °C and 85 bars.

Results show that vent mussels possess antioxidant enzymatic protection in the gills. Cd and Cu had an inhibitory effect in the enzymatic defence system, contrarily to Hg. These enzymatic systems are not completely understood in the *B. azoricus*, since reactive oxygen species might be produced through other processes than natural redox cycling, due to hydrogen sulphide and oxygen content present. Also the symbiotic bacteria may play an important contribution in the antioxidant protection of the gills.

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Menez-Gwen hydrothermal field (850 m) is located on the Mid-Atlantic Ridge (MAR) near the Azores Triple Junction and is characterized by high metal concentration, including Cd (2 nM), Cu (2 μM), Zn (2 μM), Ag (4.3 nM), high temperature (271–284 °C), low pH (4.4–4.5), high CO_2 (17–20 mmol kg^{-1}) and H_2S concentrations (1.5 mM) (Douville et al., 2002). Hg concentrations in hydrothermal vents are around 0.025 nM, although never reported from Menez-Gwen (Ando et al., 2002). The hydrothermal vents can be considered a natural pollution laboratory, where the effects of such compounds in natural inhabitant organisms can be studied to understand the adaptations of a continuous metal exposure. *Bathymodiolus azoricus* are among the most common species in Menez-Gwen. They form extensive beds surrounding the active area and live in symbiosis with chemoautotrophic bacteria (Desbruyères et al., 2000).

Although several studies on the effects of heavy metals on *B. azoricus* have been carried out (Cosson, 1997; Geret, Rousse, Riso, Sarradin, & Cosson, 1998) little is know about the antioxidant enzymatic activity and lipid peroxidation (LPO) in these organisms.

The objective of this study was to determine the effects of Cd, Cu and Hg in the antioxidant enzymes and LPO in the gills of *B. azoricus*.

B. azoricus (7.10 ± 0.81 cm) were collected in Menez-Gwen with acoustically retrievable cages during the ATOS cruise in summer 2001 and acclimated in filtered seawater collected from the Azores coastal zone and maintained at 9 °C for 48 h. The water was enriched with sulphide and methane (during the acclimation period) but, during the experiments, only methane was continuously added to avoid metal precipitation and maintain symbiotic bacteria in the gills. A group of 10 organisms was exposed separately to the following nominal metal concentrations: Cd (0.9 μM), Cu (0.4 μM) and Hg (0.1 μM) at 9 ± 1 °C and 85 atmospheres in a pressurized container IPOCAMP for 24 h. Controls were maintained in the same conditions in clean seawater. Inside IPOCAMP chamber each organism (control and exposed) were individually placed in 1 l plastic bottles. Water in each container was changed after 12 h due to oxygen and metal depletion. Pressure was re-established after 30 min. Water temperature was unaffected because it was always maintained at 9 °C. Organisms were measured and the gills dissected and immediately frozen in liquid nitrogen until further analysis.

Symbiotic bacteria were not isolated from the gills, thus the antioxidant enzymatic activities reflect the contribution from both tissue and symbiotic bacteria. SOD activity was measured by the reduction of cytochrome *c* by the xanthine oxidase/hypoxanthine system at 550 nm (MacCord & Fridovich, 1969). CAT activity was determined by the decrease in absorbance at 240 nm due to H_2O_2 consumption (Greenwald, 1985). Total and Se-dependent GPx activities were determined following the NADPH reduction at 340 nm in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (H_2O_2 or cumene

hydroperoxide respectively) (Lawrence & Burk, 1976). Protein concentrations in the supernatants were determined according with Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951). Lipid peroxidation was evaluated in terms of production of malondialdehyde and 4-hydroxyalkenals due to decomposition of polyunsaturated fatty acids (Erdelmeier, Gerard-Monnier, Yadan, & Acudiere, 1998). Analysis of variance (ANOVA) and Duncan-test were performed to the data (level of significance of 0.05).

The SOD, CAT, GPx's activities and lipid peroxidation expressed as percentage of unexposed gills of *B. azoricus* are presented in Fig. 1. Cd exposure induced a significant SOD (cytosolic and mitochondrial), CAT and total glutathione peroxidase activity inhibition ($p < 0.05$), while no significant differences in Se-dependent glutathione peroxidase ($p > 0.05$) were observed. Since Cd is a non-redox metal, it is unlikely to participate in Fenton-type reactions. Nevertheless, Cd is known to enhance the intracellular formation of reactive oxygen species and promote cellular oxidative stress. Also, Cd can compete with essential metals in protein binding sites (Pruski & Dixon, 2002) leading to the release of Fe^{2+} and Cu^{2+} ions, causing increased production of reactive oxygen species and oxidative stress.

A significant inhibition of SOD activity (both cytosolic and mitochondrial) and CAT in the gills of *B. azoricus* was also produced after Cu exposure. Cu, in contrast to Cd, is able to induce reactive oxygen species through a Fenton-like redox cycling mechanism (Halliwell & Gutteridge, 1984) and participate in the initiation and propagation of lipid peroxidation.

In the gills of mussels exposed to Hg, despite CAT that was significantly induced ($p < 0.05$), the other antioxidant enzyme activities remained unchanged. Hg, like Cd is also a non-redox metal, unable to be associated with Fenton-type reactions. Toxicity of Hg has been related to the depletion of glutathione and protein-bound sulfydryl groups, resulting in the production of reactive oxygen species. As a consequence, enhanced lipid peroxidation and DNA damage may occur.

Lipid peroxidation increased in the gills of mussels exposed to 0.9 μM Cd ($p < 0.05$), while for those exposed to 0.4 μM Cu a significant decrease in LPO levels were observed ($p < 0.05$). No significant differences between control and exposed mussels were observed after Hg exposure (Fig. 1).

These results confirm the presence of antioxidant enzymatic activity in the gills of *B. azoricus*, reflecting a physiological adaptation to continuous metal exposure in their natural environment. Cd was the only metal to produced lipid peroxidation in the gill membranes. The inhibition of most antioxidant enzymes appears to be an effect of Cd and Cu exposure. Hg appears to affect the antioxidant defence system by a different mechanism from the other two metals, since, despite CAT, no enzymatic effect occurred during Hg exposure. The antioxidant defence mechanism in *B. azoricus* is still not completely understood. The reactive oxygen species might be produced through other processes than natural redox cycling, like the combination of hydrogen sulphide with oxygen. Also the symbiotic bacteria might play an important role in the antioxidant protection of the gills.

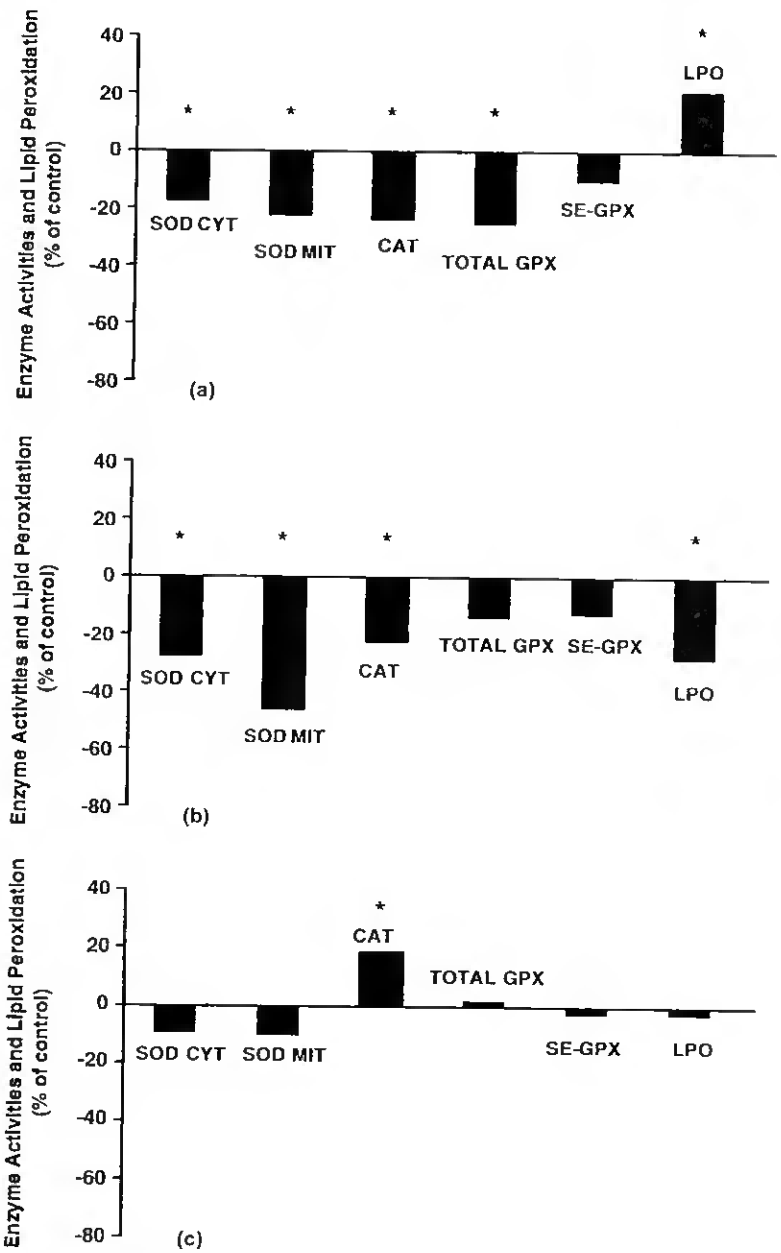

Fig. 1. The activity of SOD (cytosolic and mitochondrial), CAT, total and Se-dependent GPx, and lipid peroxidation in the gills of *B. azoricus* exposed to Cd (0.9 μM) (a), Cu (0.4 μM) (b) and Hg (0.1 μM) (c) for 24 h in IPOCAMP and expressed as percentage of unexposed gills. * Significant differences between contaminated and control antioxidant enzyme activity or lipid peroxidation levels ($p < 0.05$).

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